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# Adenosine and Acetylcholine Synthesis in a Sympathetic Ganglion

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May, 1994

"A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy"

C Anurag Tandon, 1994



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## **English Abstract**

The role of adenosine in modulating synaptic transmission in the cat superior cervical ganglion was investigated in this thesis. The first study showed that perfusion of ganglia with exogenous adenosine increased their acetylcholine (ACh) content. The effect was reduced by blockade of nucleoside transport, as if adenosine's action was mediated at an intracellular site. Isotopic labelling of the extra ACh with radiolabelled choline showed that the additional transmitter was due to increased ACh synthesis, and associated with increased choline transport. After its formation, preganglionic stimulation could release the extra ACh, but not if vesamicol, a vesicular ACh transport inhibitor, was present. Thus, the extra ACh appears to require mobilization from a reserve pool of transmitter.

Activity-dependent modulation of synaptic transmission is known to occur in sympathetic ganglia. One such form of adaptive behavior is the increase in ACh content ('rebound ACh') after high frequency preganglionic stimulation. The possibility that adenosine might play a role in the rebound phenomenon was examined in the second study. The accumulation of rebound ACh was sensitive to nucleoside transport inhibitors; dipyridamole reduced rebound ACh if it was present only after the stimulation, but not if it was present only during stimulation. After its synthesis, rebound ACh was released by preganglionic stimulation, but not if vesamicol was present, as if the extra transmitter had to be mobilized from a reserve pool. Because the dipyridamole-sensitive step occurred after the conditioning period, it seemed possible that a retrograde messenger triggered the post-stimulation change in ACh synthesis.

Thus, the final series of experiments tested whether a postsynaptic signal could alter presynaptic ACh synthesis. Antidromic stimulation increased ganglionic ACh synthesis, and, consequently, ACh content. Subsequent evoked ACh release was potentiated, as if the additional transmitter was releasable. The antidromic stimulation-induced increase in ACh content was blocked by dipyridamole suggesting that adenosine might be involved.

Overall, the results presented in this thesis are consistent with the notion that adenosine acts as a retrograde messenger after high frequency orthodromic stimulation to induce an increase in presynaptic ACh synthesis.

### Resumé Français

Lors de ce travail de thèse, nous nous sommes intéressés au rôle de l'adénosine dans la modulation de la transmission synaptique dans le ganglion supérieur cervical du chat. Les premières études ont montré que la perfusion de ganglions avec de l'adénosine produit une augmentation de leur contenu en acétylcholine (ACh). L'action de l'adénosine semble se situer au niveau d'un site intracellulaire car le blocage du transport nucléosidique provoque une diminution de cette augmentation du contenu en ACh. Des études réalisées après perfusion de choline radioactive nous ont permis de montrer que l'augmentation du contenu en ACh était due à une augmentation de la synthèse du neurotransmetteur et qu'elle était associée à une augmentation du transport de la choline. Après sa formation le surplus d'ACh formé peut être libéré par stimulation du nerf préganglionnaire. Toutefois si le vésamicol, un inhibiteur du transport de l'ACh dans les vésicules synaptiques, est perfusé, la stimulation ne permet pas la libération d'ACh. Ainsi, le surplus d'ACh formé en présence d'adénosine apparaît être présent au niveau d'un "pool de réserve" vésiculaire.

La modulation dépendante de l'activité de la transmission synaptique est connue pour avoir lieu dans les ganglions sympathiques. L'une des formes de ce type de comportement adaptatif implique une augmentation du contenu en ACh (appelée "rebound ACh") suite à des stimulations préganglionnaires à haute fréquence. La possibilité que l'adénosine puisse jouer un rôle dans la formation de ce "rebound ACh" a été étudiée dans la seconde partie de ce travail. Nous avons pu montrer que l'accumulation du "rebound ACh" était sensible aux inhibiteurs du transport nucléosidique. De plus, le dipyridamole réduit la formation du "rebound ACh" uniquement lorsqu'il est présent après la stimulation mais non pas s'il est présent durant la stimulation. Après sa synthèse, le "rebound ACh" peut être libéré par stimulation préganglionnaire en l'absence, mais non en présence, de vésamicol, montrant ainsi que l'ACh produit en excès semble être contenue dans un "pool de réserve" des vésicules. Comme la sensibilité au dipyridamole se produit après la période de conditionnement il semblait possible d'envisager qu'un messager rétrograde puisse être impliqué dans le changement post stimulatoire de la synthèse d'ACh.

Dans ce but, la dernière série d'expériences a été réalisée afin de tester si un signal postsynaptique pouvait affecter la synthèse présynaptique d'ACh. La stimulation

antidromique aumente la synthèse d'ACh ganglionnaire et de ce fait son contenu en ACh. Subséquemment la libération d'ACh est augmentée montrant ainsi que le surplus de neurotransmetteur est libérable. Cette stimulation antidromique induisant l'augmentation du contenu en ACh ganglionnaire est bloquée par le dipyridamole suggérant que l'adénosine est impliquée dans ce processus.

En conclusion, les résultats présentés lors de ce travail de thèse semblent cohérents avec l'hypothèse selon laquelle l'adénosine agit comme un messager rétrograde, après stimulation préganglionnaire à haute fréquence, afin d'induire une augmentation de la synthèse présynaptique d'ACh.

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First and foremost, I would like to thank my parents for their unfailing support and love for which I will always be grateful, and also for their bountiful care packages of Indian cuisine each weekend. Their thoughtfulness has certainly added a welcome supply of spice to my life. It is to them that this thesis is dedicated.

I had not anticipated, when I initially considered a career in science, the remarkable and pleasurable experience of meeting diverse individuals from around the world and the opportunities presented to graduate students for travelling to distant places to present our work (I still can't believe we get paid for this!). I have enjoyed this aspect of scientific life immensly, for I have been fortunate to have had the benefit of working with and befriending several talented and creative people without whom the passage of time would have seemed unbearably slow. The following paragraphs are written to convey, in my own small way, my appreciation to them for times shared.

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Rafael Cabeza and Suzanne Bayly were the first students to welcome me into the department. To one who knew little about science or scientists, they were welcome guides. After all, it was from them that I learned the 'secret pharmacology handshake', the joys of a McGill Pizzeria breakfast or a late night bottle of sake. It didn't take me long to realize Raf's 'special' character. To those who have known him will clearly understand what I mean (see acknowledgements from previous theses). Raf introduced me into the Collier gang, the research in that lab, and pushed me to become an active part of it. Sue, an 'honorary' member of the group, became a close and cherished friend with whom I shared the better part of my years as a graduate student. I will make every effort to keep in contact with them. To you, my friends, I will toast that bottle of Thomas Hardy's ale sitting on my desk when I graduate.

Collier's lab had no shortage of colorful personalities, and Serge Mykita's was more than enough to fill several shoes. Never at a loss for words, good deeds (especially to the women-folk), or flamboyant attire, Serge was always a delight to be around and to travel with. In the lab, he tutored me with lessons in surgery, and later, during that eventful trip to Portugal, he attempted to teach me the intricacies of the manual transmission. Well, at least he was successful at one. He and Christine made me feel as one of the family during my time in Strasbourg, introducing me to Alsation cuisine and, of course, their wines. I am also indebted to him for translating my thesis abstract into French. Steve Ferguson arrived shortly after I did to the lab. Once I got to know him, I realized that his gruff exterior hides a fellow who is generous and quick to a smile. Raf, Serge, Steve, and I survived many adventures on our first outing together in Europe. The most memorable was probably our collision with the police riot in Lisbon...good thing it was Serge who carried the case of beer.

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In accordance with the passage above, the text and figures included in the results section (Sections 5, 6 and 7) of this thesis are duplications of manuscipts published, in press, or submitted. Thus, the references for these are included at the end of each of their respective sections. The references for the General Introduction and the General Discussion are presented at the end of the thesis. Written permission for the reproducion of published or in press manuscripts is provided in the Appendix.

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## LIST OF ABBREVIATIONS

<b>Abbrevistion</b>	Complete Name
ACh	acetylcholine
AChE	a.cetylcholinesterase
ADA	adenosine deaminase
ADP .	adenosine 5'-diphosphate
AK	adenosine kinase
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
B <sub>max</sub>	maximum binding capacity
cDNA	complementary deoxyribonucleic acid
ChAT	choline acetyltransferase
cChAT	cytosolic choline acetyltransferase
CoA	coenzyme A
CPT	cyclopentyltheopylline
DCF	deoxycoformycin
CHA	cyclohexyladenosine
DNA	deoxyribonucleic acid
GTP	guanosine triphosphate
HC-3	hemicholinium-3
IC <sub>50</sub>	concentration for half-maximum inhibition
iChAT	ionically membrane bound choline acetyltransferase
IMP	inosine 5'-monophosphate
K <sub>d</sub> , K <sub>m</sub>	apparent affinity constants
Ki	apparent inhibition constant
Kt	apparent transport constant
kDa	kilodaltons
LDCV	large dense-cored vesicle
LTP	long-term potentiation
mChAT	non-ionically membrane bound choline acetyltransferase
mRNA	messenger ribonucleic acid
NBTI	nitrobenzylthioinosine
NMDA	n-methyl-d-aspartate
PLA <sub>2</sub>	phospholipase A <sub>2</sub>
PCh	phosphorylcholine

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SAH	s-adenosylhomocysteine
SAHH	s-adenosylhomocysteine hydrolase
SAM	s-adenosylmethionine
SCG	superior cervical ganglion
SIF	small, intensely fluorescent
TCA	trichloroacetic acid
ТРВ	tetraphenylboron
V <sub>max</sub>	maximum transport velocity

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GENERAL INTRODUCTION

The central theme of this thesis concerns the regulation of acetylcholine synthesis in sympathetic ganglia by adenosine. The following introductory section is intended to describe some of the current notions about acetylcholine and adenosine. Thus, the following passages will cover aspects of synthesis, storage, and release for both agents and point out the areas for which some contention exists.

## 1. The superior cervical ganglion as a model cholinergic system

The superior cervical ganglion (SCG) of the cat has been a valuable model for the study of cholinergic mechanisms (reviewed by Collier and Kwok, 1982). Its usefulness stems largely from its rich composition of cholinergic nerve endings and accessibility by simple surgery. Moreover, it can be perfused *in situ* through its normal vasculature and stimulated electrically through its preganglionic nerve. Thus, it is well-suited for the study of neurochemical and pharmacological aspects of acetylcholine (ACh) synthesis, storage, and release. Because some of the work presented in this thesis investigates the effects of stimulation of either the pre- or the postganglionic nerves on ACh synthesis, the following section is written to provide the reader with some basic information upon the structure of the SCG and its synaptic organization.

The earliest description of *in situ* perfusion of the cat SCG and L. suggestion that chemical transmission occurred at its synapses was provided by Kibjakow (1933); following the example of Loewi's *Vagusstoff* experiment (Loewi, 1921), Kibjakow showed that preganglionic stimulation released a chemical substance into the venous effluent which, upon introduction into the ganglion's arterial supply, stimulated the ganglion cells. The same yead, Chang and Gaddum (1933) demonstrated the presence of ACh in horse sympathetic ganglia. As the ganglion-stimulating effects of exogenous ACh were well known at that time (Dale, 1914), Chang and Gaddum (1933) suggested that Kibjakow's chemical stimulant might be ACh. The following year, measurement of evoked ACh release from cat SCG was reported by Feldberg and Gaddum (1934), but only when the perfusion medium contained eserine, an inhibitor of acetylcholinesterase (AChE). Interestingly, they were unable to reproduce Kibjakow's earlier results; his chemical transmitter appeared in the effluent in the absence of eserine, making it improbable that it was ACh, which, as is known now, does not survive the journey from the synaptic cleft without the aid of a AChE inhibitor. In a series of elegant experiments, Feldberg and Vartiainen (1934) further fortified the role of ACh as the preganglionic sympathetic neurotransmitter by showing that the source of the released ACh was indeed the preganglionic nerve endings, not the ganglion cells or the associated nodose ganglion; neither antidromic or vagal stimulation induced the appearance of ACh in the perfusate.

## 1.1 Gross and fine structure of the cat superior cervical ganglion

The cell bodies of the preganglionic neurons lie within the intermediolateral column of the spinal cord and their axons are conveyed to the ganglion by the cervical sympathetic trunk to terminate upon dendrites extending from the ganglion cells. These neurons, in turn, project ascending axons via the internal or external carotid nerves to innervate various organs or muscles of the head. The body of the ganglion, elongated and bulbous in appearance, is isolated from the surrounding tissues by a thick capsule of connective tissue that is continuous with the sheath covering the pre- and postganglionic trunks.

The cat cervical sympathetic trunk consists of both myelinated and unmyelinated fibers, the respective numbers of each vary from one animal to another (Foley and DuBois, 1940). These axons measure 1.5 to 3.5  $\mu$ m in diameter, though some of larger caliber can be found. Upon their entry into the ganglion, the preganglionic fibers bifurcate extensively forming what appears to be fine networks around ganglion cells. These mesh-like structures were first shown to be derived from preganglionic cells because they disappeared shortly after transection of the cervical sympathetic trunk (Ranson and Billingsley, 1918). This notion was later supported by electron microscopic inspection of

the ganglion (Elfvin, 1963). Early studies indicated that the quantity of axons in the preganglionic trunk was far outnumbered by the ganglion cells they innervate; Billingsley and Ransom (1918) counted approximately 4000 preganglionic fibers to 123,000 cells, giving a ratio of about 1 to 32. Foley and Schnitzlein (1957) estimated roughly twice that number of preganglionic fibers suggesting a ratio closer to 1 to 15. It is, nevertheless, clear from these values, that each preganglionic cell must transmit impulses to several ganglion cells. Moreover, the actual number of neurons innervated by single fibers is likely far greater than the ratio of pre- to postganglionic neurons since each neuron in the ganglion is supplied by 25 to 30 fibers (Blackman, 1974).

The arborization of the preganglionic axons into finer structures produces fibers of 0.1-0.3  $\mu$ m diameter that are in intimate contact with the ganglion cells through, almost exclusively, axodendritic synapses; axosomatic synapses appear to be quite rare (Elfvin, 1963). The synaptic contacts occur at variable intervals (1-6  $\mu$ m) along the length of the fibers, defined by regions dilated 3-5 times and containing a dense aggregation of various organelles. The most numerous of these are the small, clear 30-50 nm vesicles, often clustered around the presynaptic densities with an estimated concentration of about 8000/bouton (Birks, 1974). The larger, dense-cored vesicles (70-100 nm), usually located at a distance from the synaptic membrane, occur at a frequency of approximately 200/bouton [estimated from values determined by Birks (1974) and Weldon et al. (1993)]. Mitochondria are also consistently observed in the synaptic areas; these organelles appear with an average frequency of about four per bouton.

The synaptic cleft is bordered on the pre- and postsynaptic membranes by a dense material that extends into the cytoplasm 10-20 and 10-40 nm, respectively. The distance that separates the two lipid bilayers is only 6-17 nm across (Elfvin, 1963; Birks, 1974). Thus, if one compares this short distance to the diameter of the small vesicles, it becomes apparent that discharge of vesicular contents into the synapse will rapidly increase the transmitter concentration at the opposite membrane to approximately that within the lumen of the vesicle, at least temporarily.

In addition to the principal neurons and the satellite cells that surround them, there exists in ganglia a group of small, intensely fluorescent (SIF) cells, originally observed using a fluorescence method for catecholamines. These dopamine-containing cells are small in size and do not extend processes out of the ganglionic capsule (Williams and Palay, 1969; Björklund et al., 1970). The function of SIF cells in ganglia is not clear; though they are relatively few in number (Williams et al., 1977), it has been proposed that they act as inhibitory interneurons (Siegrist et al., 1968; Libet and Tosaka, 1970; Libet and Owman, 1974).

## 2. Acetylcholine Synthesis

During periods of neuronal activity, when ACh is being actively released from nerve terminals, its synthesis is increased to match the transmitter released. In this manner, the ACh stores are maintained relatively constant, even during periods of prolonged high frequency stimulation. Thus, there is clearly a close relationship between transmitter release and synthesis, and this has been the focus of many clever studies over the last 40 years. In spite of this attention on ACh synthesis and the bulk of information these studies nave provided, a number of uncertainties remain about the regulation of ACh synthesis. Since the initial work presented in this thesis (section 5) was begun as an attempt to investigate a particular mechanism that might control the activation of ACh synthesis and link it with neuronal activity, the following sections will attempt to describe briefly some of the advances and unknowns regarding the enzyme that synthesizes ACh and the acquisition of its substrates. The final part of section 2 will discuss how these components might be involved in the control of ACh synthesis.

## 2.1 Choline acetyltransferase

Choline acetyltransferase (acetylCoA:choline-O-transferase; ChAT; E.C.2.3.1.6) is

the biosynthetic enzyme that catalyzes the following reaction:

## choline + acetyl-CoA $\Leftrightarrow$ acetylcholine + CoA

ChAT, originally named choline acetylase, was first discovered in cholinergic tissue from several species by Nachmansohn and Machado (1943). It is a basic protein with a molecular weight in the range of 65,000-70,000 (Glover and Potter, 1971; Ryan and McLure, 1980). ChAT is generally accepted to be a specific marker for neuronal cells that synthesize and secrete ACh; its presence in nervous tissue coincides with that of ACh indicating its primary role in ACh synthesis (Hebb and Morris, 1969; Hildebrand et al., 1974; Rossier, 1977; Saivaterra and Fodors, 1979). It is, however, present in some non-nervous tissues such placenta (Morris, 1966), pituitary intermediate lobe (Tandon et al., 1991), and spermatozoa (Bishop et al., 1976), as well as in plant and in bacteria (White and Cavallito, 1970; Barlow and Dixon, 1973). Its significance at these sites is not yet clear, but it is possible that ACh may function as an paracrine signal.

Both substrates, choline and acetyl-CoA, are ubiquitous components of living cells and are involved in other cellular functions; their sources are discussed in the following sections below (2.2 and 2.3). The Michaelis constants for choline and acetyl-CoA are approximately 0.7-5 mM and 16-150  $\mu$ M, respectively (Glover and Potter, 1971; Morris et al., 1971; Ryan and McLure, 1980), and the forward reaction catalyzed by ChAT appears to be reversible with an equilibrium constant estimated at 5,000 (Schubert, 1966), 500 (Potter et al., 1968), or 12 (Peiklik and Guynn, 1975; Hersh, 1982), favoring the synthesis of ACh. The large variation in these numbers highlights how the differences in ionic strength of the assay medium can greatly affect enzyme activity (Rossier et al., 1977; Ryan and McLure, 1980). The value of 12 is probably closest to the true value as those authors tried to approximate physiological conditions. Kinetics studies using purified ChAT from different sources have established that the formation of ACh follows a sequential mechanism, that is, both substrates must combine in a fixed order with the enzyme before the products are released (Potter et al., 1968; Glover and Potter, 1971; Kaita and Goldberg, 1969; Morris et al., 1971; Ryan and McLure, 1980). In this scheme, acetyl-CoA binds first and is followed by choline. The products, too, are released successively, with ACh being set free first, and, subsequently, CoA, in a sequential reaction of the Theorell-Chance type. Inhibition by ACh of the forward reaction is competitive with respect to choline, but noncompetitive with respect to acetyl-CoA, while inhibition by CoA is competitive with respect to acetyl-CoA and noncompetitive with respect to choline. These data suggest that ChAT has separate binding sites for acetyl-CoA and choline. Binding of both initiates the transfer of the acetyl moiety to the hydroxyl group of choline, thus forming CoA and ACh, presumably without an acetyl-enzyme intermediate.

## 2.1.1 Delivery of ChAT to nerve endings

The presence of ChAT in nerve endings is dependent upon axonal transport following synthesis of the enzyme in the preganglionic cell bodies. The rate of its delivery to the nerve processes, as estimated by measuring its accumulation proximal to a nerve ligature, is consistent with a slow rate of axonal transport (Hebb and Waites, 1956; Hebb and Silver, 1961; Frizell et al., 1970; Saunders et al., 1973), although a fraction of the enzyme (5-20%) appears to move with greater velocity, corresponding to fast axonal transport rates (Dziegielewska et al., 1976; Fonnum et al., 1973); Fonnum et al. (1973) estimated the half-life for ChAT in rabbit tongue following delivery by the hypoglossal nerve to be approximately 16-22 days. MacIntosh and Collier (1976) suggested that ChAT could be turned over every 4 days in nerve terminals should only 6% of the enzyme move anterogradely at a fast rate (200 mm/day). This latter estimate is supported by our recent finding that selective disruption of fast axonal transport in the cervical sympathetic trunk by colchicine treatment reduced the ganglion ChAT activity by 50% in four days (Tandon, Bachoo, Weldon, Polosa, and Collier, submitted). Thus, a portion of ChAT in the cat SCG is conveyed there by a microtubule dependent mechanism and the turnover rate of the enzyme in nerve endings may be considerably faster than estimates from other cholinergic nerves.

## 2.1.2 Subcellular distribution of ChAT

To determine the size of ACh synthesis in nerve terminals, it is important to consider the distribution of ChAT within the cholinergic neuron. Information upon the subcellular distribution of ChAT was obtained in early studies by Hebb and Whittaker (1958). They demonstrated that ChAT activity was colocalized with ACh in a mitrochondrial fraction of brain homogenate following centrifugation. As fractionation techniques improved, it became clear that both ChAT and ACh were not associated with mitochondria, but concentrated in nerve endings (i.e. the synaptosomal fraction). However, it was undecided whether the ChAT was associated with synaptic vesicles (De Robertis et al., 1963; McCaman et al., 1965) or soluble in the axoplasm of nerve endings (Whittaker et al., 1964). This was resolved by the finding of Fonnum (1968) that the association of ChAT with the particulate fraction was highly dependent upon the pH and the ionic strength of the medium; association with membranes was reversibly decreased by increasing the NaCl concentration or pH, although 10-20% remained firmly bound.

More recent biochemical studies have established that the distribution of ChAT is heterogeneous. At least three forms of the enzymes exist: the major one is soluble in nerve endings (cChAT), and the two other forms are associated with the plasma membrane which, together, contribute less than half of the total enzyme activity (Benishin and Carroll, 1981; Benishin and Carroll, 1983; Badamchian and Carroll, 1985; Eder-Colli and Amato, 1985; Rylett, 1989; Salem et al., 1993). Of the latter, one is ionically associated with membranes (iChAT) and is solubilized by a high salt concentration (500 mM NaCl) while the other appears to be non-ionically membrane bound (mChAT) and requires detergent to free it. Some of the latter may be directly associated with synaptic vesicles, because Carroll (1994) has recently reported that mChAT co-purifies with synaptic vesicle. In the cat SCG, 5-10% of the total ChAT activity exists as the mChAT form (Tandon and Collier, unpublished observations).

The existence of the ChAT enzyme in different subcellular forms raises the possibility that each might be involved in the control of a different aspects of ACh synthesis. For instance, the proportional contribution to ACh synthesis of each form might vary according to whether the nerve terminals is active or resting. This notion is addressed further in the following section and in section 2.4.4.

#### 2.1.3 Some differences between the ChAT species

Benishin and Carroll (1983) have reported that the different forms of ChAT differ in some of their physico-chemical properties. cChAT activity appears to be increased by 2.5-fold with a change in pH from 6.5 to 9. This change is similar to that observed for mChAT activity, which is increased by 1.5-fold, but different from iChAT activity which is unaltered over this pH range. However, cChAT differs from mChAT in that its activity is more sensitive to denaturation by incubation at 45°C. These differences clearly indicate that the three forms of ChAT are not identical, but do not allow one to distinguish whether the differences are inherent in their structure or whether the differences arise by association with other cellular components.

Some pharmacological distinctions are also evident amongst the different forms of ChAT. The apparent affinity of mChAT for choline is similar to that of cChAT, but about 3-4 times higher than that of the iChAT (Benishin and Carroll, 1983). In addition, the sensitivity of iChAT to inhibition by 4-(1-napthylvinylpyridine) is less than that of cChAT or mChAT (Benishin and Carroll, 1983; Rylett, 1989). Furthermore, the analog of choline, homocholine, serves as a substrate for mChAT but not for either cChAT or iChAT (Benishin and Carroll, 1981; Benishin and Carroll, 1983).

This latter property is especially intriguing because homocholine is reported to be acetylated by intact synaptosomes, brain slices, and the cat SCG in an activity-dependent manner, but not by solubilized ChAT (Collier et al., 1977; Boksa and Collier, 1980b). Thus, only a small fraction of the total ChAT pool, mChAT, approximates the acetylation process of intact nerve endings. This suggests the possibility that mChAT might be preferentially involved in synthesizing ACh during neuronal activity. However, such a role for the membrane-bound ChAT has not yet been established with any certainty. The activity of the mChAT is not correlated with basal ACh synthesis (Schmidt and Rylett, 1993b), but it appears to be increased by veratridine-induced (Carroll, 1987; Schmidt and Rylett, 1993a) or electrical (Mykita and Collier, unpublished observations) stimulation. ChAT is known to be phosphorylated in vitro, but this modification is apparently not responsible for the activity-induced increase in enzyme activity (Bruce and Hersh, 1989; Schmidt and Rylett, 1993a). The phosphorylation state of the enzyme, however, appears to be inversely correlated with membrane association. Bruce and Hersh (1989) showed that phosphorylated enzyme was more readily released from membranes than the native enzyme in the presence of an increasing concentration of NaCl. This suggests that cChAT and iChAT might normally exist in equilibrium and that phosphorylation may affect the relative proportions of each by decreasing membrane association.

#### 2.1.4 Linkage of mChAT to membranes

The non-ionically bound form of ChAT in brain may be attached to membranes by an anchor in the form of a phosphatidylinositol linkage; cleavage of the anchor by phospholipase C releases ChAT into the extracellular medium (Carroll and Smith, 1990) or into the nerve terminal cytosol (Smith and Carroll, 1993) suggesting that ChAT may face outwards as well as inwards. In *Torpedo*, it is suggested that the enzyme behaves as an integral membrane protein, requiring protease treatment for dissociation (Eder-Colli et al., 1992). In support of these studies, antibodies raised to ChAT recognize an extracellular antigen (Docherty et al., 1982; Docherty and Bradford, 1986; Docherty et al., 1987), and, conversely, a monoclonal antibody recognizing cholinergic nerve terminal plasma membrane inhibits ChAT (Eder-Colli et al., 1989). The significance of the extracellular enzyme, if it truly exists as such, is unknown, although in the case of the *Torpedo* enzyme, only a portion of it may be exposed to the extracellular surface.

## 2.1.5 The ChAT gene

Application of molecular biological techniques towards the study of ChAT has resulted in its cloning from *Drosophila* (Itoh et al., 1986), pig (Berrard et al., 1987), rat (Brice et al., 1989), mouse (Ishii et al., 1990), and human (Hersh et al., 1988). There is significant conservation (80% identity) of the primary sequence across the mammalian species suggesting a common ancestral gene. The most divergent clone was that obtained from *Drosophila* which contains 8 exons coding for 752 amino acid protein; six of these exons show 60-90% homology to the rat or porcine sequence (Sugihara et al., 1991). The regulatory elements that limit expression of the gene to cholinergic neurons are located in the 5'-noncoding region (Kitamoto et al., 1992; Tajima and Salvaterra, 1992). Another level of regulation, reported for the mouse gene, appears to be provided by alternative splicing of this region to produce seven types of ChAT mRNA (Misawa et al., 1992). It is not yet known whether some of these different forms of mRNA represent genetic counterparts of the biochemical variants of ChAT.

The ready availability of ChAT cDNA has made it possible to produce the enzyme in noncholinergic cells for study. Recombinant *Drosophila* ChAT was expressed in *E. coli* and purified in sufficient quantity for analysis of its alpha-helical secondary structure and crystallized for future examination of its tertiary structure (Wu et al., 1993). More recently, a study upon the functional aspects of transfected ChAT has been reported (Misawa et al., 1994). The mouse ChAT gene was incorporated into the genomes of several different cell lines (neuronal and non-neuronal) and the synthesis of ACh measured. The ACh formed by these cells was proportional to the amount of ChAT activity and to the extracellular choline levels. The fact that most of the ACh was recovered in the supernatant after centrifugation suggests that it is not accumulated by a vesicular compartment but remains free in the cytosol after its formation. ACh release from these cells was  $Ca^{2+}$ -independent and unaffected by K+-induced depolarization.

Expression systems such as these (Wu et al., 1993; Misawa et al., 1994) will be valuable tools for future investigations because they provide the ability to study ChAT activity as it exists in the intracellular environment of cells, without contamination by other processes that might affect ACh synthesis (i.e. choline uptake, vesicular ACh uptake). Thus, the ability of mutated forms of the enzyme to synthesize ACh can be directly assessed. This would enable the characterization of possible regulatory and catalytic domains, the segments of the enzyme required for membrane insertion or association.

## 2.2 Source of acetyl-CoA for ACh synthesis

It is recognized that the immediate acetyl donor for ACh synthesis is acetyl-CoA. The CoA molecule, by virtue of its reactive thiol group, forms a thioester linkage with the acetyl moiety derived from pyruvate during oxidative decarboxylation catalyzed by the pyruvate dehydrogenase complex in the inner matrix of mitochondria. That the ultimate source of that acetyl group is glucose was first suggested by Quastel and colleagues (Quastel et al., 1936; Mann et al., 1938) and Kahlson and MacIntosh (1939) and has subsequently been confirmed in studies using radiolabelled glucose and pyruvate, and measuring the synthesis of radiolabelled ACh (Browning and Schulman, 1968; Nakamura et al., 1970; Grewal and Quastel, 1973; Tuček, 1984). In the cat SCG, Kwok and Collier (1982) showed that radiolabelled acetate can also be used as a precursor for ACh, but its incorporation into transmitter is reduced by preganglionic stimulation, whereas that of radiolabelled glucose is increased. They interpreted this observation as indicating that neuronal activity increased the rate of endogenous acetyl-CoA delivery from mitochondria, thereby diluting the radiolabelled acetyl-CoA synthesized from exogenous acetate. Thus, it would appear that under physiological conditions that require increased

ACh synthesis, the acetate derived from the extracellular hydrolysis of ACh does not serve is an immediate precursor. However, this result, coupled with reports that the efflux of acetyl-CoA from mitochondria is enhanced by increased Ca<sup>2+</sup> levels (Benjamin and Quastel, 1981; Říčný and Tuček, 1983) suggest the possibility that the availability of acetyl-CoA for ACh synthesis is increased by the Ca<sup>2+</sup>-influx that precedes transmitter release.

## 2.3 Source of choline for ACh synthesis

Choline, the other precursor required for ACh synthesis, is synthesized de novo only to a limited extent by neurons (reviewed by Blusztain and Wurtman, 1983) and, consequently, they rely upon the choline available in the extracellular medium. Some of this choline may be derived from the breakdown of membrane phospholipids. As a quaternary base, choline does not readily cross cell membranes, and, thus, requires the aid of special carriers that are present on cholinergic neurons. The notion that choline transport is an important factor in ACh synthesis was first noted by Perry (1953). He showed that ACh output during prolonged preganglionic stimulation in the presence of eserine (no exogenous choline was present in the perfusion medium) always fell to 10-15% of the initial output rate after 10 min. ACh release elicited by a second stimulation period, even after several minutes of rest, did not recover to the initial output rate of the first stimulation period. If, however, no eserine was present during the first stimulation, ACh release during the second corresponded to the maximum rate of output. Perry correctly interpreted this to indicate that the nerve terminals depend on the choline derived from hydrolyzed ACh to re-synthesize new ACh and that the presence of eserine, by preventing the destruction of released ACh, deprived them of this source. This was confirmed in studies by Birks and MacIntosh (1961) and Collier and MacIntosh (1969) who postulated that cholinergic nerve endings must be endowed with a highly efficient system for the appropriation of choline that is transiently activated by nerve impulses.

Subsequently, Collier and Katz (1974) demonstrated that approximately 50-60% of endogenously released choline is recaptured for incorporation into new transmitter.

## 2.3.1 High and low affinity choline transporters

It is generally accepted that two types of choline carriers exist on cholinergic nerve terminals, distinguished by their kinetics of transport (Yamamura and Snyder, 1972; Yamamura and Snyder, 1973; Haga and Noda, 1973; Guyunet et al., 1973; Kuhar et al., 1973). One is a low capacity, high affinity carrier that is dependent on extracellular Na<sup>+</sup> and Cl<sup>-</sup> and has a K<sub>m</sub> for choline in the range of 0.3-5  $\mu$ M. This carrier appears to be localized to cholinergic nerve terminals as demonstrated by its distribution with respect to other cholinergic markers. The high affinity transporter appears to be the primary source of choline for ACh synthesis. This was shown by experiments in which the extracellular choline concentration was increased to saturate the high affinity carrier but not the low affinity carrier. Under these conditions, choiine accumulation was increased but ACh synthesis is not. Furthermore, the activity of the high affinity system is potently inhibited by hemicholinium-3 (HC-3), a property shared with ACh synthesis (MacIntosh et al., 1956; Guyunet et al., 1973). Overall, these studies suggest that under most conditions, the high affinity choline transporter is used by cholinergic cells for provision of choline for ACh synthesis.

The other choline transport system consists of a facilitative, high capacity, low affinity carrier that is active in the absence of extracellular Na<sup>+</sup>, with a K<sub>m</sub> for choline in the range of 50-100  $\mu$ M and less sensitive to inhibition by HC-3 by three orders of magnitude compared to the high affinity transporter. The distribution of the low affinity transporter appears to be more widespread and does not correspond to the distribution of ACh; it presumably supplies choline destined for phospholipid synthesis (Collier and Lang, 1969; Diamond and Kennedy, 1969).

## 2.3.2 Regulation of high affinity choline uptake

An important characteristic required of the system that provides choline for ACh synthesis is that it increase its rate of choline delivery in response to neuronal activity when the demand for new ACh to replace released transmitter is increased. That such is the case has been clearly demonstrated in a number of studies; choline uptake is rapidly activated following an increase in ACh output during stimulation as evidenced by enhanced incorporation of radiolabelled choline into ACh (Calier and MacIntosh, 1969; Collier and Lang, 1969; Collier and Katz, 1974). The increased rate of radiolabelled ACh synthesis is clearly dependent upon activation of the high affinity carrier (Simon and Kuhar, 1975; Murrin and Kuhar, 1976; Atweh and Kuhar, 1976; Simon et al., 1976; Murrin et al., 1977; Rokoski, 1978; Antonelli et al., 1981). Furthermore, the accumulation and rest, follows the same pattern as ACh synthesis under those conditions, consistent with the activation of the choline transporter (Ilson and Collier, 1975; Collier et al., 1977; Collier and Collier, 1981; Welner and Collier, 1985).

In the sympathetic ganglion, activation of choline transport during preganglionic stimulation appears to be dependent on the presence of extracellular calcium, although barium or strontium can effectively serve as replacements (Collier and Ilson, 1977; O'Regan and Collier, 1981). This activation of transport is not reduced by increased extracellular  $Mg^{2+}$ , even though ACh release is concomitantly depressed (Collier and Ilson, 1977). Similarly, in hippocampal synaptosomes, Ba<sup>2+</sup>, Sr<sup>2+</sup>, or Mg<sup>2+</sup> can replace Ca<sup>2+</sup> during K<sup>+</sup>-induced activation of choline uptake (Murrin and Kuhar, 1976; Murrin et al., 1977). Thus, although the influx of divalent cations during depolarization are necessary for the activation of choline uptake during neuronal activity, the identity of the divalent cation is not critical. Furthermore, these results indicate that the divalent cation requirements for the activation of choline uptake are quite distinct from those of ACh release.

The affinity of the high affinity transporter for choline appears to be unaltered by stimulation, but its  $V_{max}$  is increased significantly (Simon and Kuhar, 1975; Murrin and Kuhar, 1976). It has been suggested that the increase in the  $V_{max}$  may be due, at least in part, to an unmasking of occult transporters because a parallel increase in the number of binding sites for [<sup>3</sup>H]HC-3 can be observed (Lowenstein and Coyle, 1986; Saltarelli et al., 1987). However, Ferguson et al. (1994) have recently shown that when rat hippocampal or striatal synaptosomes are pre-exposed to choline mustard aziridium ion, an irreversible inhibitor of choline transport, the subsequent stimulation-induced increase in [<sup>3</sup>H]HC-3 binding sites is prevented. They interpreted this result to indicate that the sites that were hidden to [<sup>3</sup>H]HC-3 before stimulation were accessible to the choline mustard. Because the choline mustard aziridium ion is an analog of choline, it is likely that those transporters are accessible to choline also. Therefore, Ferguson et al. (1994) concluded that increased activity of choline transporters, rather than the addition of new carriers, is the reason for increased choline uptake induced by stimulation.

The exact mechanism that promotes these changes in choline transport is not well understood. It has been proposed that activation of phospholipase A2 (PLA2) might be involved. Yamada et al. (1988) reported that exposure of striatal membranes to PLA2 decreased the Kd and increased the  $B_{max}$  of [<sup>3</sup>H]HC-3 binding. This effect could be inhibited by quinacrine, a PLA2 inhibitor, or by removal of calcium (Yamada et al., 1988; Saltarelli et al., 1990). Exposure to PLA2 also increased choline uptake in striatal synaptosomes, although not to the extent of the increase in HC-3 binding. The effect was specific to PLA2 because phospholipases B, C, or D did not mimic these changes (Yamada et al., 1989). Exogenous application of arachidonic acid, one of the products of PLA2 action, increased the affinity and the number of HC-3 binding sites in striatal membranes (Saltarelli et al., 1990). Other unsaturated fatty acids, but not the saturated ones, were also effective. To explain these effects, Saltarelli et al. (1990) proposed that the activation of PLA2 during depolarization releases its second messenger, arachidonic acid, which subsequently activates the choline transporter. However, this hypothesis does not satisfactorily explain some findings. First, choline uptake by synaptosomes is inhibited by the concentrations of arachidonic acid that increased HC-3 binding (Boksa et al., 1988; Saltarelli et al., 1990). Saltarelli et al. (1990) have suggested that this might be due to destabilization of synaptosomal membranes because arachidonic acid increased the efflux of lactate dehydrogenase, a cytosolic marker. However, Boksa et al. (1988) reported no increase in ACh release in the presence of eserine and arachidonic acid, which would be expected if the synaptosomes were disrupted. Second, while depolarization only alters the Bmax for HC-3 (Saltarelli et al., 1987), arachidonic acid or PLA2 exposure increases the affinity and the Bmax (Yamada et al., 1988; Saltarelli et al., 1990). Alternatively, the possibility exists that activation of PLA2 by stimulation is a localized effect that is not replicated by applying the enzyme *in vitro*.

Other studies have implicated a role for kinases in the regulation of choline transport. The activity of the locust high affinity carrier is stimulated by intracellular kinases A and C following activation by a cyclic AMP-dependent mechanism; the change in transport velocity is correlated with increased [<sup>3</sup>H]HC-3 binding (Breer and Knipper, 1990; Knipper et al., 1992). Chatterjee and Bhatnagar (1990) reported decreased affinity of rat striatal membranes for [<sup>3</sup>H]HC-3 in the presence of ATP and that the change could be reverted back to the high affinity state by a nonhydrolyzable analog of ATP, suggesting that a phosphorylation step may be involved. In support of this, choline uptake by synaptosomes was reduced by several kinase inhibitors. These investigators suggested that extracellular ATP co-released with ACh might be in an advantageous position to modulate transporter activity in response to transmitter release. Such a role for extracellular ATP has also been put forth for the regulation of noradrenaline (NA) uptake. NA uptake is stimulated by extracellular ATP (Hardwich et al., 1989), but reduced in the presence of a nonhydrolyzable analog of ATP, as if the transporter is subject to phosphorylation mediated by an ecto-kinase (Hendley et al., 1988; Hardwich et al., 1989). If this

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mechanism is used by cholinergic systems, it might provide some mechanism to control ACh stores during stimulation. The first study presented in this thesis (section 5) was initiated to test the possibility that ATP is involved in the activation of choline uptake during neuronal activity. The initial results were consistent with this notion because perfusion of unstimulated ganglia with ATP increased their ACh content. However, subsequent results showed that effect was not specific to ATP, and appeared to be mediated by ATP's metabolite, adenosine. Consequently, the focus of the study was shifted from investigating the role of extracellular ATP in ACh synthesis to exploring further the action of adenosine.

Further study of these biochemical mechanisms is clearly required to clarify whether they operate *in vivo* and whether they represent general, species- or tissuespecific characteristics of cholinergic tissues. Also, it will be of interest to determine whether any of these mechanisms can support the rapidity of transport activation during stimulation to account for the preferential removal of synaptic choline by uptake rather than diffusion away from the site of release. An alternate possibility that may also be considered is that such regulatory mechanisms may provide enduring changes in the ability to transport choline, and, thus, ACh synthesis during short- or long-term synaptic modulation of cholinergic function.

## 2.3.3 Molecular studies upon the high affinity choline transporter

To understand molecular aspects of structure and function of the choline transport system, several attempts have been made to isolate and characterize the high affinity carrier. Tritiated choline mustard aziridinium ion, an irreversible inhibitor of high affinity choline transport, labelled a major polypeptide of molecular weight 42 and two minor ones of 58 and 90 kDa from *Torpedo* electric organ, but not in the presence of HC-3 (Rylett, 1988). Injection of *Xenopus* oocytes with poly(A)<sup>+</sup> RNA isolated from *Torpedo* electric lobe caused expression of protein that could translocate choline only in the presence of external Na<sup>+</sup> and was potently inhibited by HC-3 (ORegan et al., 1991). Size fractionation prior to injection revealed that mRNAs 2 kilobases in length were sufficient for the activity. In locust brain synaptosomes, [<sup>3</sup>H]HC-3 was found to crosslink to a single 80 kDa protein (Knipper et al., 1989b). A similar sized protein in the insect was also recognized by monoclonal antibodies which inhibited choline transport; this 80 kDa protein, upon affinity purification and insertion into liposomes, mediated the accumulation choline in a Na<sup>+-</sup> and HC-3-sensitive manner (Knipper et al., 1989a).

A cDNA clone, isolated by its homology to the family of Na+-dependent neurotransmitter transporters, is reported to encode a 635 amino acid protein (named CHOT1) of molecular weight 71 kDa that endows injected Xenopus oocytes with Na+dependent choline transport (Mayser et al., 1992). However, the choline translocation provided by this protein is insensitive to HC-3, raising some doubt as to whether it represents the high affinity transporter associated with ACh synthesis. It is unlikely that the protein was somehow modified by the expression system because the Torpedo high affinity carrier retained its sensitivity to HC-3 following expression in Xenopus oocytes (O'Regan et al., 1991). Furthermore, Guimbal and Kilimann (1993) recently reported the cloning and expression of a Na<sup>+</sup>-dependent creatine transporter that shows 98% identity with CHOT1. Interestingly, this protein neither transports choline, nor does choline inhibit creatine transport. In addition, the tissue distribution of its mRNA differs significantly from that of CHOT1, and is not specifically confined to cholinergic tissue. The reason for these differences is unclear, but the near-identical primary structure suggests that CHOT1 is unlikely to represent the high affinity choline transporter. Furthermore, Schloss et al. (1994) have recently reported that the expression of CHOT1 increases choline transport in the host cell line by only 30%, whereas creatine uptake is increased 7-8 fold. Thus, CHOT1 appears to show greater selectivity for creatine than choline, suggesting that it most likely represents a creatine transporter. Accordingly, Schloss et al. (1994) have
proposed to rename CHOT1 to CREAT1.

# 2.4 Regulation of ACh synthesis by neuronal activity

It is evident that ACh synthesis is regulated so that in resting cholinergic nerve endings ACh formation occurs at a low rate, whereas, during activity, its rate is significantly accelerated. Since extracellular ACh does not serve as a substrate for transporters on nerve endings, and because released ACh is rapidly hydrolyzed to acetate and choline, the synthesis of new ACh during periods of ACh release is necessary so as not to deplete ACh stores (Birks and MacIntosh, 1961; Collier and MacIntosh, 1969). In this way, the amount of ACh synthesized is closely related to that released. However, the mechanism that links ACh release with its synthesis is not yet clear. Although, several different hypotheses with merit have been proposed, none are entirely satisfactory on an individual basis; it is possible that they operate in combination *in vivo*.

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# 2.4.1 Regulation of ACh synthesis by ACh

A simple manner in which junctional ACh levels could be regulated is by product inhibition. This hypothesis postulates that in the absence of nervous activity, the concentration of ACh present in the cytosol is sufficient to inhibit the association of choline with ChAT to the extent that it would only replace ACh lost to intracellular degradation or to basal release. During nerve stimulation, ACh discharged by exocytosis is replenished by the transport of cytosolic ACh into vesicles. As a consequence, the local cytosolic ACh concentration is lowered enough to relieve the inhibition of ChAT, which can then restore the level of transmitter to its normal complement before the next impulse. However, maximal inhibition of ACh synthesis by ACh is not greater than 50%, at least *in vitro* (Kaita and Goldberg, 1969; Glover and Potter, 1971), and requires 10-100 mM ACh to achieve it. According to Tuček (1985), the cytosolic concentration of ACh is in the range of 0.2-0.4 mM. Thus, this mechanism is unlikely to contribute much towards maintaining a low rate of ACh production during rest.

#### 2.4.2 Equilibrium model of ACh synthesis

As described in section 2.1, the reaction catalyzed by ChAT is considered to be reversible, and may be close to equilibrium in the nerve terminals. It is, therefore, governed by the law of mass action, where perturbation of the concentration of the substrates (choline or acetyl-CoA) and of the products (ACh or CoA) will cause a readjustment of their relative levels to re-establish the equilibrium. This equilibrium model for the regulation of ACh synthesis was first suggested by Potter et al. (1968) and Glover and Potter (1971) and has been reviewed by Tuček (1984; 1985). The primary impetus for a shift in the equilibrium towards ACh synthesis is a decrease in the cytosolic ACh concentration. This could occur as a result of basal or stimulus-evoked ACh release; movement of ACh out of the cytosolic compartment and into a vesicular one during evoked ACh release shifts the reaction towards the formation of new ACh.

There is evidence, however, that appears to be inconsistent with this equilibrium model of regulation. Blockade of the removal of ACh from vicinity of ChAT by vesamicol, an inhibitor of the vesicular ACh transporter, should, according to this hypothesis, remove the driving force for ACh synthesis by maintaining the cytoplasmic transmitter concentration. Yet, ACh synthesis during preganglionic stimulation of ganglia was unaltered by the presence of vesamicol (Collier et al., 1986). The drug markedly reduced ACh release and increased ACh content by the amount that would normally have been released. These results suggest that it is unlikely that the principle of mass action guided the increased rate of acetylation of choline, as the level of cytoplasmic ACh probably increased in the presence of vesamicol. An interesting feature this work is that ACh release, *per se*, was not a requirement for increased ACh synthesis during activity.

## 2.4.3 Dependence of ACh synthesis upon high affinity choline uptake

The most accepted mechanism for regulating ACh synthesis is provided by the high affinity choline transporter. The rate of choline transport by this system is significantly enhanced by nerve stimulation, as is ACh synthesis, and the choline delivered by this mechanism is preferentially used for acetylation (see section 1.2.3). The high affinity carrier is commonly regarded as the rate limiting step in ACh synthesis and it is accepted that the capture of choline is tightly coupled to ACh synthesis. The basis for this notion comes from experiments done by Barker and Mittag (1975) who showed that acetylation of the choline analogs, pyrrolcholine and monoethylcholine, was unrelated to their affinity for ChAT, but dependent on their ability to be transported by the high affinity uptake system. It was concluded that, following their transport, the analogs are not free in the cytosol, but are delivered to ChAT for acetylation. Thus, the kinetic parameters for ACh synthesis match those of choline transport and not acetylation by ChAT, at least as reflected by properties of the soluble enzyme. Moreover, inhibitory effects of low Na<sup>+</sup> or HC-3 on choline transport correspond well to the inhibition of ACh synthesis by the same conditions (MacIntosh et al., 1956; Guyunet et al., 1973; Simon and Kuhar, 1975; Simon et al., 1976).

It is important to note, however, that increased choline transporter activity is not always associated with increased acetylation. As mentioned in section 2.3.2, the requirement for divalent cations for the activation of choline transport during nerve stimulation differs from that for the activation of the release process. Consequently, the accumulation of choline analogues can be increased by stimulation in the absence of increased ACh release when extracellular  $Ca^{2+}$  is replaced by other divalent cations (Collier and Ilson, 1977; O'Regan and Collier, 1981). If choline uptake controlled ACh synthesis under these conditions, it would be expected that ACh content would be increased. This was not so: when choline, rather than its analogs, was available during such conditions, no extra ACh was formed. Thus, it is possible that the acetylation process depends not only upon the activity of the choline transporter, but may also require that  $Ca^{2+}$  influx precede or accompany the choline entry. In support of this notion, Welner and Collier (1985) have shown that the activity-induced acetylation of diethylhomocholine by sympathetic ganglia appears to require the presence  $Ca^{2+}$  in the perfusion medium. In addition, exposure of hippocampal tissue to vasoactive intestinal peptide was shown to increase ACh synthesis in a  $Ca^{2+}$ -dependent manner; this effect did not rely upon increased choline uptake by the high affinity system, but, apparently, on increased ChAT activity (Lapchak and Collier, 1988).

Overall, these results indicate that although choline transport is critical for ACh synthesis, choline transport and acetylation may only be coupled when the depolarizing stimulation is accompanied by  $Ca^{2+}$  influx.

# 2.4.4 Regulation of ACh synthesis by ChAT

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Since acetylation can be differentiated from choline transport under certain experimental conditions as indicated above, the two processes appear not to be coupled in the strictest sense. It is likely that optimal transmitter production requires activation of both. This implies that ChAT may also have a regulatory function in ACh synthesis, a notion that does not generally receive widespread support. This stems from the fact that the level of total ChAT activity that can be observed *in vitro* following solubilization, is far in excess of that necessary to keep pace with ongoing ACh synthesis, and does not appear to be affected by activity (reviewed by Hebb, 1972). However, ChAT activity is generally evaluated with saturating substrate concentrations that are unlikely to exist *in vivo*. Thus, such measures possibly represent the rate at which the enzyme can function, not necessarily the rate at which it does at any given moment.

The lack of effective inhibitors that can function in intact cells has hindered experimentation upon the role of ChAT *in vivo*. The conditions closest to representing selective inhibition of ChAT come from temperature-sensitive *Drosophila* mutants whose ChAT activity is reduced by increasing ambient temperature. When these flies are housed at 32°C, the decrease in ACh content is closely correlated to that of ChAS activity, as if enzyme activity has some role in determining transmitter levels (Salvaterra and McCaman, 1985). We have also recently found that ACh content is similarly matched to ChAT activity in the cat SCG (Tandon, Bachoo, Weldon, Polosa, and Collier, submitted). In these experiments, the fast axonal transport in the cervical sympathetic was disrupted by a 2 hr exposure to colchicine. The resulting decrease in ganglionic ChAT activity was closely correlated to the decrease in ACh content; both were reduced by 50%. Are 4 days. This was not due to a loss of nerve endings as inspection of treated and control ganglia by electron microscopy showed no significant difference in the number of boutons or their presynaptic specializations.

In the last few years, numerous studies have reported the existence of three forms of ChAT (see section 2.1.2); the non-ionically membrane bound form, representing 5-12% of the total enzyme activity, has been suggested to have privileged access to incoming choline (Benishin and Carroll, 1981; Benishin and Carroll, 1983; Badamchian and Carroll, 1985; Eder-Colli and Amato, 1985; Rylett, 1989; Salem et al., 1993). If this form of ChAT is preferentially involved in ACh synthesis, then the argument that ChAT activity is in excess becomes irrelevant, for the membranous enzyme represents only a minor fraction of the total ChAT activity. Its substrate specificity is closer to that the acetylation machinery of synaptosomes or intact tissue such as ganglia (Collier et al., 1977; Boksa and Collier, 1980b; Benishin and Carroll, 1981) as compared to the other forms of ChAT and it shows increased activity following depolarization by veratridine (Carroll, 1987; Schmidt and Rylett, 1993a) or electrical stimulation (Mykita and Collier, unpublished observations). Furthermore, the activity of mChAT is reduced selectively by the removal of Ca<sup>2+</sup> from extracellular medium (Schmidt and Rylett, 1993a), a feature that is similar to the requirement for Ca<sup>2+</sup> for the acetylation of transported choline (section 2.4.3).

The convenient location of this mChAT at the plasma membrane suggests that it is

in close enough proximity to the choline transport system to provide some molecular basis for the kinetic coupling of transport and acetylation. This also applies to mChAT that might be associated with synaptic vesicles (Carroll, 1994): recycling vesicles might be supplied with newly-synthesized ACh by this enzyme when they are close to the choline transporters in the presynaptic membrane. Such a notion is consistent with the results of Mykita and Collier (1989) which showed that the increased ACh content following stimulation in the presence of vesamicol, a consequence of decreased ACh output but normal ACh synthesis, was prevented by picrylsulfonic acid, an impermeant agent that reacts covalently to primary amine groups, which, on its own, did not affect ACh release or synthesis. The interaction between intracellular vesamicol, bound to the vesicular ACh transporter, and extracellular picrylsulfonic acid to prevent ACh synthesis likely occurred during the process of vesicle fusion. Because choline transport was unaffected in these experiments, it was suggested that activation of mChAT was, in some way, impaired by the combination of the two drugs.

### 2.4.5 Summary of ACh synthesis control

Under various conditions, experimental or otherwise, the control of ACh synthesis in vivo may rely on more than one of the mechanisms described above for cholinergic neurons to operate efficiently. For instance, during periods of inactivity, ACh synthesis might be regulated by mass action kinetics (equilibrium model) involving, predominantly, the soluble form of ChAT, whereas during activity, the control of transmitter production could be shifted to activation of choline capture through increased transporter activity and activation of the membranous form of ChAT for increased acetylation. However, these issues remain unresolved, as are the mechanisms that govern the activation of either of these two processes during neuronal activity.

## 3. Acetylcholine storage and release

One of the key results presented in section 5 is that the exposure of sympathetic ganglia to exogenous adenosine induces an increase in their ACh content. It was of interest in that study to determine whether the additional transmitter is subsequently available for release and which intracellular compartment of transmitter it combined with. Thus, the following passages are intended to summarize the current notions about the compartmentalization, mobilization and release of ACh.

## 3.1 Quantal ACh release and the vesicle hypothesis

In the early 1950s, the notion was advanced that mammalian neuromuscular transmission occurred in a quantal manner, a consequence of multimolecular packets of ACh released from presynaptic nerve endings activating ACh receptors on postsynaptic structures and resulting in miniature end-plate potentials (MEPPs) (Fatt and Katz, 1951; Fatt and Katz, 1952; Del Castillo and Katz, 1954). The subsequent application of electron microscopy to the study of synaptic ultrastructure revealed the presence of clusters of small, clear vesicles, heavily concentrated in the vicinity of the presynaptic membrane (De Robertis and Bennett, 1955). This location for these organelles provided a simple presynaptic morphological explanation for the quantal nature of ACh action as measured by electrophysiological methods and has led to the concept that vesicles with a fixed quantity of transmitter, upon stimulation, empty their content of ACh into the synaptic cleft following fusion with the presynaptic membrane. Formally coined as the vesicle hypothesis, this notion is generally accepted by most neurobiologists, although it has not been proven conclusively. Some experimental observations that cannot be easily explained by the vesicle hypothesis has led some to assert that an extravesicular, cytoplasmic pool of ACh is the source of released transmitter (reviewed by Tauc, 1982). This 'cytoplasmic' theory of ACh release is discussed further in section 3.3.5. Most evidence concerning evoked ACh release, nonetheless, is supportive of the vesicle hypothesis, albeit a modified

version of the original, and is less compatible with the idea of nonvesicular release.

# 3.2 Storage of ACh

If the vesicle hypothesis for ACh release was to be readily accepted, it was necessary to test whether that these structures contain ACh. Biochemical studies of isolated vesicle preparations showed that to be the case; some of the ACh in nerve endings appeared to be 'bound' in vesicles following differential and density-gradient centrifugation of ruptured synaptosomes (De Robertis et al., 1963; Whittaker et al., 1964). The remaining unbound transmitter was considered to be free in the cytosol. The estimated number of ACh molecules in a quantum is approximately 6000, in general agreement with the estimated number of molecules in mammalian cholinergic vesicles (in the range of 5000-10000), giving a lumenal transmitter concentration of approximately 0.16-0.26 M (Kuffler and Yoshikami, 1975; Whittaker, 1984); this high concentration appears to be in fluid form as determined by its proton NMR spectrum. Another major component of cholinergic vesicles is ATP, also present as free solute (Dowdall et al., 1974; Füldner and Stadler, 1982); it's concentration is 10-20% of that of ACh. The release of ACh and ATP have been shown to occur with a stoichiometry that reflects their molar ratios in synaptic vesicles (Silinsky, 1975; Richardson and Brown, 1987).

### 3.2.1 Vesicular ACh uptake

ACh, a quaternary amine, does not readily cross phospholipid bilayers. Thus, synaptic vesicles require a specific transporter for the uptake of ACh into the vesicle lumen from the cytosol. Because the ACh within synaptic vesicles is concentrated approximately 1000-fold compared to the cytosol, the translocation mechanism is clearly powered by some form of thermodynamic energy. This energy is provided by a cytoplasmically oriented ATPase, stimulated by Ca<sup>2+</sup>, Mg<sup>2+</sup>, bicarbonate and ACh (Breer et al., 1977; Rothlein and Parsons, 1979; Michaelson and Ophir, 1980; Rothlein and

Parsons, 1980; Diebler and Lazereg, 1985), and is used to pump protons into vesicles to create an acidic and positively charged interior (Michaelson and Angel, 1980; Toll and Howard, 1980; Anderson et al., 1982). Active ACh transport is coupled to this proton gradient as an increase in the extravesicular pH increases both transport activity and its selectivity for ACh. Of the total ATPase activity associated with vesicles, less than half appears to be necessary for ACh transport (Yamagata and Parsons, 1989); the function of other 50-85% is not yet defined. Cytosolic ATP, but not intravesicular ATP, is the substrate for both ATPases.

The rich cholinergic innervation of the *Torpedo* electric organ has been the best source of synaptic vesicles for the characterization of the ACh transporter. Concentrative uptake of ACh can be reconstituted *in vitro* by vesicle preparations isolated from this tissue when MgATP and bicarbonate ion are provided as cofactors (Parsons and Koenigsberger, 1980). This transport is extremely sensitive to inhibition by vesamicol, which has an IC50 of ~41 nM (Toll and Howard, 1980; Anderson et al., 1983; Anderson et al., 1986), an action consistent with its presynaptic, use-dependent inhibition of neuromuscular transmission reported much earlier (Brittain et al., 1969; Marshall, 1970a; Marshall, 1970b; Gandiha and Marshall, 1973a; Gandiha and Marshall, 1973b). The drug binds to its receptor on the extravesicular portion of the ACh transporter, but apparently not at the ACh recognition site as kinetic studies indicate that inhibition is noncompetitive with respect to ACh (Bahr and Parsons, 1986; Kornreich and Parsons, 1988).

The pharmacological specificity of the ACh transporter has been examined in a series of studies *in vitro* (Bahr et al., 1992; Clarkson et al., 1992; Clarkson et al., 1993). Though it shows little selectivity for ACh as compared to its analogs, none of the classical noncholinergic transmitters (serotonin, noradrenaline, glutamate) appears to be actively transported. However, Clarkson et al. (1993) observed that noradrenaline was a competitive inhibitor of [<sup>3</sup>H]ACh binding, as was dopamine for [<sup>3</sup>H]vesamicol binding. In this respect, it is interesting that the *unc*-17 gene recently cloned from *C. elegans* is

proposed to code for the vesicular ACh transporter, ostensibly a 58 kD protein (Alfonso et al., 1993). Its sequence shows approximately 40% identity with mammalian synaptic vesicle monoamine transporter and its product appears to be localized to synaptic vesicles in cholinergic neurons. This suggests the possibility that vesicular transporters for ACh and catecholamines may have evolved from a single ancestral gene.

## 3.3 Heterogeneity of ACh stores and their contribution to transmitter release

It was first demonstrated by Collier (1969) that newly synthesized ACh was preferentially released from the cat SCG by preganglionic stimulation. This followed from the observation that stimulation in the presence of radiolabelled choline resulted in the labelling of almost all ganglionic ACh (Collier and MacIntosh, 1969). If ganglia thus treated were subsequently stimulated during perfusion without radioactive tracer, the specific radioactivity of released ACh was lower than that in the tissue shortly after the onset of activity, as if it was derived from choline that was recently transported. This conclusion, at first, appeared to be at odds with the vesicle hypothesis which predicted that pre-existing ACh would be liberated first, and later replaced by newly synthesized transmitter. Collier (1969) speculated that the new ACh might have been incorporated into vesicles close to the synaptic membrane, and, thus, available for immediate exocytosis.

#### **3.3.1 Distinct vesicular fractions**

The notion that vesicular ACh stores are metabolically and physically heterogeneous is supported by several studies (Barker et al., 1972; Zimmermann and Whittaker, 1977; Zimmermann and Denston, 1977a; Zimmermann and Denston, 1977b; Von Schwarzenfeld, 1979; Àgoston et al., 1985). At least two vesicle fractions exist in the *Torpedo* electric organ, VP1 and VP2 (nomenclature of Whittaker and colleagues), for which analogous forms can be isolated from mammalian cholinergic tissue. The VP1 vesicles have the same size and density as ACh containing vesicles in resting tissue and only slowly accept new transmitter when nerve terminals are active. The VP<sub>2</sub> vesicles appear to be derived from the VP<sub>1</sub> fraction and become apparent during stimulation, distinguished by their smaller diameter and greater density on sucrose gradients; these are considered to be located near the synaptic membrane. The smaller organelles are more metabolically active than the VP<sub>1</sub> as they rapidly and preferentially accumulate newly synthesized ACh during nervous activity. The specific radioactivity of released ACh is close to that of the VP<sub>2</sub>, and bears little resemblance to the ACh associated with the nonoccluded or VP<sub>1</sub> fractions (Suszkiw et al., 1978). Furthermore, uptake of extracellular dextran, a membrane impermeable agent, is associated with the VP<sub>2</sub> but not VP<sub>1</sub> fraction, indicating that the lumen of the VP<sub>2</sub> vesicles is exposed to the extracellular space during neuronal activity. Increasing the frequency of stimulation recruits more of the VP<sub>1</sub> to the VP<sub>2</sub> fraction.

The role of these vesicle types has been reviewed by Whittaker (1984). In this model, the vesicles of the VP2 fraction are those that support exocytotic ACh release and undergo recycling. The VP1 fraction acts as the reserve pool from which ACh needs to be mobilized for release. The vesicles of this fraction attain characteristics of the VP2 fraction with increasing activity.

An intriguing result from experiments of Gracz et al. (1988) indicates that the ratio of ACh transporter activity to vesamicol receptor of the VP2 fraction is 4-7-fold higher compared to that of the VP1 fraction, suggesting the possibility that ACh uptake by vesicles may be regulated according to their functional state. The difference in that ratio is consistent with the faster incorporation of newly synthesized ACh into the VP2 vesicles. The mechanism by which the ACh transporter might be regulated is not known, but it is possible that it undergoes covalent modification during the transition of VP1 vesicles to the VP2 pool resulting in a change in transport activity.

# 3.3.2 The readily releasable and reserve ACh fractions

The concept that ACh stores are not homogeneous was known well before it was realized that different vesicle populations existed. Kinetic analysis of evoked ACh release from the cat SCG by Birks and MacIntosh (1961) had led them to suggest that not all molecules of ACh were equally releasable. Of the total ganglionic ACh, about 15% was not released by nerve impulses. This fraction, presumably located in preganglionic axons, was termed 'stationary ACh'. The other 85%, representing the releasable portion of tissue ACh was called 'depot ACh'. This fraction, when released by activity, behaved as if it was composed of more than one compartment: 20 Hz stimulation was initially accompanied by a high rate of ACh efflux that declined exponentially during the first 5 min, after which the output stabilized to approximately 60-80% of the initial value. To account for this pattern of ACh release, Birks and MacIntosh (1961) proposed that the depot ACh consisted of a small, readily releasable compartment, approximately 15% of total ACh content, from which transmitter was first liberated and a larger, less readily releasable fraction, 70% of the total tissue stores, that contributed to the sustained, steady-state output.

Another compartment of ACh, somewhat artifactual, doubles the tissue stores when ganglionic acetylcholinesterase is inhibited by eserine. The characteristics of this pool have been studied by Collier and Katz (1971). Though the location of this 'surplus ACh' appears to be presynaptic as it is not formed by decentralized ganglia, it is not mobilized for release by preganglionic stimulation. Surplus ACh accumulates more slowly in ganglia exposed to less permeant anticholinesterases, suggesting that it is susceptible to hydrolysis by intracellular cholinesterases.

# 3.3.3 Vesamicol and the readily releasable pool of ACh

The presence of a fraction of tissue ACh with properties resembling those of the readily releasable compartment is apparent when ganglia are stimulated in the presence of vesamicol, a vesicular ACh uptake inhibitor (reviewed by Parsons et al., 1993). Vesamicol

did not affect the rate of transmitter output during the initial few minutes of stimulation, but attenuated it if stimulation was prolonged (Collier et al., 1986). Furthermore, the total evoked ACh release in those experiments amounted to some 14% of ganglionic ACh content in the presence of vesamicol independent of the rate of stimulation, a value remarkably close to the size of the readily releasable pool measured by Birks and MacIntosh (1961). Collier et al. (1986) suggested that the ACh released in the presence of vesamicol represented pre-existing transmitter bound by vesicles preferentially involved in initial transmitter output, docked at release sites or close to the synaptic membrane in anticipation of release. Once emptied of their contents, inhibition of ACh transport by vesamicol prevents re-filling. Thus, the ACh released in vesamicol's presence is considered to reflect the contents of the readily releasable fraction. Furthermore, since this compartment represents only a portion of the total vesicular ACh stores in nerve terminals, it suggests that vesamicol also impairs mobilization of ACh from reserve vesicles.

Cabeza and Collier (1988) reported that the ACh synthesized in the presence of vesamicol that was not released by nerve impulses could, however, be released from a vesicular source in the presence of black widow spider venom, the active component of which is  $\alpha$ -latrotoxin, which promotes vesicle fusion but prevents recycling. Exposure to ouabain, too, increased ACh output from ganglia whose impulse-releasable pool of ACh had been exhausted by stimulation in the presence of vesamicol (Prado et al., 1993). Thus, some vesicles, presumably constituents of the less readily releasable compartment, are less sensitive to inhibition by vesamicol and can accumulate ACh, but the presence of the drug renders them unable to transfer their transmitter to the readily releasable fraction or cannot themselves reach the limited number of release sites as those might already be occupied by empty vesicles. In accord with this is the observation that vesamicol appears to preferentially inhibit ACh uptake by recycling vesicles in the rat and snake neuromuscular junctions (Seari et al., 1990; Searl et al., 1991). If the inhibition of transmitter mobilization by vesamicol results from preventing vesicles from recycling, then the ACh liberated by

the subsequent application of spider toxin or ouabain raises the possibility that these toxins might make use of vesicle release sites not normally available during impulse-induced ACh release.

The resistance of some vesicles to vesamicol is peculiar to intact preparations as it has not been observed in preparations of isolated vesicles. In fact, the concentration of vesamicol or one of its analogs to inhibit ACh transport in isolated vesicles by half is significantly less than that required to saturate half of its binding sites (Kaufman et al., 1989). This suggests that a large portion of vesamicol binding sites may not represent functional transporters. Moreover, these functional and nonfunctional transporters might distinguish themselves by their affinity for vesamicol because Rogers et al. (1993) have recently provided evidence suggesting the occurrence of high and low affinity vesamicol receptors. It is not known whether both forms may be situated on the same vesicle or whether they are mutually exclusive.

## 3.3.4 Contribution of cholinergic false transmitters to the vesicle hypothesis

Additional support for synaptic vesicles as the source of released ACh during activity comes from studies upon choline analogs that are precursors to false transmitters. Increased accumulation of homocholine and of mono-, di-, and tri-ethyl analogs of choline is observed during stimulation of brain slices and sympathetic ganglia (Ilson and Collier, 1975; Collier et al., 1975; Collier and Ilson, 1977; Collier et al., 1977; Boksa and Collier, 1980a; Boksa and Collier, 1980b). The subcellular distribution of the acetylated metabolites of these analogs in brain tissue and their Ca<sup>2+</sup>-dependent, fractional release from ganglia, respectively, is similar to that of ACh during rest or activity (see review by Collier, 1988). In contrast, acetyldiethylhomocholine is poorly incorporated into the vesicular fraction following its synthesis from diethylhomocholine in synaptosomes and high K<sup>+</sup> does not enhance its rate of release above that of its spontaneous efflux (Welner and Collier, 1984). Similarly, acetyldiethylhomocholine is synthesized by sympathetic ganglia, but its release is unrelated to preganglionic stimulation (Welner and Collier, 1985). Altogether, these results indicate that only those acetylated molecules that meet the requirements for passage into vesicles are released from nerve terminals in an activity-dependent fashion. Exclusion from these organelles permits them a role in basal release only.

A similar conclusion is reached when a comparison is made of the molar ratios of ACh/false transmitter compartmentalized in various tissue fractions to that released by stimulation. Von Shwarzenfeld (1979) reported that the acetylated derivatives of radiolabelled pyrrolidinecholine or homocholine were incorporated into the vesicular fractions of guinea pig brain, preferentially into the denser, more metabolically active pool. During stimulation, the ratio of ACh/false transmitter was matched closely to that recovered in the dense vesicle fraction, but not the lighter vesicle fraction or the nonoccluded fraction. Interestingly, unstimulated tissue released ACh and false transmitter in a ratio that approximated the nonoccluded fraction. Comparable results were reported by Luqmani et al. (1980) after comparison of the [14C]ACh to [3H]acetylhomocholine ratio of subcellular fractions of *Torpedo* electric organ to that in the effluent. The simplest interpretation to be made from these results is that the denser, more active vesicles are the source of released transmitter during stimulation; at rest, however, spontaneous transmitter output is supported by the cytoplasmic fraction.

# 3.3.5 The mediatophore

Despite the popularity of the vesicular hypothesis and the experimental evidence in its favor, it has not yet been proven. Several groups have argued that some data cannot be easily explained by this model and have proposed that evoked ACh release originates from a cytoplasmic pool of ACh. Critical support for the idea of nonvesicular ACh release was provided by the isolation of a protein and capable of translocating ACh (Israël et al., 1984; 1986; 1988; 1990). When inserted into ACh-containing proteoliposomes, this protein (mediatophore) can facilitate the release of ACh in a Ca<sup>2+</sup>-dependent manner, thus providing strong evidence for the existence of cellular machinery that can release ACh in the absence of vesicles. The mediatophore has been isolated from a variety of cholinergic tissues: electric organ of Torpedo, mammalian brain and neuromuscular junction. It is a multimeric protein of molecular weight 210 kDa, composed of 15 kDa subunits that appear to form a pentameric channel when visualized by electron microscopy. Cloning of the 15 kDz subunit by Birman et al. (1990) revealed a sequence highly homologous to a gap junction protein, a protonophore of bovine chromaffin granules, and a subunit of the V-type ATPase found in synaptic vesicles (reviewed by Israēl and Dunant, 1993).

The importance of the mediatophore to the release process has been shown by using *Xenopus* oocytes injected with  $poly(A)^+$  mRNA from the *Torpedo* electric lobe. These oocytes subsequently express ChAT activity and choline uptake and are able to synthesize, store, and release ACh (Gundersen et al., 1985; O'Regan et al., 1991). Release from these cells, however, is reduced or abolished if antisense sequences of the 15 kDa mRNA coding for the mediatophore protein accompany the initial injection of total poly(A)<sup>+</sup> mRNA (Cavalli et al., 1993), as if the production of the mediatophore protein is critical for transmitter release.

Other studies have strengthened the argument that the mediatophore might be involved in the evoked release of ACh. Rapid freeze-fracture studies of ultrastructural changes in nerve terminals of the *Torpedo* electric organ during single nerve impulses reveal a transient increase in the number of intramembranous particles (Muller et al., 1987). The time course of the occurrence of these particles was fast and corresponded to that of the postsynaptic current. In contrast, profiles of vesicle fusion were not observed during synaptic transmission. Moreover, there was no increase in the number of these particles if stimulation was done in the presence of low extracellular Ca<sup>2+</sup>. Thus, the appearance of these particles, and not vesicle fusion, matched the rapidity of synaptic transmission. The Ca<sup>2+</sup>-induced appearance of intramembranous particles also occurs in proteoliposomes embedded with the mediatophore, suggesting that these particles represent an 'activated' form of the mediatophore molecule (Brochier et al., 1992).

To incorporate some of the evidence obtained from supporters of the vesicular hypothesis, Israël and Dunant (1993) have proposed that synaptic vesicles might be maneuvered close to mediatophore molecules by some form of docking mechanism. Calcium influx would promote ACh to flow out of the vesicles and through the pore formed by the mediatophore into the synaptic cleft. Thus, vesicles could act as the source of transmitter, but without the need for fusion with the plasma membrane. This model takes into account the shortcomings of both the vesicular and nonvesicular hypotheses: the relatively slow appearance of vesicle fusion profiles as compared to ACh release and the quantal nature of ACh release, respectively. However promising, there are several questions that need to be tested for this model to gain further acceptance. For example, do vesicles become associated with the mediatophore? Does the mediatophore allow the passage of cholinergic false transmitters across presynaptic membranes as has been observed in intact tissue? What are the structural limitations for permeants of this 'pore'? To this end, the mediatophore is reported to show 5-fold greater preference for releasing ACh as compared to ATP (Birman et al., 1986). This, however, does not explain the observation that the ratio of ACh/ATP released is similar to that in vesicles (Silinsky, 1975; Richardson and Brown, 1987). In addition, the effect of physiological stimuli (ie. electrical impulses) on ACh release by the mediatophore is not known. The tests so far have been limited to the use of the calcium ionophore A23187 because proteoliposomes, composed of artifical membranes, lack the machinery for action potentials.

# 3.3.6 Summary of ACh compartmentalization and release

Following its synthesis in the cytoplasm, ACh apparently has several possible destinations; it may remain free in the cytosol, or it can be sequestered into a pool of synaptic vesicles that make it available for immediate release in a  $Ca^{2+}$ -dependent manner

or to vesicles where it will await future mobilization for release. The factors affecting this compartmentalization are not completely understood, but it seems clear that movement into one of these compartments is not necessarily irreversible for any individual transmitter molecule, as some interchange between compartments appears to occur.

# 3.4 Modulation of ACh stores and release by activity: "Rebound ACh"

As mentioned earlier, the results presented in section 5 indicate that exogenous adenosine increases ganglionic ACh content. This effect of adenosine is reduced in the presence of dipyridamole, an inhibitor of nucleoside transport. To test whether endogenous adenosine might have some role in modulating ACh synthesis and content, a dipyridamole-sensitive component of ACh synthesis was sought (section 6). It was found that dipyridamole prevents the induction of a well-known phenomenon that occurs after high frequency preganglionic stimulation and results in an increase in the ACh content of a sympathetic ganglion. These results suggested that adenosine might be an endogenous signal that initiates the synthesis of this extra transmitter after presynaptic conditioning. The characteristics of this phenomenon and its induction by high frequency stimulation are outlined below.

Adaptive changes in the ganglionic ACh content were first reported by Rosenblueth et al. (1939). They described a reduction in transmitter content during the first few minutes of 60 Hz stimulation which gradually recovered back to normal if activity was prolonged. Moreover, cessation of the tetanic stimulation followed by a brief rest resulted in a gradual increase in ganglionic ACh content compared to the contralateral unstimulated ganglion. This was the first indication that the transmitter storage capacity of nerve terminals in the cat SCG was not normally maintained at its maximum level, but could be modulated by preganglionic stimulation. The extent of the rebound increase was, however, not quantified in that study and the phenomenon was not investigated further until some thirty years later. Subsequent studies confirmed those earlier observations and provided a quantitative description of the effect. The ACh content increases progressively to its maximal level 15-20 min after the conditioning stimuli (Friesen and Khatter, 1971; Birks and Fitch, 1974; Bourdois et al., 1974). If the conditioning is sufficiently intense, extra transmitter may accumulate during the period of activity (see fig. 1 of Bourdois et al., 1975). The extra transmitter is aptly referred to as 'rebound ACh' and is clearly the result of increased ACh synthesis following stimulation. The synthesis is associated with an activation of choline transport as there is enhanced incorporation of radiolabelled choline into ACh as well as increased accumulation of choline analogs during the rest period (O'Regan and Collier, 1981; Collier et al., 1983). Ganglia appear not to maintain the extra transmitter as it disappears from the tissue with an approximate half-life of 2 hrs without further stimulation, presumably as a consequence of intracellular turnover (Bourdois et al., 1974).

# 3.4.1 Induction of rebound ACh

Comparison of figures from different studies suggests that the extent of the potentiation is related to the duration and frequency of the preceding activity. The shortest duration of continuous stimulation tested that induces the appearance of rebound ACh is 4 min, though this requires a stimulation frequency of 60 Hz to produce an increase of 30%, as neither 5 nor 20 Hz stimulation for this duration increase ACh content significantly (Friesen and Khatter, 1971). Stimulation at 50 Hz for a somewhat longer period (15 min) increased transmitter content by only 19%, but further lengthening of the stimulation period to 60 min increased rebound ACh to 60% of the initial content (Bourdois et al., 1974). In contrast, the effect of 20 Hz stimulation following a similar protocol (60 min stimulation plus 20 min rest) was to elevate ACh stores by 40% (Birks and Fitch, 1974). Thus, induction of rebound ACh requires lengthy periods of intense activity that are unlikely to occur physiologically.

Continuous stimulation, however, is not the only form of activity which can increase ACh content, Birks (1978) found that burst-patterned stimuli, perhaps more likely to occur in vivo, also induced the synthesis of rebound ACh. He demonstrated that short trains (0.5-1 sec) of 10-80 Hz stimulation applied to the preganglionic nerve at intervals of 2-180 sec increased ACh content by 30-70% as effectively as uninterrupted stimulation. The increase in transmitter stores was proportional to the frequency and duration of the trains, with maximal potentiation reached at rates between 40-60 Hz. Interestingly, conditions that increase sympathetic discharge were also found to increase the ganglionic ACh level. Anesthetized cats breathing a hypoxic air mixture increased their ganglionic ACh content by 30%. This increase was dependent on invasion of nerve endings by preganglionic impulses, because ganglia denervated just prior to the experiment showed no change in transmitter levels (Birks, 1978). To account for this, Birks postulated that preganglionic sympathetic activity may be coded as a means of regulating ganglionic ACh content, enabling it to be responsive to environmental influences. It is revealing, in this respect, that the accumulation of rebound ACh was greatest in cats with the lowest initial ganglionic ACh content (Birks, 1977) and that housing the animals in controlled conditions for 1-2 weeks significantly decreased the variation between cats by reducing the number of animals with very high ganglionic content (Birks, 1978), as if the ganglionic ACh stores of those animals were under modulatory influence.

# 3.4.2 Compartmentalization and release of rebound ACh

Rebound ACh appears to incorporate, for the most part, into the releasable fraction of ganglionic stores. This conclusion is supported by several lines of evidence. First, after inducing the synthesis of this extra transmitter, stimulation in the presence of HC-3 causes most of the rebound ACh, along with depot ACh, to be lost (Birks and Fitch, 1974; Bourdois et al., 1974). Friesen and Khatter (1971) showed that a second bout of intense activity (60 Hz for 4 min) following that which induced the rebound phenomenon

could reduce ACh content to the original level, though it is not certain whether the rebound ACh formed all or any part of the mobilized transmitter. Low frequency stimulation (4 Hz) during the formation of rebound ACh did not affect its accumulation, but increased the rate at which it was lost (Birks, 1977).

Second, measurement of evoked ACh release following induction of rebound ACh suggests that it supplements the releasable compartment. Bourdois et al. (1974) reported increased ACh output from conditioned ganglia, but only after 2000 impulses were delivered. To explain the delay, they suggested that the extra transmitter was incorporated into the less readily releasable compartment after its synthesis and required mobilization for release. In contrast, examination of this issue by Birks (1977) did not reveal a lag in increased output; ACh release evoked by 5 Hz was enhanced in proportion to the increased store size. In neither of these studies, did the antecedent conditioning affect the rate of basal transmitter release.

Third, Collier et al. (1983) directly assessed the releasability of the extra transmitter by providing ganglia with [3H]choline during the rest period after conditioning, and, thus, allowing the incorporation of radiolabel into rebound ACh. Subsequent preganglionic stimulation released [3H]ACh with a specific radioactivity very close to that of the tissue stores, indicating that rebound ACh was as releasable as the pre-existing stores, possibly after having mixed with the pre-formed ACh.

These studies, however, do not provide information on which ACh compartment accommodates the bulk of the extra transmitter or whether it distributes equally among vesicular and extravesicular sites, which would suggest that some vesicles may normally hold less than their full potential. The compartmentalization of rebound ACh and its subsequent release are investigated in section 6.

### 3.4.3 Mechanism of induction of rebound ACh

The mechanism by which the synthesis of rebound ACh is activated is not known.

It is similar to the activation of ACh production during neuronal activity in that it requires increased choline transport activity. Enhanced transport of choline analogs is evident shortly after the tetanic activity and blockade of the high affinity choline transporter during the rest period by replacement of Na<sup>+</sup> by Li<sup>+</sup> prevents the formation of the extra transmitter (ORegan and Collier, 1981). However, the regulation of rebound ACh synthesis probably differs from that of ACh synthesis during stimulation since the latter serves only to replace lost transmitter whereas rebound ACh, by definition, augments transmitter stores well above their norm. Rebound ACh is not formed if the prior preganglionic stimulation is done in the absence of Ca<sup>2+</sup>, suggesting that impulse invasion of nerve terminals is not a sufficient trigger and that coincident transmitter release may be important (Collier et al., 1983). But transmitter release is also not the key event as transmitter release induced by high K<sup>+</sup> was unable to induce the synthesis of rebound ACh. These results suggest that both aspects of high frequency stimulation, the repetitive depolarization and the concomitant transmitter output, are critical factors.

The accumulation of the rebound increase has been tested in the presence of various pharmacological agents (Bourdois et al., 1974). Ganglia stimulated and rested following injection of atropine accumulated an equivalent amount of extra transmitter as in atropine's absence. Phenoxybenzamine partly reduced rebound ACh. In contrast, the presence of hexamethonium or tubocurarine completely suppressed the appearance of rebound ACh suggesting that ACh action at nicotinic receptors may be important in its formation. It is important to note, however, that this study employed high doses of these drugs, given by i.v. injection or applied directly onto the ganglionic tissue, which might have had effects other than on nicotinic receptors. For instance, direct inhibitory effects upon choline transport by these nicotinic antagonists have been reported (Holden et al., 1975; Simon et al., 1975); an obvious consequence of this would be the inhibition of ACh synthesis. Indeed, Bourdois et al. (1974) observed that ganglia conditioned with hexamethonium, but allowed no rest, had lost 35% of their initial ACh. Furthermore, re-

examination of this issue showed that a lower concentration of tubocurarine, sufficient to block nicotinic transmission as judged by the absence of nictitating membrane contraction during preganglionic stimulation, did not reduce the accumulation of radiolabelled homocholine during the post-stimulation rest period or the synthesis of rebound ACh (Collier et al., 1983). Thus, transmitter release, but not its action at muscarinic or nicotinic receptors, appears to be necessary for the induction of the rebound phenomenon. An additional argument against released ACh as the trigger for the synthesis of the extra ACh is that the rebound effect is clearly frequency-dependent between 10-60 Hz, whereas the amount of ACh secreted per impulse decreases progressively at stimulation rates greater than 16 Hz such that the minute output rate evoked by 16 Hz is similar to that by 64 Hz (Birks and MacIntosh, 1961). Consequently, an alternative hypothesis proposing that factors co-released with ACh might be involved in activating the synthesis of extra transmitter appears to be more plausible (see General Discussion).

## 3.4.4 Role of rebound ACh in sympathetic function

Long-lasting modification of synaptic transmission by previous impulse traffic is a feature of many synapses in the central and peripheral nervous systems of a variety of organisms; it is considered to represent the underlying basis for different forms of learning, memory and behavior. The role of rebound ACh in sympathetic function, if the phenomenon exists *in vivo*, is not known and can only be theorized. Because of the highly arborized structure of preganglionic fibers, small changes in transmitter output as a result of rebound ACh could result in enhanced transmission by increasing the recruitment of postganglionic neurons (see discussion by Birks, 1977). It may represent a means of magnifying autonomic transmission during stressful conditions to strengthen or weaken responses from various tissues. Thus, ganglia are more than simple relay stations for conveying information to peripheral organs and muscles, they represent important processing units which adapt to external conditions. Furthermore, sympathetic ganglia

might serve as useful experimental models for studying use-dependent changes in the efficacy of synaptic transmission that occur at other synapses; their internal arrangement and pre- and postganglionic nerves provide well-defined structures for investigating regulatory alterations in synaptic communication and the contribution of pre- or postsynaptic structures to such changes.

# 4. Adenosine and Neuronal function

As mentioned earlier, the three studies presented in this thesis (section 5, 6 and 7) investigate the role of adenosine in modulating the rate of ACh synthesis in a sympathetic ganglion. The following sections will therefore focus on the synthesis, storage and release of adenosine and its role in the control of neurotransmission.

Purine nucleosides and nucleotides are basic constituents of all living cells, involved in a multitude of intracellular functions ranging from energy metabolism and catalysis of biochemical reactions to storage of genetic information and cell replication. The universal character of these compounds has, in effect, hampered the elucidation of their role in neurotransmission. This difficulty has been overcome primarily by the development of pharmacological agents during the last two decades that enable the dissociation of various purine actions. However, there still exists much uncertainty about the identity of extracellular purines, their movement across biological membranes, and the mechanisms for their removal. These are all important considerations if one wishes to understand the intracellular and extracellular roles of purines.

### 4.1 A Brief History of Purines

The earliest description of the powerful pharmacological actions of adenosine on cardiovascular function was by Drury and Szent-Györgyi (1929). In the 30 years that followed, studies upon adenosine were limited to its physiological effects on various organs. Adenosine was shown to have negative inotropic and chronotropic effects on the

heart and caused potent vasodilatation in all vascular beds, except in the kidneys (reviewed by Olssen and Pearson, 1990). In the central nervous system, administration of adenosine or ATP was followed by sedation and hypnosis, suggesting direct effects on brain function (Feldberg and Sherwood, 1954; Maitre et al., 1974).

A role for purines as possible neurotransmitters was suggested on the basis of stimulation-induced efflux of ATP from sensory nerves (Holton and Holton, 1954; Holton, 1959) and the notion of regulation by adenosine of cardiac function was revived by Berne (1963) who measured the release of the endogenous nucleoside and its metabolites from cardiac tissue during hypoxic conditions. Two important reports in the early 1970s had a major impact on the study of purines. Sattin and Rall (1970) reported the involvement of adenosine cyclic 3',5'-monophosphate (cyclic AMP) as a second messenger for adenosine action and the antagonism of this action by xanthine derivatives. Burnstock (1972) put forth the purinergic hypothesis which postulated a role for ATP in non-holinergic, nonadrenergic autonomic neurotransmission. A few years later, purinergic receptors were classified into P1 and P2 subtypes on the basis of nucleoside and nucleotide potencies (Burnstock, 1978), and these were subdivided into further categories (Van Calker et al., 1979; Londos et al., 1980; Burnstock and Kennedy, 1985). The classification scheme is discussed further in section 4.5. In the 1980s, investigations into purine effects evolved to molecular characterization of adenosine and ATP receptors and the enzymes involved in purine metabolism.

# 4.2 Biochemical pathways of adenosine synthesis and degradation

Adenosine is composed of the purine b. se, adenine, linked to the 5-carbon sugar d-ribose. Phosphoesterification of the ribose by inorganic phosphate catalyzed by adenosine kinase creates the nucleotide, adenosine 5'-monophosphate (AMP); the opposite reaction is effected by 5'-nucleotidase. Sequential addition of phosphate moieties during oxidative phosphorylation or glycolysis yields the high energy compounds, adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP); the chemical energy stored in the phosphoester bonds is used by cells to drive energy-requiring reactions. It is adenosine and ATP that are generally portrayed as the extracellular regulators of physiological function, though both AMP and ADP are agonists at particular purine receptors.

## 4.2.1 Adenosine formation

Adenosine can be produced in mammalian cells following de novo synthesis of 5'inosine monophosphate (IMP) from ribose-5-phosphate. IMP is subsequently converted to AMP. However, this route is energetically taxing, requiring 5 molecules of ATP to produce the IMP, and is not considered to contribute a major fraction of intracellular adenosine (Allsop and Watts, 1983). According to Stone and Simmonds (1991), de novo synthesis of purines in humans, under normal conditions, replaces those purines that are excreted (approximately 2-4 mmol/day). This amount represents only a small portion of the purines that actually undergo metabolism each day as most purines are efficiently salvaged and recycled. Much of the endogenous purine production appears to occur in the liver and in skeletal muscle. De novo purine synthesis is an important feature of the developing brain, but appears to be quite limited in the adult (see Stone and Simmonds, 1991). Dietary purines are not generally considered to represent an important source of purines, as most of these are excreted in the form of uric acid (see Stone and Simmonds, 1991). The phosphoribosylation of adenine to AMP and the subsequent dephosphorylation to adenosine also represents a minor pathway. Fig 4.1 illustrates some of the biochemical pathways which lead to the formation and metabolism of adenosine.

### 4.2.1.1 Adenosine from the transmethylation pathway

A potentially more important source of adenosine appears to be that supplied from the transmethylation pathway. The loss of the methyl moiety from s-adenosylmethionine (SAM) forms s-adenosylhomocysteine (SAH), which is a potent inhibitor of transmethylation reactions, and consequently, must be maintained at low levels. It is cleaved by SAH hydrolase to form adenosine and homocysteine (De la Haba and Cantoni, 1959; Snyder, 1985; De la Haba et al., 1986) in a reversible reaction with an equilibrium in favor of SAH. However, the reaction normally proceeds in the direction of hydrolysis because the products are rapidly metabolized further, which, in the case of adenosine would be catalyzed by adenosine kinase to form AMP, or adenosine deaminase to form inosine.

Although during normoxic conditions this pathway may supply a third of the total adenosine (Deusson et al., 1989), there is no evidence that SAH hydrolase activity is altered by energy status or nervous activity, suggesting that its role, in the present context, is limited to provision of a basal level of adenosine (Lloyd et al., 1988). However, this reaction does have some interesting experimental applications. Accumulation of SAH by tissue during exposure to excess homocysteine provides a measure of cytosolic adenosine levels, i.e., adenosine availability is rate limiting for SAH formation (Deusson et al., 1988). During normoxic conditions, SAH accumulation by guinea pig heart increased linearly and indicated an intracellular free nucleoside concentration of about 80 nM. Hypoxic conditions resulting in accelerated SAH accumulation indicated that the adenosine concentration increased 25-fold to approximately 2 µM. A similar increase in SAH content can also be observed in cortical tissue during hypoxia or excitation with high K<sup>+</sup> in the presence of homocysteine (McIlwain and Poll, 1986). As well, exposure of brain tissue to homocysteine reduces the adenosine efflux normally observed during hypoxia or K+induced depolarization (McIlwain and Poll, 1985; Lloyd et al., 1993); it was concluded from these studies that a large portion of the adenosine released by such conditions was normally the result of intracellular formation of adenosine, not from the extracellular hydrolysis of released ATP, and that in the presence of excess homocysteine, the adenosine thus formed was "trapped" intracellularly as SAH.

### 4.2.1.2 Adenosine from ATP consumption

The major source of free intracellular adenosine appears to be that formed during ATP metabolism. As ATP consumption by a variety of cellular processes increases, ADP is formed. This nucleotide is converted by myokinase to AMP, which is subject to dephosphorylation by cytoplasmic 5'-nucleotidase, producing adenosine (Arch and Newsholme, 1978; Newby et al., 1983; Worku and Newby, 1983). The endo-5'-nucleotidase appears to exist primarily as a cytosolic protein in brain (Montero and Fes, 1982; Nagata et al., 1984). In this way, the cellular adenosine concentration is closely related to the energy status of a tissue and the balance between ATP synthesis and utilization; increased tissue workload or impaired ATP synthesis leads to an increase in the adenosine concentration (reviewed by Newby, 1984). Because this pathway is directly linked to cellular metabolism, its proportional contribution to adenosine production is increased significantly during neuronal activity in comparison to that supplied by the other pathways mentioned above.



Figure 4.1 Intracellular metabolic pathways for the synthesis and degradation of adenosine. Abbreviations: APRT, adenine phosphoribosyltransferase; ADA, adenosine deaminase; AK, adenosine kinase; ribose-5-p, ribose-5-phosphate; HPRT, hypoxanthine-guanine phosphoribosyltransferase; 5'-N, 5'-nucleotidase; NT, nucleoside transporter; PNP, purine nucleoside phosphorylase; SAH, s-adenosyl homocysteine; SAHH, s-adenosyl homocysteine hydrolase; SAM, s-adenosyl methionine.

# 4.2.1.3 Adenosine from hydrolysis of released ATP

Adenosine is also formed extracellularly following the release of ATP which is compartmentalized in synaptic vesicles (see section 3.2). The sequential action of ecto-ATPases or ecto-kinases and ecto-ADPases produces ADP and AMP (Ehrlich et al., 1986; Nagy et al., 1986; Cunha and Sebastião, 1991; James and Richardson, 1993). An ecto-5'-nucleotidase subsequently generates adenosine from the available AMP (Kreutzberg et al., 1978; Lee et al., 1986; James and Richardson, 1993). Thus, the decrease in adenosine release effected by inhibition of this enzyme is generally accepted to represent adenosine derived from extracellular ATP. In the cat SCG, the existence of 5'nucleotidase at synaptic junctions has been demonstrated by cytochemical means (Nacimiento et al., 1991). Its ready access to released nucleotides offers the potential for modulation of synaptic transmission. If one makes the assumption that mechanisms to destroy released transmitters are generally found near their sites of action in order to limit prolonged action at receptors, the enzyme may exist there for two reasons: to reduce the action of released ATP and to promote the formation of adenosine. Both may have important consequences on transmission.

### 4.2.2. Adenosine metabolism by adenosine kinase and adenosine deaminase

At least two enzymes constitute important pathways for the metabolism of adenosine. These are adenosine kinase (AK) and adenosine deaminase (ADA). AK is an intracellular enzyme that phosphorylates adenosine with high affinity ( $K_m = 0.2-2 \mu M$ ; Arch and Newsholme, 1978; see also Geiger and Nagy, 1990) but with low capacity (Phillips and Newsholme, 1979) to form AMP. Though AK is a widely distributed, its activity varies from one tissue to another. It is likely that such regional differences reflect its proportional contribution to adenosine removal in comparison to other pathways for adenosine metabolism. For example, the critical role of AK for maintaining low adenosine levels in brain is suggested by two recent studies. Adenosine efflux collected by

microdialysis from rat caudate nuclei was significantly enhanced by an inhibitor of AK activity, iodotubercidin (Sciotti and Van Wylen, 1993a). The effect of EHNA, an ADA inhibitor, on adenosine efflux was not as great. Also, experimentally-induced cortical seizure activity in rats was reduced by application of exogenous adenosine (Zhang et al., 1993); this effect of the nucleoside was mimicked to a greater extent by inactivation of AK than by inactivation of ADA, indicating a role for endogenous adenosine that is predominantly reduced by the action of AK. In cardiac tissue, however, AK is reported to be less important in controlling adenosine levels when the concentration of the nucleoside is raised (Newby et al., 1983). It is possible that enzyme activity becomes saturated with the excess substrate and its relative contribution to adenosine removal is reduced.

The other degradative pathway produces inosine following removal of the an amine moiety from adenosine by adenosine deaminase (ADA). This enzyme is present intracellularly, free in cytosol or associated with the plasma membrane, and at extracellular sites, bound to the external face of cells through the aid of a ADA-binding protein (Van der Wyden and Kelley, 1976; Phillips and Newsholme, 1979; Franco et al., 1986). In contrast to AK, ADA has a lower affinity (Km =  $17-100 \mu$ M) for adenosine and a greater  $V_{max}$  (Geiger and Nagy, 1990). Accordingly, its activity may be more important in comparison to AK when adenosine levels are increased; at low concentrations, adenosine is predominantly phosphorylated, whereas at higher concentrations, an increasingly greater proportion of the nucleoside is deaminated (Schrader et al., 1972; Lewin and Bleck, 1979). Though ADA activity is widely distributed, its immunohistochemical localization in brain is surprisingly discrete and bears some resemblance to that of adenosine transporter sites (Nagy et al., 1984; Geiger and Nagy, 1990), perhaps indicating the importance of maintaining low concentrations of extracellular adenosine at its targets. However, there appear to be species-specific variations in ADA distribution, at least when determined by immunohistochemistry (Schrader et al., 1987; Patel and Tudball, 1986; Yamamoto et al., 1987), implying that this method may not detect all forms of the enzyme. ADA activity is

effectively inhibited by 2'-deoxycoformycin (DCF) and erythro-9-(2-hydroxy-3nonyl)adenine (EHNA); these agents prolong the inhibitory actions of adenosine on neuronal firing, suggesting that ADA is necessary for metabolizing endogenous extracellular adenosine (reviewed by Geiger et al., 1991). An important physiological function for this enzyme is also clearly implied by the association of severe combined immunodefiency (SCID) disease with a deficiency of ADA (Hirschhorn et al., 1979).

Thus, as depicted in fig. 4.1, the level of adenosine found in various tissues appears to be dependent on the balance between the activities of enzymes that synthesize the nucleoside (5'-nucleotidase and SAH hydrolase) and the enzymes that inactivate it (adenosine kinase and adenosine deaminase).

#### 4.3 Nucleoside transport

A major mechanism for controlling extracellular adenosine levels, in addition to extracellular metabolism, is by cellular uptake. Since adenosine and its metabolites do not easily cross biological membranes, they are transported into or out of cells by passage through nucleoside carriers. These proteins are found in virtually all cell types, emphasizing their importance not only in regulating the amount of extracellular nucleoside, but also their role in purine salvage. Some cells do not possess the machinery for *de novo* purine synthesis and are entirely dependent upon extraneous sources, e.g. erythrocytes.

Over the last three decades, at least three separate components of nucleoside movement across membranes have been elucidated: a nonsaturable, passive diffusion mechanism, a carrier-mediated facilitated diffusion system, and a concentrative, Na+dependent mechanism. All three have been shown to exist together within individual cells; the first two mechanisms of transport are more clearly established and better understood than the latter, which was the last to be described. This is because the active transport of nucleosides appears to represent only a small component of the total nucleoside uptake and is somewhat more difficult to distinguish, requiring more elaborate experimentation. For some tissues, its significance is not yet understood.

## 4.3.1 Passive diffusion

The passive transport of nucleosides is nonsaturable and proportional to the nucleoside concentration. Because the passive transport of nucleosides tends to be sluggish, it is most unlikely that such movement plays any meaningful role in terminating the extracellular actions of the nucleosides. For experimental purposes, nevertheless, it can account for a substantial portion of the total nucleoside uptake if the extracellular nucleoside concentration saturates the carrier mediated processes (Roos and PflegGr, 1972; Plagemann and Richey, 1974). As the concentration of nucleosides is increased, the proportional contribution of passive uptake is also increased. Thus, when measurements of nucleoside transport are made, the amount of nucleoside transport that occurs in the presence of transport inhibitors is considered to represent passive diffusion. This value is subtracted from the total uptake to provide an estimate of facilitated transport.

## 4.3.2 Facilitated diffusion

The carrier-mediated, nonconcentrative nucleoside transport generally contributes the largest portion of nucleoside uptake. These transporters equalize the concentration of the nucleosides on either side of the plasma membrane, supporting efflux as well as influx. The contribution of these transporters to adenosine release is discussed below (see section 4.4.2). Generally, the intracellular pathways for metabolism of adenosine described above (phosphorylation or deamination) are sufficiently active to remove recently transported adenosine from the vicinity of the carriers by forming AMP or inosine. These modifications efficiently trap adenosine products into an intracellular pool, and maintain an inward gradient for adenosine, giving facilitated transport the appearance of a

concentrative system. Consequently, an important distinction between transport and uptake is necessary (reviewed by Plagemann et al., 1988; Hertz, 1991). Transport refers only to the passage of nucleosides through the membrane without the aid of subsequent metabolism or efflux. Thus, when one measures the transport rate of radiolabelled substrates, only the initial rate of entry reflects that of the transport process; the longer the period after exposing cells to the substrate, the greater the likelihood that it will be either metabolized or transported out. Uptake, on the other hand, refers to the amount of radiolabel accumulated without regard to metabolic processes, and, thus, its rate over the period of minutes to hours will reflect, not transport kinetics, but rather the rate at which the radiolabel is incorporated into other molecules. It was demonstrated by Kuroda and McIlwain (1973) that very quickly after transport, most of the radiolabelled adenosine is incorporated into nucleotides. Consequently, inactivation of AK significantly reduces adenosine accumulation (Shimizu et al., 1972; Gu and Geiger, 1992; Gu et al., 1993). In general, such difficulties are avoided by making measurements of adenosine accumulation after 15-60 seconds of exposure to the radiolabelled adenosine (Bender et al., 1981a; Geiger et al., 1988) or with nucleosides that are less quickly metabolized than adenosine such as uridine (Paterson et al., 1980; Woffendin and Plagemann, 1987; Lee and Jarvis, 1988b), formycin B (Plagemann and Aran, 1990b; Plagemann et al., 1990) or the Ladenosine enantiomer (Gu et al., 1991; Gu and Geiger, 1992; Casillas et al., 1993).

## 4.3.2.1 Kinetics of adenosine transport

Nucleoside transport kinetics, studied in a variety of tissues and in dissociated or cultured cells, closely follow Michaelis-Menten kinetics (Plagemann et al., 1988). The present discussion will be confined primarily to those investigations using neuronal cells; results from these are mostly in agreement with characteristics of nucleoside transport in non-neuronal tissues (reviewed by Plagemann et al., 1988). Early studies measuring adenosine uptake by mammalian synaptosomes showed an initial, rapid phase of transport and, subsequently, a slower rate that continued linearly for tens of minutes (Banav-Schwartz et al., 1980; Bender et al., 1980; Barberis et al., 1981); the latter has a Kt of about 1-20 µM. This component is saturated with adenosine concentrations greater than 10-100 µM, and abolished by low temperature, suggesting some form of facilitated diffusion. Examination of the rapid component during 30 sec incubations with radiolabelled adenosine shows a saturable uptake with a Kt for adenosine of about 1 µM and a maximum velocity 3-4 times more than that of the slower influx (Bender et al., 1981a; Bender et al., 1981b). The slower component is more sensitive to omission of sodium or calcium from the medium than the fast one, possibly reflecting its dependence on adenosine metabolism as the driving force. Approximately 70% of the adenosine accumulated after 30 sec remains unaltered, whereas 50-90% is changed after 10-15 min (Bender et al., 1980; Banay-Schwartz et al., 1980; Bender et al., 1981a). A small sodiumdependent component of nucleoside transport has been measured in rat brain (Johnston and Geiger, 1989), and it is possible that part of the decreased transport measured in these studies was not a result of decreased metabolism, but inhibition of a Na+-dependent carrier.

Extremely short-scale (15 sec) estimates of adenosine transport kinetics, during which time there is very little transformation of transported adenosine, have been reported by Geiger et al. (1988) and Johnston and Geiger (1990) using dissociated brain cells obtained from rat, guinea pig or mouse. The rapidity of these measurements was enabled by centrifugation of the transport mixture into an inhibitor cocktail consisting of the nucleoside transport inhibitors, dipyridamole and dilazep. These studies revealed that adenosine accumulation occurred with two components of high ( $K_t$ =0.3-1.5  $\mu$ M) or low ( $K_t$ =300-500  $\mu$ M) affinity. The V<sub>max</sub> of these components were quite divergent, ranging from 3-9 pmoles/mg prot/15 sec to 600-3400 pmoles/mg prot/15 sec, respectively, depending on the species. The preferred permeant for these carriers is adenosine, but related compounds are tolerated. Because the preparation utilized in these studies was a

heterogeneous population of dissociated brain cells containing neurons and glia, it is difficult to interpret whether these properties were representative of more than one transporter on one or several cell types. The kinetics of the neuronal adenosine transporter are similar to those measured in cultured astrocytes (Hertz, 1978; Lewin and Bleck, 1979), suggesting that glial cells have an uptake system not unlike that of neurons, and may represent important sites for the removal of extracellular nucleosides. However, since transport studies using immortalized cell lines containing a homogeneous population of cells suggest that individual cells can be endowed with multiple nucleoside transport systems (Belt, 1983a; Belt, 1983b; Chello et al., 1983), it seems possible that neuronal cells also have several different facilitated adenosine transporters.

## 4.3.2.2 Heterogeneity of facilitated adenosine transport

The earliest suggestion that different nucleoside transport systems could also be distinguished on the basis of inhibitor sensitivity was provided by Lauzon and Paterson (1977); they compared the amount of nitrobenzylthioinosine (NBTI) required to saturate its binding sites to that required for inhibition of nucleoside transport in HeLa cells. It was found that even after saturation of these binding sites with NBTI, a significant portion (25%) of nucleoside transport was unaffected. This remaining fraction of uptake was not effected by simple diffusion because it could be reduced by a further 100-fold increase in NBTI concentration. To explain this, Lauzon and Paterson (1977) proposed that different facilitated transporters had differential sensitivity to NBTI. This hypothesis was supported by several other studies using a variety of different cell lines which reported NBTI-sensitive and -resistant components of nucleoside transport (Belt, 1983a; Belt, 1983b; Belt and Noel, 1985; Plagemann and Wohlheuter, 1985). Inhibition by NBTI is biphasic and the proportion of NBTI-sensitive to -resistant transport varies between different cell types, irrespective of their species of origin. In some cell lines, the presence of one or the other transport system is exclusive. Both types of transport are, however, equally sensitive to
inhibition by dipyridamole as it shows a monophasic dose-response curve. The substrate specificity of these two transport systems are reported to be similar, preferring adenosine as the permeant in comparison to other nucleosides (Belt, 1983a). However, Hammond's (1991) analysis of NBTI-sensitive and -resistant transporters of Ehrlich ascites tumor cells indicates distinct substrate specificity; guanosine, cytidine, their 2'-deoxy derivatives, and 2'-deoxyadenosine showed greater affinity for the NBTI-sensitive transporter than the resistant form. This suggests that the transport mechanism might be similar, but not identical. Overall, these observations most likely reflect two independent carrier systems, although the possibility that each represents a different conformation of the same transporter cannot be ruled out.

#### 4.3.2.3 Heterogeneity of adenosine facilitated transport in neuronal cells

NBTI-sensitive and -resistant binding sites also appear to exist in neuronal cells. Binding studies indicate that [<sup>3</sup>H]dipyridamole specifically labels 2-2.5 times as many sites in guinea pig brain as does [<sup>3</sup>H]NBTI, and that NBTI is unable to inhibit more than 50% of the dipyridamole binding (Marangos et al., 1985; Marangos and Deckert, 1987; Deckert et al., 1988). It is unlikely that this is the result of more than one dipyridamole binding site per transporter because Marangos and Deckert (1987) have reported that the ratio of the B<sub>max</sub> of dipyridamole compared to the B<sub>max</sub> of NBTI varies widely between different tissues (heart 2.3; testis 1.7; lung 1.6; liver 3.3; kidney 6.7), and the distribution of labelling by [<sup>3</sup>H]dipyridamole in guinea pig brain is different from that of [<sup>3</sup>H]NBTI (Deckert et al., 1988). Furthermore, upon subcellular fractionation, the largest portion of these dipyridamole binding sites in brain appear to be as ociated with the purified synaptosomal fraction suggesting their primary function at nerve endings (Marangos and Deckert, 1987) whereas, NBTI binding sites are distributed equally between the synaptosomal and myelin fractions (Hammond and Clanachan, 1984).

These dipyridamole and NBTI binding sites appear to represent components of

functional transporters because nucleoside uptake into rat or guinea pig synaptosomes is comprised of two components whose sensitivity differs with respect to NBTI (Lee and Jarvis, 1988a; Lee and Jarvis, 1988b; Shank and Baldy, 1990). Inhibition of transport by NBTI is clearly biphasic in both species, though the rat has a greater proportion of NBTIresistant uptake than the guinea pig. Regional variations are also evident in both species: the cerebellum has a greater amount of NBTI-resistant transport than the cortex. Dipyridamole inhibits both components with equal apparent affinity. Although the transport inhibition curves for dipyridamole are monophasic in all studies, the shape of the curves vary from shallow and flat (Geiger et al., 1988; Lee and Jarvis, 1988a) to sigmoidal (Lee and Jarvis, 1988b; Shank and Baldy, 1990; Jones and Hammond, 1992), suggesting a complex interaction with the nucleoside transporter. Dipyridamole is a competitive inhibitor of NBTI binding and, conversely, NBTI competitively inhibits dipyridamole binding, suggesting an interaction with the transporter at a mutually exclusive site. However, the NBTI-resistant binding of dipyridamole is poorly inhibited by other agents that inhibit transport or by permeant substrates (Jones and Hammond, 1992) indicating that it is a noncompetitive interaction, presumably via an allosteric site. Kinetic analysis of adenosine transport in synaptosomes from guinea pig and rat cerebral cortex suggests the existence of 3 distinct facilitated nucleoside transport systems (Shank and Baldy, 1990). Two of these appear to be insensitive to NBTL and one is sensitive. The NBTI-resistant forms show high (IC50= ~5-50 nM) and low (IC50= ~120-200 nM) sensitivity to dipyridamole. Whether these pharmacological differences represent different states of the same carriers or whether they indicate individual, non-interconvertible proteins is not clear.

Various benzodiazepines have been shown to inhibit nucleoside transport (Phillis et al., 1981; Hammond and Clanachan, 1984; Shank and Baldy, 1990) and specific binding of NBTI or dipyridamole (Verma et al., 1985; Marangos and Deckert, 1987; Jones and Hammond, 1992) and some of these agents are useful in distinguishing between the two transporters. In particular, Lee and Jarvis (1988a) have demonstrated that the NBTIsensitive and -resistant transporters show stereoselectivity with respect to inhibition by two benzodiazepine stereoisomers, RO 11-3624 and meclonazepam (previously called RO 11-3128). Both these agents inhibit the NBTI-sensitive component with near equal potency (Ki = 36 and 27  $\mu$ M, respectively), but RO 11-3624 is almost 50 times more potent than meclonazepam at the NBTI-resistant transporter (Ki = 11 and 500  $\mu$ M, respectively). In the present studies (sections 5 and 6), the action of exogenous adenosine or high frequency stimulation on ACh synthesis was blocked by dipyridamole, but not NBTI, suggesting an important role for NBTI-resistant transporters in adenosine action. This interpretation was strengthened by the use of the two benzodiazepine stereoisomers, RO 11-3624 and meclonazepam, to distinguish between NBTI-sensitive and -resistant carriers. Various other agents have been reported to have Ki values between NBTIsensitive and resistant transport that differ by 2-5-fold (Hammond, 1991).

#### 4.3.2.4 Regulation of facilitated nucleoside transport

Studies by Miras-Portugal and co-workers have used chromatin cells as a model to study the modulation of adenosine transport by a variety of agents. Exposure of chromaffin cells to nerve growth factor results in a 2-3-fold increase in transport capacity per cell and a minor decrease in the affinity of the transporter for adenosine (Torres et al., 1987). Transport was also increased by the action 5'-(N-ethylcarboxamido)adenosine, ostensibly mediated by A2 receptors (Delicado et al., 1990). This effect did not appear to be the result of increased formation of cyclic AMP, because forskolin, an activator of adenylyl cyclase, reduced transport capacity. Increased transport was correlated to an increased number of NBTI binding sites, suggesting the possibility that an occult pool of transporters was mobilized. These studies suggest that the activation of these intracellular carriers may be under regulatory influence which is reflected in the number of functional molecules. Nucleoside transporters in chromaffin cells reportedly undergo a cycle of internalization and externalization, with some transporters being consumed in the process (Torres et al., 1992). Also, Liang and Johnstone (1992) have reported the existence of an intracellular pool of nucleoside transporters in mammalian red blood cells; specific binding of NBTI was 20-50% greater in lysates than in intact cells. It is possible that this process can be modulated to increase or decrease the number of functional transporters.

Several agents also reduce the amount of nucleoside transport by chromaffin cells; forskolin or a cyclic AMP analog decreased transport capacity in a concentration dependent manner, with a concomitant reduction in NBTI binding but no change in the apparent affinity of NBTI for its receptor (Sen et al., 1990). NBTI binding in homogenates was also decreased, indicating that if the transporters were internalized, they were in addition modified so as to be unrecognizable by NBTI. A similar inhibition of nucleoside transport is effected by various secretagogues; nicotinic agonists, the calcium ionophore A23187, or high K<sup>+</sup> reduced transport 20-50% (Delicado et al., 1991). Activation of protein kinase C by phorbol esters or P2y agonists or application of exogenous protein kinase C to chromaffin cell membranes reduces adenosine transport and NBTI binding, respectively (Delicado et al., 1991; Sen et al., 1993). Thus, adenosine transport and NBTI binding seem to be under the regulatory control of more than one neurotransmitter receptor and various second messenger systems.

This type of control has not been studied a great deal with respect to the NBTIresistant transport. The only such report on this matter is by Shank and Baldy (1990) who observed a significant decrease in NBTI-sensitive and -resistant adenosine transport during depolarization of rat cortical synaptosomes by veratridine or high K<sup>+</sup>. Interestingly, veratridine was more efficacious as an inhibitor of transport than was K<sup>+</sup>, even though the K<sup>+</sup>-induced stimulation caused a greater degree of depolarization. This would seem to imply that depolarization, *per se*, was not the cause of the reduced transport, but that it was probably governed by intracellular biochemical messengers. Overall, these results suggest that facilitated nucleoside transport is a highly regulated process, and that its capacity is affected by cellular activity and external agents.

#### 4.3.3 Active nucleoside transport

Concentrative nucleoside transport systems has been reported to function in many tissues; e.g. kidney (Le Hir and Dubach, 1985; Plagemann and Aran, 1990b), spleen (Darnowski et al., 1987; Plagemann et al., 1990), liver (Holstege et al., 1991), intestinal epithelium (Vijayalakshmi and Belt, 1988; Jarvis, 1989), blood cells (Plagemann and Aran, 1990a), and leukemia cells (Crawford et al., 1990; Dagnino et al., 1991b; Dagnino et al., 1991a). It is coupled to the Na<sup>+</sup> gradient across cell membranes to provide a driving force; one sodium ion is co-transported with each adenosine molecule into the cell interior (Jarvis, 1989; Dagnino et al., 1991a). Thus, concentrative uptake is sensitive to sodium replacement or to the presence of compounds such as ouabain which alter the Na+ gradient by inhibiting the Na<sup>+</sup>/K<sup>+</sup>-ATPase. Since the substrate specificity of the uptake system in various tissues are not identical, it is assumed that they represent several subtypes of the same transporter, having evolved to suit the needs of each tissue. Indeed, more than one active nucleoside transport system may exist in certain cell types (Williams and Jarvis, 1991), while others have none (Plagemann and Aran, 1990b; Plagemann and Aran, 1990a; Plagemann, 1991). The active transporters are quite insensitive to inhibitors of the facilitated nucleoside transport systems such as NBTI or dipyridamole. Consequently, active transport can be evaluated in the presence of these agents by comparing uptake in absence or presence of sodium in the assay medium.

Recently, a Na<sup>+</sup>-dependent nucleoside transporter protein of 672 amino acids was cloned from a rabbit renal cDNA library by its homology to the Na<sup>+</sup>-dependent glucose transporter (Pajor and Wright, 1992). Several Na<sup>+</sup>-dependent nucleoside transport systems have also been expressed following microinjection of oocytes with mRNA from rat jejunum (Huang et al., 1993). These techniques might allow further characterization of concentrative uptake without contamination by facilitated transport. In primary cultures of neurons, active nucleoside uptake can be observed when intracellular adenosine metabolism is inhibited (Hertz, 1991). Johnston and Geiger (1989) have also reported a Na<sup>+</sup>-dependent component of nucleoside uptake in dissociated rat brain cells. This uptake was inhibited by ouabain or by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, and showed low sensitivity to the inhibitors of the facilitative transporters, NBTI and dipyridamole. However, the Na<sup>+</sup>-dependent component represented only a minor portion of the total uptake, leaving its physiological significance unclear. It is possible that this transport, if constitutively active, is important to clear low concentrations of adenosine from the extracellular space.

Although it has not received any experimental attention as yet, an alternative role for the Na<sup>+</sup>-dependent adenosine transporter might be to mediate depolarization-induced adenosine release. There is increasing experimental support for the notion that biogenic amines and amino acid transmitters can be released by a carrier-mediated mechanism (reviewed by Adam-Vizi, 1992; Levi and Raiteri, 1993; Atwell et al., 1993). This form of release is initiated when the Na<sup>+</sup> gradient across cells membranes is decreased or reversed during depolarization. Consequently, the active transport systems coupled to the sodium gradient that normally co-transport Na<sup>+</sup> ions and their respective transmitter into the interior of the cell, may be reversed such that both substrates are transported outwards. This release process is Ca<sup>2+</sup>-independent and Na<sup>+</sup>-dependent, and shows sensitivity to inhibitors of active transport. Thus, the 'uptake' systems function as a mechanism to release cytosolic transmitter. In principle, this reversed transport could operate for any Na<sup>+</sup>-dependent carrier, including the Na<sup>+</sup>-dependent adenosine carrier.

## 4.4 Adonosine release

One of the fundamental difficulties in investigations of purinergic mechanisms has been, and continues to be, the elucidation of the origins and identities of extracellular purines. There is clearly no argument among investigators that these compounds exist outside of cells, and that they play a role in cell communication by exerting distinct pharmacological actions on various targets. The debate lies in which purines are released and the mechanism by which they are released. Accurate measurement of released nucleotides and nucleosides is confounded by their rapid extracellular metabolism and removal. Consequently, conflicting results arise when measures are made with different time scales or extracellular removal inhibited by different procedures. Additional confusion is introduced by the fact that neurons are endowed with more than one route to effect purine release, each regulated individually. These pathways are described below.

## 4.4.1 Vesicular adenine nucleotides: source of extracellular adenosine

Richardson and Brown (1987) showed that exposure of purified cholinergic nerve terminals to radiolabelled adenosine results in a rapid accumulation of the nucleoside. However, within 30 s, only about 40% of the label is recovered as free adenosine. Furthermore, after 5 min, only 10% of the label remains as free adenosine. The fate of 75% of the nucleoside is conversion to nucleotides. This rapid metabolism of adenosine suggests that the intracellular adenosine concentration is kept low, at least during periods of inactivity. Consequently, there does not appear to be a store of free adenosine that might be accessed for release during activity. Thus, the nucleoside released by neuronal activity is presumably derived from one of adenosine's metabolites. One of these is the ATP stored in synaptic vesicles (see section 3.2). Once released, this ATP is available for sequential dephosphorylation to adenosine by a series of ecto-enzymes (Cunha and Sebastião, 1991; James and Richardson, 1993). Another possible source of extracellular adenosine is from hydrolysis of diadenosine polyphosphates. These compounds, consisting of two adenosine groups connected by a string of 4 or 5 phosphate moieties (Ap4A or Ap5A, respectively), are co-stored with ATP in neuronal vesicles and in chromaffin granules and are available for  $Ca^{2+}$ -dependent evoked release (Rodriguez del Castillo et al., 1988; Pintor et al., 1991; Castillo et al., 1992). They are also released from brain

synaptosomes (Pintor et al., 1992); ATP and diadenosine polyphosphates are liberated in molar ratios that match their synaptosomal content. In addition, application of exogenous Ap4A or Ap5A inhibits hippocampal synaptic transmission by an action on A1 receptors (Klishin et al., 1994), suggesting the possibility that they are hydrolyzed extracellularly to form adenosine. However, ATP is 20 times more abundant than the polyphosphate compounds, indicating that the diadenosine polyphosphates provide only a small portion of extracellular adenosine, even when their two adenosine residues are accounted for. Most studies which have measured purine efflux have not attempted to quantify the diadenosine polyphosphates released, and, so, the present discussion will concentrate upon ATP release.

Electrical or K+-induced stimulation of cortical synaptosomes, pre-exposed to radiolabelled adenosine or adenine, increased radiolabelled adenine nucleotide and adenosine efflux in a Ca2+-dependent and tetrodotoxin-sensitive manner (Kuroda and McIlwain, 1974; Bender et al., 1981a). These compounds represented 6-10% and 45-80% of the total radiolabelled purine output, respectively. Since no inhibitors of ATPases were used in these studies, the proportional contribution of nucleotides was probably greater. Indeed, MacDonald and White (1985) found that inhibition of 5'-nucleotidase reduced evoked adenosine efflux by almost half, indicating that much of it was from nucleotides hydrolyzed after release. Using the more rapid luciferin-luciferase detection assay for ATP, White (1977) reported increased release of the nucleotide from rat brain synaptosomes as a result of depolarization by K<sup>+</sup> or veratridine. A similar Ca2+dependent, depolarization-induced discharge of ATP has been reported from hippocampal synaptosomes (Terrian et al., 1989). Richardson and Brown (1987) have demonstrated stimulation-evoked ATP release from affinity purified cholinergic nerve terminals. The ATP release in that study was Ca2+-dependent and the ratio of ACh/ATP was 9, a value significantly greater than their molar ratio in nerve terminals of ~2.5, but closer to that of the vesicular fraction (~7). Thus, at least a portion of the ATP liberated by depolarization

in the studies mentioned above originated from cholinergic nerve endings, most likely from a vesicular source.

A number of investigations have studied ATP release at the mammalian neuromuscular junction. Silinsky (1975) observed Ca<sup>2+</sup>-dependent ATP and ADP efflux during stimulation of the phrenic nerve. The nucleotides did not appear to originate from the hemidiaphragm since stimulation with tubocurarine present did not prevent their release, and direct electrical or carbachol stimulation of the muscle did not induce their release. It was concluded that the nucleotides were of neuronal origin, released together with ACh. Silinsky (1975) estimated that the synaptic ATP concentration after a single impulse might approach 80  $\mu$ M transiently.

Nucleotide released by nerve stimulation of the rat extensor digitorum muscle was shown to be frequency dependent (Smith, 1991). Surprisingly, in this study, nucleotide release was equivalent to ACh output on a molar basis. It is generally considered that cholinergic synaptic vesicles normally contain less ATP than ACh. However, there is no information upon the ratio of ACh to ATP in vesicles of this preparation, and it is possible that some of the extracellular nucleotide in this study may have originated from another source. Indeed, Cunha and Sebastião (1993) recently reported that the adenine nucleotides released during nerve stimulation of the frog sartorius muscle originated from motor nerve terminals and from the muscle they innervate. Approximately half of the total evoked nucleotide release occurred in the presence of tubocurarine; this efflux was Ca2+dependent. In addition, direct stimulation of the muscle also evoked a similar amount of nucleotide release, even in the presence of low extracellular Ca<sup>2+</sup>. Thus, two components of nucleotide release, pre-and postsynaptic, can be distinguished at amphibian neuromuscular junctions, each contributing equally. This conclusion, however, leaves unanswered as to how the postsynaptic adenine nucleotides make their way out of the cells. It is not known whether there is a specific mechanism for this movement or whether simple diffusion can account for it, nor is it clear whether it occurs at a localized site (i.e.

the endplate) or whether it is a general phenomenon over the surface of the cells.

## 4.4.2 Adenosine efflux

The second mechanism to elevate the extracellular adenosine concentration is mediated by its outward movement through bidirectional facilitated carriers following intracellular formation (refer to sections 4.2.1 and 4.3.2 for information upon intracellular adenosine production and adenosine transport, respectively). This system depends solely on the increased cytosolic concentration of the nucleoside. As mentioned above, since the concentration of free adenosine is normally very low, activity dependent increases in adenosine release must rely upon recent and local production of the nucleoside. In general, adenosine efflux that cannot be accounted for by the hydrolysis of released nucleotides (i.e. that occurs during inhibition of 5'-nucleotidase), is ascribed to this mechanism of membrane passage.

Because adenosine efflux is mediated by the same carriers that transport adenosine into cells, it, too, is sensitive to the effects of transport inhibitors. This has raised some controversy as to how agents suc!: as dipyridamole can increase the extracellular adenosine concentration (Fredholm et al., 1982; Ballarin et al., 1991; Mitchell et al., 1993; Pazzagli et al., 1993; Fredholm et al., 1994), when, in some studies, they reduce its release (Daval and Barberis, 1981; Maire et al., 1982; Jonzon and Fredholm, 1985; Sweeney et al., 1993). Newby (1986) proposed a multicompartment model to explain the actions of uptake inhibitors on adenosine influx and efflux in cardiac tissue. This model, which has recently received experimental support from the study of Fredholm et al. (1994) with rat hippocampal slices, assumes at least two cell populations: net adenosine producing cells and net adenosine inactivating cells. The net rise in cytosolic adenosine in the adenosineproducing cells results in its efflux, which is reduced in the presence of a transport inhibitor; a noncompetitive inhibitor is predicted to be more effective than a competitive one with a progressive increase in the adenosine concentration. Thus, in the absence of adenosine-inactivating cells, one would observe a net reduction of adenosine output in the presence of dipyridamole. However, the inhibition by dipyridamole of the removal of interstitial adenosine by adenosine-inactivating cells is quantitatively greater than the inhibition of the transport out of the adenosine producers, thereby, increasing the interstitial adenosine concentration in the presence of dipyridamole. An increasing contribution of metabolizing enzymes in the adenosine-forming population would effectively decrease the adenosine efflux, although at higher nucleoside concentrations, these enzymes gradually become saturated. Simply put, despite that fact that adenosine efflux is constrained by them, transport inhibitors increase the level of extracellular adenosine by limiting its metabolism following uptake by neighboring cells.

Ceballos et al. (1994) have recently shown that the activities of purine metabolizing enzymes are very different when their levels in neurons are compared to those in glia, at least when determined with primary cultures of these cells. Glia appear to have markedly greater levels of enzyme activities involved in the degradation of adenosine. These results, when combined with the scheme of Newby (1986) and Fredholm et al. (1994), suggest that neurons and glia may play the roles of adenosine-producing cells and adenosine-inactivating cells, respectively.

#### 4.4.2.1 Adenosine release induced by stimulation

Activity- or depolarization-dependent release of adenosine and its metabolites has been observed in numerous studies (Kuroda and McIlwain, 1974; Hollins and Stone, 1980b; Daval and Barberis, 1981; Bender et al., 1981a; Maire et al., 1982; MacDonald and White, 1985; Jonzon and Fredholm, 1985; Hoehn and White, 1990; Pedata et al., 1990; Lloyd et al., 1993; Pazzagli et al., 1993; Fredholm et al., 1994). In general, this release in Ca2+-dependent, though Ca2+-independent efflux can also be observed with certain secretagogues (White, 1977; Cahill et al., 1993a; Cahill et al., 1993b; Cahill et al., 1993c). Differences in the relative proportion of adenosine to its metabolites in the effluent are reported for different tissues, presumably reflecting the importance of various metabolic pathways. Lloyd et al. (1993) have shown that adenosine release from electrically stimulated hippocampal slices is reduced by more than half in the presence of homocysteine, which traps intracellularly formed adenosine as SAH (see section 4.2.1.1), consistent with a cytosolic elevation in adenosine levels induced by activity. However, it is not always obvious that adenosine, as opposed to its deaminated metabolites, is released. A portion of these metabolites are probably formed intracellularly prior to release.

The time course of evoked adenosine output is somewhat unusual; electrical or K<sup>+</sup>-induced release is slow and the bulk of the nucleoside output occurs after the end of the depolarizing stimulation (Kuroda and McIlwain, 1974; Hollins and Stone, 1980b; Jonzon and Fredholm, 1985; Eder-Colli et al., 1989; Hoehn and White, 1990; Pedata et al., 1990; Lloyd et al., 1993). It is unlikely that the appearance of adenosine in the effluent was impeded in these studies by its transit out of the tissue, because the release of other transmitters coincided with the stimulation and lengthening the stimulation period further delayed the adenosine discharge. In addition, the interval over which adenosine was released was 2-5 times longer than the duration of stimulation, and, in some instances, adenosine release was apparent some 20-30 min after the stimulus. This pattern of release clearly suggests that adenosine formation and release is distinct from that of classical neurotransmitters, and compatible with the notion that it is produced by metabolic processes that consume ATP.

Newby (1984) has applied the term 'retaliatory metabolite' to adenosine, referring to its cardiovascular role and implying that its production and release are directly related to the energy status of overworked tissue. This reference is applicable to nervous tissue as well, where its production appears to be correlated with increased workload, i.e. increased frequency and intensity of stimulation (Jonzon and Fredholm, 1985). Exposure to hypoxic conditions or metabolic inhibitors is also an effective stimulus for its neuronal release (Maire et al., 1985; Pedata et al., 1993; Fredholm et al., 1994). This release is Ca2+- independent and is reduced in the presence of homocysteine (Lloyd et al., 1993; Sciotti and Van Wylen, 1993b). Overall, these results suggest that adenosine is formed when ATP usage is increased or when ATP synthesis is reduced.

#### 4.4.2.2 Regulation of adenosine release

An additional factor in the delayed output of adenosine by activity pertains to the modulation of transporter activity by stimulation (see section 4.3.2.4). Regulated decreases in carrier velocity or capacity might reduce efflux as effectively as nucleoside influx. Thus, activation of various second messenger systems by pre- or postsynaptic neuronal activity might represent important determinants of adenosine release, themselves subject to activity dependent phenomena such as synaptic potentiation.

#### 4.4.2.3 Adenosine release from sympathetic ganglia

Information upon purine release from sympathetic ganglia is quite sparse. Kato et al. (1974) failed to detect any activity-dependent release of endogenous ATP in the perfusate from the cat SCG. Whether this result reflects the absence of release or the inability of ATP to remain unaltered as it passes from the interstitial space into the ganglionic vasculature is not clear. However, it has been demonstrated that high levels of 5'-nucleotidase exist within the synaptic areas of the cat SCG (Nacimiento et al., 1991), suggesting the possibility that the enzyme is there to reduce the concentration of extracellular nucleotides.

Studies measuring the radiolabelled purine release following exposure of ganglia to tritiated adenosine have met with some success. McCaman and McAfee (1986) reported that preganglionic stimulation of rat SCG, pre-exposed to radiolabelled adenosine, increased the Ca<sup>2+</sup>-sensitive release of radioactivity in the form of ADP, adenosine and its metabolites. Hypoxia also increased the efflux of radioactivity, and this was unaffected by low Ca<sup>2+</sup>. However, this study did not investigate whether the release originated from

pre- or postsynaptic cells. Rubio et al. (1988) showed that amphibian ganglia, pre-loaded with radiolabelled adenosine, released radioactivity in a frequency dependent manner upon stimulation of the preganglionic nerve. About 60% of the radiolabel was associated with adenosine. Interestingly, the effect of preganglionic stimulation was blocked by the addition of nicotinic and muscarinic antagonists, and mimicked by carbachol, a cholinoceptor agonist, suggesting that adenosine release was the result of ACh action. Furthermore, antidromic stimulation of the ganglia promoted the release of radiolabel. This action was frequency-dependent indicating that postsynaptic cells in sympathetic ganglia appear to possess a mechanism which enables them to release adenosine upon activation. Cultured sympathetic neurons obtained from rat SCG and pre-incubated with [ $^{3}H$ ]adenosine, released radiolabel upon stimulation with elevated K<sup>+</sup> (Wolinsky and Patterson, 1985). The tritium was associated with increased ADP, inosine, adenosine and hypoxanthine in the culture dish. However, because these cells develop processes with nerve terminal-like specializations in culture, it is difficult to establish whether that release occurs from the cells or from their terminals, or the relative contribution of each.

The results presented in section 6 suggest that endogenous adenosine might be the involved in activating the synthesis of rebound ACh after high frequency orthodromic stimulation. It seemed possible that the source of the adenosine might be the postganglionic cells, and that the synthesis of rebound ACh was the result of retrograde transmission. Thus, the experiments described in section 7 test the possibility that postganglionic cells can release a diffusible retrograde messenger to activate presynaptic ACh synthesis. This was done by testing the effect of antidromic stimulation on ACh content.

#### 4.5 Adenosine receptors

Comparison of the distinct actions of adenosine and ATP led Burnstock (1978) to propose that different receptors existed for each, termed P1 and P2, the former having greater affinity for the less phosphorylated compounds (order of potency for P1: adenosine > AMP > ADP > ATP). The P1 receptors were further delineated on the basis of opposing actions of adenosine on adenylyl cyclase activity (Londos and Wolff, 1977; Van Calker et al., 1979; Londos et al., 1980). These studies described three sites at which adenosine could interact to decrease (A1, P-site) or increase (A2) cAMP production. In addition, the A2 subtype is subdivided into A2a and A2b categories.

The A1 and A2 receptors are integral membrane proteins with their agonist binding sites facing the extracellular space, awaiting activation by extracellular adenosine. The P-site, on the other hand, has an intracellular location upon the catalytic subunit of adenylyl cyclase (Yeager et al., 1986). More recently, a novel adenosine receptor with distinct agonist potency ratios was cloned from a rat cDNA library by Zhou et al. (1992), and has been named the A3 receptor, not to be confused with the hypothetical A3 subtype proposed by Ribeiro and Sebastião (1986). Several recent reviews describe these receptors and their second messengers in detail (Jacobson et al., 1992; Curruthers and Fozard, 1993; Collis and Hourani, 1993).

#### 4.5.1 A1 and A2 receptors

Methodical studies by Londos and Wolff (Londos and Wolff, 1977) formed the basis for distinguishing adenosine receptors that required integrity of the ribose moiety (R sites). These receptors could be further differentiated by their actions on the activity of adenylyl cyclase; R<sub>i</sub> and R<sub>a</sub> inhibited or promoted, respectively, the formation of cAMP (Londos et al., 1980). About the same time, Van Calker et al. (1979) named these two adenosine receptors A1 (inhibitory) and A2 (stimulatory) receptors. Methylxanthines are competitive antagonists at both these receptors (Sattin and Rall, 1970; Daly et al., 1981; Daly et al., 1983). With an increasing number of second messenger systems associated with these receptors, agonist potency ranking is considered to be the most appropriate method of distinguishing the receptors (reviewed by Williams, 1990; Olsson and Pearson, 1990; Collis and Hourani, 1993); for A1: cyclohexyladenosine (CHA) > R-phenylisopropyladenosine (R-PIA) > N-phylicarboxamudoadenosine (NECA) > S-PIA > CGS 21680, and for A2: NECA = CGS 21680 > R-PIA > CHA > S-PIA. The A2 receptors are further subdivided into A2a (high affinity) and A2b (low affinity) forms which differ in their sensitivity to adenosine (Daly et al., 1983).

Both A1 and A2 receptors have been cloned from several species, showing high homology to each other (reviewed by Linden et al., 1991). Their primary structures show similarity to those proteins which belong to the seven transmembrane, G-protein linked super family of receptors. This is consistent with the biochemical and pharmacological studies indicating the involvement of GTP-binding proteins in their transduction mechanisms (Dolphin and Prestwich, 1995; Trussell and Jackson, 1987; Freissmuth et al., 1991). Although the most-studied second messenger system for these receptors is adenylyl cyclase, other systems such as  $K^+$  and Ca<sup>2+</sup> channels, phospholipase A2 and C, and guanylyl cyclase have also been described (reviewed by Olsson and Pearson, 1990).

#### 4.5.2 Regulation of transmitter release by adenosine action at A1 and A2 receptors

The depressant action of adenosine on transmitter release has been the subject of numerous studies over the last two decades. Increased K+ current and decreased Ca<sup>2+</sup> conductance appear to be the key effects of adenosine that lead to reduced neuronal excitability (reviewed by Stone, 1981; White, 1988; Dunwiddie, 1990; Greene and Haas, 1991). This inhibitory effect of adenosine or related analogs is not selective for any particular transmitter as the evoked release of ACh (Pedata et al., 1983; Spignoli et al., 1984; Jackisch et al., 1984), noradrenaline (Wakade and Wakade, 1978; Harms et al., 1978; Jonzon and Fredholm, 1984), dopamine (Harms et al., 1979; Myers and Pugsley, 1986),  $\gamma$ -aminobutyric acid (Hollins and Stone, 1980a; Kirk and Richardson, 1994), glutamate (Dolphin and Archer, 1983; Dolphin and Prestwich, 1995), and serotonin (Feuerstein et al., 1985) are reduced. In general, these inhibitory effects are mediated by

receptors that show a pharmacology consistent with that of the A1 receptor. In some instances, however, an inhibitory adenosine receptor with a unique agonist profile appears to be involved, and, as a result, a putative A3 receptor subtype has been proposed (Ribeiro and Sebastião, 1986).

In addition to the reduction of transmitter release, a facilitatory action of adenosine receptors on transmitter release has also been reported. By using drugs that show some selectivity between A1 and A2 receptors, it is evident that activation of A2 receptors on certain nerve endings can potentiate ACh release. Stimulation of adenosine receptors with a non-selective agonist during selective blockade of A1 sites, or by direct stimulation of A2 receptors with the selective A2 agonist, CGS 21680, increased evoked ACh release from striatal (Brown et al., 1990; Kirk and Richardson, 1994) and hippocampal (Sebastião and Ribeiro, 1992) synaptosomes, and from a neuromuscular junction (Correia-de Sá et al., 1992). Direct application of adenosine to these synapses shows a biphasic effect: at low concentrations (nanomolar range), an inhibitory component is evident, whereas higher adenosine concentrations (micromolar range), the inhibition is reduced. This observation has been explained on the basis that the A1 receptor has greater affinity than the A2 type for adenosine, and is, therefore, activated by lower concentrations. As the concentration of adenosine is increased, the facilitatory A2 receptors are also stimulated, thus counteracting the inhibitory effect. Consistent with this argument, CGS 21680, the A2 selective agonist, potentiates ACh release when applied at a low concentration, but inhibits it at higher concentration, when it is in sufficient concentration to act upon A1 receptors (Correia-de Sá et al., 1992). Thus, it is apparent that the effect of adenosine upon evoked transmitter release at nerve terminals that possess both A1 and A2 subtypes will be a balance of the effects of both receptors. However, it is important to note that in none of these studies was adenosine shown to potentiate ACh release, because the A1 effect was always predominant. This suggests that the physiological role of the A2 receptor, at least with respect to transmitter release, might be to limit the extent of presynaptic inhibition produced by the A1 receptor. In this manner, the effect of endogenous adenosine on transmitter release could be maintained within certain limits established by the relative proportions of A1 and A2 receptors. This, however, does not preclude other independent actions of the A2 receptors on nerve terminal function that could be mediated by a variety of second messengers.

## 4.5.3 The P-site

Londos and Wolff (1977) labelled this receptor the P-site because of the requirement that its agonists contain an intact purine ring to induce inhibition of adenylyl cyclase. This site is accessible to intracellular, but not extracellular, adenosine (Haslam and Rosson, 1975). Its physiological relevance to adenosine action is unclear. However, because of the possibility that the effect of adenosine on ACh synthesis described in this thesis is mediated through an intracellular site, it is important to discuss some of the studies that have investigated the P-site.

One of the earliest reports that suggested an action of adenosine at an intracellular site was by Haslam and Rosson (1975) who showed that exposure of human *j*-atelets to adenosine activated adenylyl cyclase at low concentrations (less than 20  $\mu$ M), but inhibited activity at higher levels (more than 40  $\mu$ M). The inhibitory phase could be alleviated by the addition of nitrobenzylthioguanosine, a nucleoside transport inhibitor, as if adenosine needed to be transported into the cells prior to its inhibitory action. The pharmacology of this site has been studied in several subsequent reports. In contrast to A1 and A2 receptors, adenosine action at the P-site is not antagonized by methylxanthines or mimicked by A1 and A2 agonists (Londos and Wolff, 1977; Florio and Ross, 1983; Yeager et al., 1986). Studies with purified adenylyl cyclase suggest that the P-site is located on the catalytic subunit, but not at the ligand binding site, because adenosine inhibition is noncompetitive with respect to ATP (Florio and Ross, 1983; Yeager et al., 1986; Johnson et al., 1989; Marone et al., 1990). The rank order of potency of various

adenosine metabolites to inhibit cAMP production via the P-site is as follows: 2',5'-dd-3'AMP > 2'-d-3'AMP > 2',5'-dd-adenosine > 3'AMP > 2'-d-adenosine > adenosine, where dd and d refer to dideoxy or deoxy derivatives, respectively (Johnson et al., 1989; Bus'afield and Johnson, 1990). Prior activation of adenylyl cyclase by forskolin or other stimulators greatly enhances the potency of these agents. It is generally assumed that because the IC50 of adenosine is quite high (~80  $\mu$ M), it is unlikely to act as a regulatory component through this site, and, consequently, its deoxy derivatives are considered to be more important physiological agonists. Nevertheless, it is possible that transient elevations in adenosine concentration may affect adenylyl cyclase activity, particularly when the enzyme is activated and the potency of adenosine at the P-site is increased. Overall, the physiological role of this P-site in adenosine action awaits further clarification. This could be greatly facilitated by the discovery of a specific antagonist for the site.

#### Statement of the Problem

As mentioned in the Introduction, the synthesis of ACh by cholinergic nerves is tightly regulated so as to keep pace with the fluctuations of ACh release during rest and during neuronal activity. Because released transmitter is quickly replenished by the synthesis of new ACh, the ACh content of cholinergic nerve endings does not vary greatly. There is no clear consensus in the literature upon the mechanism that governs the rate of the ACh synthetic machinery. Because the rate of choline uptake is correlated with the rate of ACh synthesis, and pharmacological blockade of high affinity choline transporter results in a similar inhibition of ACh synthesis, at least part of the control of ACh synthesis appears to dictated by the activity of the high affinity choline transporter. This premise, however, has a similar limitation in that the mechanism that increases choline transport for ACh synthesis is not well-understood.

A simple manner in which choline transport could be controlled by the release process is that one of the released substances acts upon the transporter to initiate transport. Chatterjee and Bhatnagar (1990) have provided some evidence to suggest that ATP is involved in a phosphorylation-dependent conversion of HC-3 binding sites from a high- to a low-affinity state. The possibility that these affinity states represent functional states of the choline transport suggest that ATP, co-released with ACh, might determine the level of activation of choline transport, and, consequently, ACh synthesis. Some evidence for this form of control of transporter activity is reported for the noradrenaline carrier (Hendley et al., 1988; Hardwich et al., 1989). These studies showed that noradrenaline uptake was increased by ATP and reduced by a nonhydrolyzable analog of ATP, as if a phosphorylation step mediated by an ecto-kinase activated the transporter.

The initial experiments tested the possibility that exposure of sympathetic ganglia to ATP might increase their rate of ACh synthesis as a result of increased choline uptake. It was predicted that if extracellular ATP could increase ACh synthesis when nerve endings were not actively releasing transmitter, the transmitter content of the tissue would be increased accordingly. Thus, ganglia were perfused with a medium containing ATP and the ACh content was measured. The preliminary results were consistent with the notion of increased ACh synthesis induced by ATP. However, as the effect was characterized further, it became apparent that the ATP metabolite, adenosine, was responsible for the activation of ACh synthesis. Such an action of adenosine had never been reported before. Consequently, the following studies examine the role of adenosine in modulating ACh synthesis and synaptic transmission. The specific questions asked were: i) What is the source of choline for the increased ACh synthesis? ii) What is the pharmacology of the adenosine effect? iii) Is the extra transmitter releasable, and if so, in which ACh compartment is it stored? RESULTS

# Increased Acetylcholine Content Induced by Adenosine in a Sympathetic Ganglion and its Subsequent Mobilization By Electrical Stimulation

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Abbreviations used: ACh, acetylcholine; NBTI, nitrobenzylthioinosine; ChAT, choline acetyltransferase; TCA, trichloroacetic acid; TPB, tetraphenylboron; HC-3, hemicholinium-3; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; AMP, adenosine 5'-monophosphate; Appcp, By-methyleneadenosine 5'-triphosphate; PCh, phosphorylcholine; vesamicol, 2-(4-phenylpiperidino)cyclohexanol; meclonazepam, (S)-5-(2-chlorophenyl)-1,3-dihydro-3-methyl-7-nitro-2H-1, 4-benzodiazepin-2-one; RO 11-3624, (R)-5-(2-chlorophenyl)-1,3-dihydro-3-methyl-7-nitro-2H-1, 4-benzodiazepin-2-one.

## 5.1 Abstract

The present study was initiated to examine the effects of ATP on acetylcholine (ACh) synthesis. The exposure of superior cervical ganglia to ATP increased ACh stores by 25%. but this effect was also evident with ADP, AMP, and adenosine, but not with Bymethylene ATP, a nonhydrolyzable analog of ATP, or by inosine, the deaminated product of adenosine. Thus, we attribute the enhanced ACh content caused by ATP to the presence of adenosine derived from its hydrolysis by 5'-nucleotidase. The adenosineinduced increase of tissue ACh was not the consequence of an adenosine-induced decrease of ACh release. The extra ACh remained in the tissue for more than 15 min after the removal of adenosine but it was not apparent when ganglia were exposed to adenosine in a Ca2+-free medium. Incorporation of radiolabelled choline into [3H]ACh was also enhanced in the presence of adenosine suggesting an extracellular source of precursor. Moreover, the synthesis of radiolabelled forms of phosphorylcholine and phopholipid was not reduced in adenosine's presence, suggesting that the extra ACh was not likely derived from choline destined for phospholipid synthesis. Aminophylline did not prevent the adenosine effect to increase ACh content; it was blocked by dipyridamole, but not by nitrobenzylthioinosine (NBTI). In addition, two benzodiazepine stereoisomers known to stereoselectively inhibit the NBTI-resistant nucleoside transporter displayed a similar stereoselective ability to block the effect of adenosine. Together, these results argue that adenosine is transported through a NBTI-resistant nucleoside transporter to exert an effect on ACh synthesis. The extra ACh accumulated as a result of adenosine's action was releasable during subsequent preganglionic nerve stimulation, but not in the presence of vesamicol, a vesicular ACh transporter inhibitor. We conclude that the mobilization of ACh is enhanced as a result of adenosine pretreatment.

Key words: Acetylcholine; nucleotide; adenosine; NBTI-resistant nucleoside transport. Running title: Increased ACh Content Induced by Adenosine

## **5.2 Introduction**

It is clear that choline transport into cholinergic nerve terminals is regulated in response to activity. Thus, stimuli that induce the release of acetylcholine (ACh) from nerve terminals also increase choline uptake (see review by Collier, 1988). This activation of choline uptake is considered important in regulating ACh synthesis (see also review by Tuček, 1985), but the mechanism by which neuronal activity or transmitter release activates choline transport is not well understood. The present experiments were initiated as an attempt to study this item by testing the hypothesis that extracellular adenosine 5'-triphosphate (ATP) levels regulate choline uptake.

This notion of a potential role for ATP in regulating choline transport was formulated on the basis of its effect on the affinity of the binding of hemicholinium-3 (HC-3) to the choline transporter (Chatterjee and Bhatnagar, 1990); they showed that ATP induced a low affinity state HC-3 binding site and proposed that this represented a necessary prerequisite for the activation of choline uptake. A somewhat similar mechanism might also control other neuronal uptake processes: an effect of ATP to increase norepinep<sup>th</sup>: ine uptake is evident from studies with rat cortical synaptosomes and PC12 cells (Hendley et al., 1988; Hardwich et al., 1989). Thus, these studies suggested the possibility that the co-release of the nucleotide with the neurotransmitter at cholinergic nerve terminals (see review by Stone, 1981) acts presynaptically to activate the synthesis of new ACh, thereby enabling the cells to match synthesis with release.

To test that hypothesis, we perfused the cat superior cervical ganglion with ATP and, indeed, measured increased ACh content. However, further investigation revealed that the effect of the nucleotide was likely due to its metabolite, adenosine. Thus, our subsequent experiments had two objectives: (i) to characterize the effect of adenosine on ACh content and (ii) to test whether the extra ACh was incorporated into a releaseable pool of transmitter. The evidence we present here leads us to propose a novel mode of action of adenosine: the nucleoside is transported to an intracellular site through a nitrobenzylthioinosine-resistant nucleoside transporter to stimulate an increase in the synthesis of a releasable store of ACh. To our knowledge, this is the first report of such an influence of adenosine on neurotransmitter synthesis and release in an intact preparation. A preliminary account of this work has been presented in abstract form (Tandon and Collier, 1991).

#### 5.3 Materials and Methods

Materials used: [y-32P]ATP (10 Ci/mmol), [3H]choline chloride (80 Ci/mmol), [3H]acetylcholine iodide were purchased from New England Nuclear (Boston, MA, USA): hemicholinium-3 hydrate and butyronitrile were obtained from Aldrich Chem. Co. (Milwaukee, WI, USA); acetylcholine chloride was from Hoffmann-LaRoche (Basel, Switzerland); meclonazepam and RO 11-3624 were generous gifts from Hoffmann-LaRoche (Mississauga, Canada); EGTA was obtained from Eastman Kodak (Rochester, NY, USA); aminophylline was from Research Biochemicals Inc. (Natick, MA, USA); Amberlite CG-400 (chloride form), choline chloride, ATP, ADP, AMP, adenosine, inosine, By-methylene ATP, dipyridamole, nitrobenzylthioinosine, tetraphenylboron (sodium salt),  $\alpha$ -chloralose, acetylcholinesterase (EC 3.1.1.7, ACh hydrolase, type V-S), choline kinase (EC 2.7.1.32, ATP:choline phosphotransferase), and physostigmine sulfate were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vesamicol was a gift of Dr. S.M. Parsons (University of California at Santa Barbara) or was synthesized by Dr. T.H. Chan (McGill University) by reacting 4-phenylpiperidine (a gift of Dr. M. Peel, Glaxo) with cyclohexene oxide. OptiPhase HiSafe II and all other chemicals and reagents were obtained from Fisher Scientific (Montreal, Que., Canada).

#### 5.3.1 Experimental procedures

Anaesthesia was induced in cats of either sex (1.5-4.0 kg) with a 2:1 mixture of N<sub>2</sub>O/O<sub>2</sub> with 2% halothane and maintained with an i.v. injection of  $\alpha$ -chloralose (80mg/kg). Surgical preparation of the superior cervical ganglia was done according to the method of Kibjakow (1933) as described by Collier and Kwok (1982). All animal use procedures were in strict accordance with the guidelines of the Medical Research Council of Canada and the Canadian Council on Animal Care and were approved by a the local Animal Care Committee. One or both ganglia were perfused (0.2-0.4 ml/min) with a Krebs

solution of the following composition (mM): NaCl 120, KCl 4.6, CaCl<sub>2</sub> 2.4, KH<sub>2</sub>PO4 1.2, MgSO4 1.2, NaHCO<sub>3</sub> 25, and dextrose 10. Choline chloride (10  $\mu$ M) was always added, unless indicated otherwise. The medium was filtered and subsequently equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> to maintain a pH of 7.4 at 37°C. In some experiments, the perfusion medium also contained one or more of the following: [3H]choline chloride (155-240 mCi/mmol), physostigmine sulfate (15  $\mu$ M), hemicholinium-3 (10  $\mu$ M), or vesamicol (10  $\mu$ M). In Ca<sup>2+</sup>-free experiments, CaCl<sub>2</sub> was omitted from the Krebs solution and EGTA (0.1 mM) was added.

To test the effects of purines or inhibitors on ACh synthesis, the drugs were added to the Krebs solution just before perfusion. In such experiments, the ganglia were not stimulated during exposure to the drugs. These ganglia, and the untreated contralateral ganglia, were excised and each placed in 1 ml of 10% trichloroacetic acid (TCA) for 1 hour on ice. The TCA was removed by extraction with water-saturated ether and the aqueous layer was assayed for ACh content.

When measuring evoked release of ACh, ganglia were stimulated via their preganglionic sympathetic trunk with square wave pulses (8-10V, 0.5ms, 5 or 20 Hz) using a platinum electrode. During periods of prolonged stimulation, the electrode was moved a few millimeters proximally along the nerve every 10 minutes. The perfusate was collected in 2 or 5 minute periods into pre-chilled tubes and stored on ice for not more than one hour. Samples were stored overnight at -20°C for assay the following day.

# 5.3.2 Measurement of ACh, [3H]ACh, [3H]phosphorylcholine and [3H]phospholipid

ACh and labelled ACh were obtained from the aqueous tissue extracts and the perfusate samples by the method of Fonnum (1969) as described Welner and Collier (1984). Briefly, the samples were extracted by 400  $\mu$ l of tetraphenylboron in butyronitrile (TPB/butyronitrile; 10 mg/ml). The choline esters were removed from the organic phase by 150  $\mu$ l of AgNO3 (20 mg/ml); excess silver was precipitated with 10  $\mu$ l of MgCl2 (1

M). Samples were lyophilized to dryness.

The ACh content of the samples was determined according to the radioenzymatic method of Goldberg and McCaman (1973). Choline was phosphorylated by choline kinase and ATP. ACh was hydrolyzed to choline with acetylcholinesterase and phosphorylated with  $[\gamma$ -32P]ATP. The phosphorylated choline was separated from the labelled ATP by anion-exchange chromatography and the radioactivity measured.

The labelled ACh content was measured by incubating the dried samples with choline kinase and ATP. The reaction was stopped with cold water and the labelled choline esters were extracted with TPB/butyronitrile (10 mg/ml) leaving the phosphory-lated choline in the aqueous phase. Aliquots of the organic layer were measured for radio-activity. Tritiated standards were processed in parallel to correct for recovery of labelled products.

The incorporation of radiolabelled choline into phosphorylcholine was estimated by measuring the tritium content in the aqueous phase after the initial tissue extract (TCA-soluble) was extracted with TPB/butyronitrile; most of the radioactivity in this fraction is in the form of phosphorylcholine (Collier and Lang, 1969). Radiolabelled phopholipid content was determined by extraction of TCA-insoluble tissue tritium with 2:1 methanol/choloroform (Collier and Lang, 1969).

Radioactivity was measured by liquid scintillation spectrometry. 32P and 3H were measured in 10 or 5 ml OptiPhase HiSafe II with an counting efficiency of 99% and 30%, respectively.

## 5.3.3 Statistical analysis

All statistical comparisons were done using two-tailed Students t-test for paired samples. This analysis was considered appropriate because all comparisons were between the two ganglia of each animal: one was used as the test preparation and its contralateral served as control. The values are presented as percent or amount of change; each such value was calculated for each pair of ganglia.

## 5.4 Results

## 5.4.1 Effects of purine compounds on ACh stores

The initial experiments were designed to test if extracellular ATP could increase ACh content (see Introduction). Thus, we exposed ganglia to choline and measured the effect of ATP on total tissue ACh. Since the two superior cervical ganglia of a cat normally contain similar amounts of ACh (Feldberg, 1943; Birks and MacIntosh, 1961), the contralateral ganglion enabled an estimate of the initial ACh content of the test ganglion. Ganglia exposed to control medium showed no change in total ACh content, whereas stores were increased by  $400 \pm 100$  pmoles during a 45 min exposure to ATP (figure 5.1).



Figure 5.1: Effect of ATP on ganglionic ACh stores. Ganglia were perfused with a Krebs containing solution no ATP (hatched bar) or 100 µM ATP (solid bar) for 45 minutes. The contralateral ganglia in each experiment were excised without perfusion and assayed for ACh Bars represent ACh content. stores of the perfused ganglion as a percent of the contralateral ganglion value (mean contralateral ACh content was  $1668 \pm 143$ pmol). Each bar represents the mean  $\pm$  SEM of 4 experiments, \*p<0.05.

To test whether this apparent effect of ATP to increase ACh content was an ATP effect, or was likely caused by its hydrolysis products, a nonhydrolyzable analog of ATP and metabolites of ATP were tested for their effect on tissue ACh content. Ganglia were perfused with Krebs medium containing 100  $\mu$ M solutions of  $\beta\gamma$ -methylene ATP (Appcp), ADP, AMP, adenosine, or inosine (table 5.1). Appcp did not significantly change the ACh

stores. The less phosphorylated metabolites of ATP, with the exception of inosine, increased ACh stores by  $350 \pm 85$  pmoles (20-25%). These results suggest that adenosine, which can be made available by the action of ecto-nucleotidases on ATP but not from Appcp, is likely the active compound. Thus, the rest of the experiments reported here focus on the effects of adenosine on ACh content.

<u></u>	Treatment	<u> </u>	Percent of Contralateral
Control		7	$104 \pm 4$
Аррср		6	109 ± 7
ADP		4	122 ± 7*
AMP		5	119 ± 11 <sup>a</sup>
Adenosine		5	124 ± 7*
Inosine		4	$105 \pm 5$
Adenosine	(no Ca <sup>2+</sup> ; EGTA)	6	$111 \pm 5$
Adenosine	(1 μM)	3	91 ± 8
Adenosine	(10 μM)	3	96 ± 7
Adenosine	(250 µM)	3	$120 \pm 7^*$

Table 5.1 Effects of purines on ACh content

Ganglia were perfused with compounds indicated for 45 minutes. Results are expressed as percent  $\pm$  SEM of the ACh content of the unperfused contralateral ganglion for each individual experiment. The purine concentration in these experiments was 100  $\mu$ M, unless otherwise indicated. The mean contralateral ACh content in these experiments was 1583  $\pm$  62 pmol.  $\pm$  p<0.05, a 0.05<p<0.1.

To test whether the accumulation of the extra ACh is dependent on extracellular calcium, six ganglia were exposed to adenosine in Ca<sup>2+</sup>-free medium (with EGTA and no added calcium). The increase in ACh stores was  $111 \pm 68$  pmoles, much less than that measured in the presence of adenosine in Ca<sup>2+</sup>-containing medium (table 5.1) and the change did not reach statistical significance (0.2>p>0.1).

Three other concentrations of adenosine were tested for their effect on ACh content (table 5.1); the nucleoside had no significant effect on ACh stores at either 1  $\mu$ M

or 10  $\mu$ M concentrations and 250  $\mu$ M did not increase ACh stores to a greater extent than did 100  $\mu$ M adenosine. Thus, the effect does not have a clear dependence on concentration, although our tests were limited.

The ACh content of ganglia was measured after they were treated with 100  $\mu$ M adenosine for 45 minutes and then without adenosine for 15 minutes or 75 minutes to provide some information about the stability of the extra ACh. The extra ACh was still present 15 minutes after four ganglia were treated with adenosine; ACh stores of the test ganglia were 125 ± 5% of the contralateral ganglia. However, in two further experiments, only about half of the extra ACh remained 75 minutes after adenosine exposure; the extra ACh measured 11% and 12% of the contralateral ACh content.

Adenosine could increase the ACh content of tissue as a consequence of a decreased ACh release. To test this point, AC:: efflux was measured from ganglia in the absence and presence of adenosine. Basal ACh output from ganglia perfused with control medium (Krebs buffer with 10  $\mu$ M choline and 15  $\mu$ M physostigmine) was 3.8 ± 0.6 pmoles/min and in the presence of 100  $\mu$ M adenosine it was 3.9 ± 0.8 pmoles/min (n=5) indicating that adenosine did not affect basal ACh release. Thus, the increased ACh content of ganglia that results from exposure to adenosine appears due to an increase in the rate of ACh synthesis rather than a decrease in the rate of ACh release.

If adenosine increases ACh synthesis, this could be by increasing the acetylation of endogenous choline or by increased utilization of extracellular choline. To distinguish between these possibilities, ganglia were perfused with 100  $\mu$ M adenosine and [<sup>3</sup>H]chcline (10  $\mu$ M; 155 mCi/mmol) for 45 min while the contralateral control ganglia were perfused with [<sup>3</sup>H]choline alone. As shown in table 5.2, total ACh and [<sup>3</sup>H]ACh were both increased by adenosine and the change was similar for both measures suggesting that the source of choline for the adenosine-induced increase in ACh content was probably mostly extracellular. Furthermore, this result confirms that the extra ACh accumulated in adenosine's presence was indeed the result of increased synthesis, not reduced turnover. Choline captured by nerve terminals can be metabolized by either of two pathways: incorporation into ACh, or into phosphorylcholine (PCh) leading to phospholipid synthesis. To address the possibility that adenosine might inhibit the latter route and shunt the extra choline for conversion into ACh, we measured the effect of adenosine on the incorporation of  $[^{3}H]$ choline into PCh and phospholipid. The adenosine certainly did not reduce incorporation of  $^{3}H$  into these choline metabolites; while the incorporation of radiolabelled choline into PCh or phospholipid appeared to be increased to a similar extent as that of ACh (about 20%; table 5.2), these changes did not reach statistical significance (0.2>p>0.1). These results demonstrate that the adenosine-induced increase in ACh synthesis was not at the expense of the synthesis of PCh from choline. In these experiments, the total  $[^{3}H]$  incorporation for test and control ganglia was  $498 \pm 42 \times 10^{3}$ dpm and  $439 \pm 29 \times 10^{3}$  dpm, respectively (0.05 .

	Percent of control
ACh (pmol)	$124 \pm 4^*$
[ <sup>3</sup> H]ACh (dpm)	120 ± 5*
[ <sup>3</sup> H]Phosphorylcholine (dpm)	$121 \pm 12^{a}$
[ <sup>3</sup> H]Phospholipid (dpm)	$119 \pm 12^{a}$

 Table 5.2 Incorporation of [<sup>3</sup>H]choline in the presence of adenosine

Results are expressed as the percent  $\pm$  SEM of the values measured in the contralateral gauglion for each separate experiment (n=5); \*p<0.05, \*0.1<p<0.2. The mean values for the contralateral ganglia were: for ACh content 1514  $\pm$  134 pmoles, for [<sup>3</sup>H]ACh 47  $\pm$  6.7 x 10<sup>3</sup> dpm, for [<sup>3</sup>H]PCh 195  $\pm$  17 x 10<sup>3</sup> dpm, and for [<sup>3</sup>H]phospholipid 108  $\pm$  34 x 10<sup>3</sup> dpm.

## 5.4.2 Pharmacology of adenosine-induced increase in ACh synthesis

An adenosine-induced increase in the synthesis of ACh has not been described previously and, thus, it was of interest to characterize this phenomenon. Extracellular adenosine is known to mediate its effects through two different types of cell surface receptors, A1 and A2, and both are blocked by aminophylline. The action of adenosine is terminated by its uptake into cells; two mechanisms exist, one is blocked by nitrobenzylthioinosine and both by dipyridamole. Thus we tested the effects of adenosine in the presence of these drugs. Ganglia were exposed for 45 minutes to adenosine (100  $\mu$ M) and one of the following drugs: aminophylline (100  $\mu$ M), dipyridamole (10  $\mu$ M), or nitrobenzylthioinosine (NBTI; 100  $\mu$ M). The results are summarized in figure 5.2. Aminophylline had no effect on the adenosine-induced increase in ACh stores; ganglionic ACh content was increased by 333 ± 80 pmoles over the initial content. The effects of the nucleoside uptake inhibitors on the accumulation of the extra ACh were surprising. In the presence of dipyridamole, adenosine did not increase the ACh store size, but the adenosine-induced increase of ACh content was not blocked by NBTI; ACh content increased by 350 ± 40 pmoles in the presence of adenosine and NBTI, not different from the group that was exposed to adenosine alone.



Figure 5.2: Pharmacology of adenosine-induced increase in ACh synthesis. Ganglia were pefused with a Krebs solution for 45 minutes containing adenosine (100  $\mu$ M) or adenosine with one of the following: aminophylline (100  $\mu$ M), dipyridamole (10  $\mu$ M), NBTI (100  $\mu$ M), RO 11-3624 (100  $\mu$ M), or meclonazepam (100  $\mu$ M). Bars show the change in ACh stores as a percent of the unperfused contralateral ganglion value (mean contralateral ACh content was 1531 ± 74 pmol). Bars represent mean ± SEM of 4 to 6 experiments, \*p<0.05.

The possibility that dipyridamole might inhibit ACh synthesis by affecting processes c her than nucleoside transport and, thus, offset the synthesis of the extra ACh in the presence of adenosine was tested. Five ganglia were exposed to 10  $\mu$ M dipyridamole alone for 45 minutes; the ACh level in the presence of dipyridamole (1555 ± 58 pmoles) was maintained at a level similar to that of the contralateral ganglion (1502 ± 100 pmoles), suggesting that the effects of dipyridamole are limited to an antagonism of adenosine.

These results suggest a novel mode of action for adenosine; uptake of the purine through dipyridamole-sensitive, NBTI-resistant nucleoside transporters to an intracellular site which stimulates the synthesis of ACh. To test this notion, we used two benzodiazepine stereoisomers which affect the NBTI-sensitive and -resistant transporters differentially. The benzodiazepine meclonazepam (formerly called RO 11-3128) and its stereoisomer RO 11-3624 both inhibit the NBTI-sensitive adenosine transport but the NBTI-resistant mechanism shows stereoselectivity with meclonazepam being approximately 50-fold less potent than RO 11-3624. Ganglia were perfused with adenosine (100  $\mu$ M) in the presence of either RO 11-3624 or mecionazepam (100  $\mu$ M). The presence of RO 11-3624 completely inhibited the adenosine-induced increase in ACh stores (figure 5.2). In contrast, perfusion with adenosine in the presence of meclonazepam increased ACh stores by 17%. These results support the conclusion that adenosine is delivered by NBTI-resistant nucleoside transporters to an intracellular site for its effect on ACh synthesis.

#### 5.4.3 Releasability of the extra ACh

To test if the extra ACh synthesized in the presence of adenosine can be mobilized into a releasable pool of neurotransmitter upon stimulation of the preganglionic sympathetic trunk, the left and right ganglia of six animals were prepared for perfusion; one ganglion of each pair was exposed to adenosine and [3H]choline (10  $\mu$ M; 240 mCi/mmol) for 45 minutes, while its control ganglion received only the radiolabelled choline. Subsequently, the perfusion medium was changed to one free of added choline but containing physostigmine (15  $\mu$ M) to prevent the hydrolysis of secreted ACh and HC-3 (10  $\mu$ M) to prevent any further ACh synthesis by limiting the entry of choline into the nerve terminals. Following another 10 minutes of rest to wash out residual [<sup>3</sup>H]choline, the preganglionic nerves were stimulated (20 Hz) for 75 minutes and the effluent was collected every 5 minutes. This protocol allowed us to measure the releasable pool of total ACh and of the radiolabelled ACh synthesized during the exposure to adenosine.



Figure 5.3: Releasability of the extra ACh. Both ganglia of each animal were perfused with a Krebs solution containing [<sup>3</sup>H]choline (240 mCi/mmol) for 45 minutes; the test ganglion was also exposed to adenosine (100  $\mu$ M) for this period. The medium was changed to a Krebs solution containing HC-3 (10  $\mu$ M), physostigmine (15  $\mu$ M), but without added choline. Following another 10 min. of rest, the ganglia were stimulated at 20 Hz. (a) The release of ACh from control ( $\Box$ ) and from adenosine-treated (**m**) ganglia for a 75 min stimulation period. Stimulation was commenced at 5 minutes. (b) The release of [<sup>3</sup>H]ACh from control ( $\Box$ ) and from adenosine pre-treated (**m**) ganglia during a 75 min stimulation period. Stimulation was commenced at 5 minutes. (c) Specific radioactivity of released ACh from control ( $\Box$ ) and from adenosine-treated (**m**) ganglia Results are the mean ± SEM of 6 experiments. \*p<0.05

The release of total ACh is shown in figure 5.3a. The basal release before
preganglionic nerve stimulation was similar whether or not the ganglia had been pretreated with adenosine  $(2.5 \pm 1.4 \text{ and } 2.8 \pm 1.4 \text{ pmoles/min}$ , respectively). Stimulation of the sympathetic trunk clearly released ACh; evoked relase was maximal during the first collection period and declined thereafter as the consequence of HC-3's presence. The initial release of ACh during stimulation of the adenosine-pretreated tissues was statistically greater (p<0.05) than that from control ganglia; in the first two collection periods, some 75% more ACh was released from the test ganglia than from their contralateral controls. After this, the release from both ganglia was similar. The total evoked ACh output for the 75 minute stimulation period was  $1582 \pm 205$  pmoles from ganglia pre-conditioned with adenosine and  $1222 \pm 95$  pmoles from their control ganglia; the difference (p<0.05) is similar to the value of the adenosine-induced increase in ACh stores measured in the earlier experiments.

The release of  $[{}^{3}H]ACh$  is illustrated in figure 5.3b which shows that the release pattern of radiolabelled ACh was closely matched to that of the total ACh release; an early peak was followed by a decline in the output rate. The  $[{}^{3}H]ACh$  output from the adenosine-treated ganglia was significantly (p<0.05) greater than the controls during the first 10 minutes of stimulation. Thus, the extra ACh synthesized in the presence of adenosine is mobilized into a releasable pool upon electrical stimulation. The specific radioactivity of the released ACh was slightly, although not significantly, greater from adenosine-treated ganglia than from the controls groups (figure 5.3c), indicating that the ACh synthesized during the exposure to adenosine mixed almost completely with the preexisting stores.

#### 5.4.4 Releasability of the extra ACh in the presence of vesamicol

In the presence of vesamicol (formerly called AH5183), a drug which interferes with the transfer of ACh into vesicles (see review by Marshall and Parsons, 1987), preganglionic stimulation liberates only some 14% of the total ACh content, which is considered to represent a readily-releasable fraction of the ganglionic ACh stores (Collier et al., 1986; Cabeza and Collier, 1988). The results of the previous experiments above (figure 5.3) indicated that most of the adenosine-induced extra ACh and [<sup>3</sup>H]ACh was released within the first 10 minutes of stimulation, either reflecting a change in the size of the readily releasable pool or an increase in the rate of mobilization for release. To distinguish these possibilities, six ganglia were exposed to adenosine for 45 minutes, while the contralateral ganglia were perfused without the nucleoside. The medium was changed to one with physostigmine (15  $\mu$ M) and vesamicol (10  $\mu$ M) and, following ten minutes of perfusion at rest, the preganglionic nerve was stimulated at 5 Hz for 30 minutes. The perfusate was collected every two minutes for the first ten minutes, and subsequently every five minutes.



Figure 5.4: Effect of vesamicol on the release of the extra ACh. Both ganglia were perfused with Krebs solution for 45 minutes: one side was also exposed to adenosine (100 µM) during this time. The medium was changed to Krebs solution containing vesamicol (10  $\mu$ M) and physostigmine (15  $\mu$ M). Following another 10 minutes of rest, 5 Hz stimulation was commenced and the ACh output was measured. Results are illustrated as ACh release from control (D) and adenosine-treated (**■**) ganglia. Shown are the means

of 6 experiments. Error bars have been omitted for clarity (the SEM for any point was less than 15%). Inset: total evoked release from control (open bar) and adenosine-treated (closed bar) in the presence of vesamicol. Evoked release was calculated as the total ACh release minus the mean basal output measured during 15 min prior to stimulation.

Three outcomes were possible for this experiment: the extra ACh augments the readilyreleasable pool by about 360 pmoles, only some 25% more ACh is released in the presence of vesamicol, or there is no change in the size of the readily-releasable pool. The last was clearly the case; the pattern of ACh output in the presence of vesamicol from adenosine-treated ganglia was rather similar to that from those not pre-treated with adenosine (figure 5.4). Moreover, the total stimulus-evoked ACh release from the adenosine-treated tissue,  $256 \pm 55$  pmoles, was not different from the  $242 \pm 42$  pmoles released from the control ganglia (figure 5.4, inset). This result indicates that the size of the readily releasable pool of ACh is unaffected by the adenosine treatment, and that the extra ACh is presumably stored in the remaining fractions of the tissue ACh stores.

#### 5.5 Discussion

There is a substantial body of evidence demonstrating the diverse roles of purine nucleotides and nucleosides in the nervous system (see reviews by Williams, 1987; White, 1988); these compounds serve as locally acting extracellular modulators of neurotransmission in addition to their roles in intracellular energy metabolism. Evidence reported by Chatterjee and Bhatnagar (1990) suggested the possibility that the role of ATP might extend to the modulation of the choline transporter, as has been implied for norepinephrine uptake (Hendley et al., 1988; Hardwich et al., 1989). In those studies, ATP appeared to exert its effects on the respective transporters by a phosphorylation event, ostensibly mediated by an ecto-kinase. The initial aim of the present study was to test the effects of extracellular ATP on ACh synthesis in a sympathetic ganglion. The prediction was that ATP would increase ACh synthesis but that Appcp, a nonhydrolyzable analog would not. The prediction held, but the ATP effect was shared by ADP, AMP, and adenosine. Since these metabolites do not support phosphorylation, the possibility that the increased ACh content effected by ATP is mediated by an external phosphorylation event on the choline transporter appears to be unlikely. The simpler explanation is that the nucleotides are metabolized to adenosine by ecto-nucleotidases present in the synaptic cleft (Nacimiento et al., 1991), and that adenosine then mediates the increase of ACh stores. The stability of the methylene group in the Appcp molecule prevents the hydrolysis of the terminal phosphate moiety, and it is, thus, resistant to degradation by 5'nucleotidase. That adenosine is the key purine in the induction of ACh synthesis in our experiments is supported by the observation that inosine, the deaminated metabolite of adenosine, did not increase ACh content.

Most of the extracellular actions of adenosine are mediated through surface receptors that can interact with a variety of intracellular second messenger systems (see review by Olah and Stiles, 1992). These receptors mediate the much-studied inhibitory effect of adenosine on evoked transmitter release (White, 1988) and the most plausible explanation for the adenosine-induced increase of tissue ACh might appear to be that reduced ACh release caused the increased content. But adenosine did not affect ACh release under the experimental conditions where it increased ACh content and block of extracellular receptors with a methylxanthine, aminophylline, did not inhibit the effect of adenosine to increase ACh content. Thus, adenosine did not increase ACh content as an indirect consequence of its ability to inhibit release. Moreover, the inability of aminophylline to prevent the effect of adenosine in the present study indicates that adenosine did not produce its effect by interacting with one of the extracellular receptors identified so far.

The transport of adenosine across cell membranes occurs through specific components of cell membranes; these can be concentrative (Vijayalaksimi and Belt, 1988; Plagemann and Aran, 1990) or facilitated diffusion transporters (Bender et al., 1980; Barberis et al., 1981; Bender et al., 1981a; Bender et al., 1981b). It is the latter mechanism that is considered to mediate the largest portion of nucleoside flux and it is inhibited by dipyridamole or NBTI. Pharmacological heterogeneity amongst the facilitated nucleoside transporters was first noted by Lauzon and Paterson (1977) with a tumour cell line and the phenomenon has been described for a variety of tissues, including neuronal cells (Marangos and Deckert, 1987; Lee and Jarvis, 1988a; Lee and Jarvis, 1988b; Shank and Baldy, 1990); these transporters are equally sensitive to dipyridamole but can be distinguished on the basis of their sensitivity to NBTI. In the present tests, dipyridamole but not NBTI prevented the effect of edenosine to increase ACh content. These nucleoside transport inhibitors are not known to block receptor function at the concentrations used, and generally potentiate the extracellular actions of adenosine by restricting its removal from the extracellular space (see review by Wu and Phillis, 1984). Thus, the action of adenosine on ACh content appears to be directed through a specific group of transporters, those with low sensitivity to NBTL, and this conclusion was supported by the use of the benzodiazepine stereoisomers, meclonazepam and RO 11-3624, that have stereoselective actions on the NBTI-resistant nucleoside transport mechanism (Lee and

Jarvis, 1988b). These compounds also exhibited a stereoselective ability to block the adenosine induced increase in ACh synthesis.

Thus, overall, the pharmacological tests discussed above support the idea that for adenosine to effect an increase of ACh content, it must be transported into the cholinergic nerve terminals; the nucleoside presumably acts intracellularly. Our experiments have not attempted to identify this intracellular site of action, although we have eliminated the most obvious possibility. Adenosine can inhibit choline kinase activity, at least *in vitro* (Wecker and Reinhardt, 1988), and thus, it appeared possible that such an effect, if manifest in intact tissue, could shunt newly-transported choline away from the pathway leading to the formation of phosphorylcholine and phospholipid, thereby increasing the availability of precursor for ACh synthesis (Wurtman et al., 1990). However, the incorporation of radiolabelled choline into these other forms was not reduced by the exposure of ganglia to adenosine under conditions where its incorporation into ACh was increased. Indeed, in this experiment, the incorporation of [3H]choline into all choline-containing species tended to increase in adenosine-treated tissue, as if choline uptake were augmented. Considering that choline delivery is the most popular candidate for regulating ACh synthesis (see review by Tuček, 1985), it appears possible that adenosine acts at this level.

A possible location at which intracellular adenosine might interact is the P-site, designated as such by Londos and Wolff (1977) because of its intolerance to modifications in the purine ring. This site appears to be on the catalytic subunit of adenylyl cyclase and adenosine inhibits enzyme activity (Florio and Ross, 1983; Yeager et al., 1986; Johnson et al., 1989; Marone et al., 1990). It is, however, not clear how inhibition of adenylyl cyclase might translate into an increase in ACh synthesis. It would be necessary to postulate a cyclic AMP-dependent event that is inhibitory to ACh synthesis and that this control is relieved in the presence of adenosine. There is little information to support this, although it is reported that forskolin, a potent activator of adenylate cyclase, inhibited the synthesis of [<sup>3</sup>H]ACh by 20% in hippocampal slices (Lapchak and Collier, 1988). If such an event occurs, the question is whether cyclic AMP alters choline transport or ChAT activity or both. Changes in intracellular cyclic AMP levels can alter choline transport dynamics, but increased transport appears to result from increased cyclic AMP (Breer and Knipper, 1990). With respect to ChAT, it has been suggested to exist as a phosphoprotein, but the phosphorylation does not involve cyclic AMP-dependent kinase (Bruce and Hersh, 1989).

The adenosine-induced potentiation of the transmitter pool appeared to be Ca<sup>2+</sup>dependent. Since facilitated nucleoside transport is not particularly sensitive to low Ca<sup>2+</sup> (Banay-Schwartz et al., 1980), it is likely that this property reflects that of a second messenger system involved in the adenosine phenomenon. Unfortunately, this property does not distinguish the possibility that adenosine increases ACh synthesis by activating the choline transporter or ChAT. While choline uptake appears not dependent on extracellular Ca<sup>2+</sup> (Simon and Kuhar, 1976), its activation during electrical stimulation appears to be so (Collier and Ilson, 1977). Also, the acetylation of choline may be dependent on an influx of Ca<sup>2+</sup> during stimulation; Welner and Collier (1985) reported that the acetylation of a choline analogue, diethylhomocholine, but not its transport, was reduced when the extracellular Mg<sup>2+</sup> was elevated so as to compete with the influx of Ca<sup>2+</sup>.

The extra ACh synthesized in the presence of adenosine was eventually lost in the absence of adenosine, possibly by intracellular destruction, but it was retained for more than 15 min following the removal of the adenosine and this feature allowed us to test the releasability of the extra ACh. The change in transmitter levels was associated with a change of evoked release; ACh and radiolabelled ACh output during preganglionic stimulation was increased substantially from ganglia that had been pre-treated with adenosine and [<sup>3</sup>H]choline. The resulting specific radioactivity of the liberated ACh was somewhat similar to that of the control, suggesting that the extra ACh had mixed relatively freely with the pre-existing stores. The difference in ACh output between the control and treated ganglia was similar to the increased store size, implying that most, or all, of the

extra ACh was mobilized for release upon electrical stimulation. The functional consequences of enhanced ACh release on ganglionic transmission may be significant (Birks, 1977); because of the remarkable divergence of presynaptic neurons (Billingsley and Ranson, 1918), even modest increases in output can have notable effects on recruitment of postganglionic neurons, and therefore, on transmission.

Interestingly, in these experiments that measured ACh release, the fractional release of transmitter was augmented from the ganglia that had been treated with adenosine; while ACh stores were increased by 25%, the initial output rate was almost 75% greater than from the control ganglia. This suggested that the extra ACh might have been incorporated, in whole or in part, into the readily releasable fraction of the preexisting stores. However, the evoked release of ACh from adenosine-treated ganglia in the presence of vesamicol, a drug which appears to inhibit the movement of ACh into the readily releasable pool of ACh (Collier et al., 1986), was no different from the control ganglia, suggesting that the adenosine had increased the size of the less readily releasable portion of the ganglionic ACh stores, either vesicular or extravesicular, from which it was mobilized to increase release in vesamicol's absence. Consequently, it would appear that the transfer of ACh to the more readily releasable  $\leq$  .ction is more efficient after ganglia have been exposed to adenosine.

The present results raise the possibility that endogenous adenosine might be a factor that modulates transmission in the superior cervical ganglion. One likely source of synaptic adenosine is from the ATP co-stored in cholinergic vesicles. The ATP:ACh content of such vesicles has been estimated to be in the range of 1:5 in a variety of preparations (Dowdall et al., 1974; Richardson and Brown, 1987). Estimates of the intravesicular ACh and ATP concentrations are about 500 mM and 100 mM, respectively (for review see Whittaker, 1982). The concentration of ACh in the synaptic cleft after the release of a single quantum is estimated as 0.3 mM (Kuffler and Yoshikami, 1975), so if vesicles exocytose their total content, ATP would reach 60 µM. Indeed, Silinsky (1975)

observed a 1:5 ratio of ATP: ACh in the efflux from the rat phrenic nerve during electrical stimulation and estimated that the synaptic concentration of ATP resulting from a single nerve impulse may be 80 µM, and possibly higher during periods of repetitive stimulation. These values are similar to the purine concentration used in the present experiments. Relatively few studies, however, have examined the release of purines from sympathetic ganglia. Kato et al. (1974) were unable to detect any evoked release of endogenous nucleotides, but subsequent studies with ganglia pre-exposed to [3H]adenosine have shown the evoked release of tritiated purine compounds, either as adenosine metabolites (McCaman and McAfee, 1986) or as adenosine (Rubio et al., 1988). Part of the uncertainty surrounding the identity of the released purines is likely due to the extensive mechanisms for their extracellular metabolism. For example, high levels of ecto-5'nucleotidase, the rate limiting step in the degradation of released ATP to adenosine (Richardson et al., 1987; Richardson and Brown, 1987), have been localized to the synaptic cleft in sympathetic ganglia (Nacimiento et al., 1991), and adenosine deaminase, the enzyme which converts adenosine to inosine, is reportedly associated with certain preganglionic neurons (Senba et al., 1987; see review by Nagy et al., 1990). The presence of such comprehensive means for the destruction of released purines suggests that these substances may be important in regulating neurotransmission in ganglia.

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#### **Preface to Section 6**

The results presented in Section 5 indicate that exogenous adenosine can modify ACh synthesis and release in a sympathetic ganglion. The lack of a close correlation between the concentration of exogenous adenosine and its effect on ACh content suggested that it was unlikely that adenosine was the controlling factor in the rate of ACh synthesis during neuronal activity. However, it remained possible that endogenous adenosine, if released by repetitive presynaptic activity, might achieve synaptic concentrations in the range of those used in section 5, and, consequently, play a role in modulating ACh synthesis. Repetitive preganglionic stimulation is known to promote significant changes in ganglionic ACh content and the efficacy of synaptic transmission. Thus, the aim of the following study was to examine whether endogenous adenosine might have a role in determining ACh synthesis after high frequency preganglionic stimulation. The nucleoside transport inhibitors used in the previous section provided the means to address this question.

### The Role of Endogenous Adenosine in a Post-Stimulation Increase in the Acetylcholine Content of a Sympathetic Ganglion

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Abbreviations used: ACh, acetylcholine; NBTI, nitrobenzylthioinosine; TCA, trichloroacetic acid; TPB, tetraphenylboron; CHA, cyclohexyladenosine; CPT, cyclopentyltheophylline; vesamicol, 2-(4-phenylpiperidino)cyclohexanol; meclonazepam, (S)-5-(2-chlorophenyl)-1,3-dihydro-3-methyl-7-nitro-2H-1, 4-benzodiazepin-2-one; RO 11-3624, (R)-5-(2-chlorophenyl)-1,3-dihydro-3-methyl-7-nitro-2H-1, 4-benzodiazepin-2-one.

#### 6.1 Abstract

Previous experiments showed that exposure of sympathetic ganglia to exogenous adenosine increased acetylcholine (ACh) content and its subsequent release. This effect was not mediated through extracellular adenosine receptors, but at an intracellular site following its uptake through nitrobenzylthioinosine (NBTI)-resistant nucleoside transporters. We postulated that endogenous adenosine may play a role in modulating synaptic transmission in the superior cervical ganglion. The present study tested whether adenosine is involved in the activation of acetylcholine synthesis that occurs during a rest period following prolonged presynaptic tetanic activity. Conditioning of ganglia with high frequency stimulation (15 Hz) for 45 min followed by a 15 min rest increased their ACh content by 45%. The appearance of this "rebound ACh" showed sensitivity to nucleoside transport inhibitors; it was prevented by dipyridamole, but not by NBTI or meclonazepam, and it was reduced in the presence of RO 11-3624 suggesting an involvement of NBTI-resistant transporters. The effect of dipyridamole was specific for the synthesis of rebound ACh in that it did not inhibit ACh release or ACh synthesis during stimulation. The inhibitory action of dipyridamole on the synthesis of rebound ACh was not evident if it was present only during the tetanic stimulation but it was if dipyridamole was present during the rest period following it, suggesting that adenosine's presence after tetanic stimulation is of importance. This conclusion was strengthened by experiments showing that the presence of cyclopentyltheophylline, an antagonist at inhibitory adenosine receptors, increased ACh output evoked by test stimulation immediately following tetanic activity, as if endogenous adenosine was available at that time to activate the adenosine receptors that inhibit transmitter release. ACh release from conditioned ganglia was 44% greater than that from the controls. However, the rebound ACh was not mobilized in the presence of vesamicol, a vesicular ACh transporter inhibitor. These results suggest that endogenous adenosine released after tetanic stimulation activates ACh synthesis which results in an increase of ganglionic ACh that is available for subsequent mobilization and release.

#### 6.2 Introduction

In a recent paper, we reported that adenosine can, under particular experimental conditions, increase the acetylcholine (ACh) content of superior cervical ganglia (Tandon and Collier, 1993). This effect was manifest when adenosine was perfused through a ganglion at rest; it appeared not to result from an action of adenosine on the classical extracellular adenosine receptors in that it was not blocked by adenosine receptor antagonists, but it was prevented by agents like dipyridamole that block adenosine uptake. These properties clearly distinguish the effect of adenosine to increase ACh stores from its well-studied effect to decrease transmitter release (see review by White, 1988); the latter effect is only manifest during stimulation, and it is blocked by receptor antagonists.

We showed in that initial report that the extra ACh synthesized in adenosine's presence was releasable after adenosine's removal, but, otherwise, its potential physiologic significance was not explored. The present work attempted to do so by testing if the adenosine phenomenon is related to a well-reported example of presynaptic adaption that is reflected as an increase in the ACh content of a sympathetic ganglion following a conditioning paradigm of high frequency, long duration stimulation of its preganglionic input.

This post-stimulation increase in ACh stores was first reported by Rosenblueth et al. (1939) and has been described subsequently in greater detail by Freisen and Khatter (1971), Birks and Fitch (1974), Bourdois et al. (1974), O'Regan and Collier (1981), and Collier et al. (1983). It results from an accelerated rate of ACh synthesis, possibly due to increased choline uptake (O'Regan and Collier, 1981; Collier et al., 1983), during a period of rest that follows the conditioning stimulation, and can increase tissue ACh by up to 70% depending upon the frequency and duration of the conditioning stimuli. The extra transmitter, which we will refer to as 'rebound ACh', is incorporated into a releasable pool (Birks, 1977; Bourdois et al., 1974; Collier et al., 1983), but there is some uncertainty about the particular intraterminal store that is augmented by the extra ACh synthesized. Although it is not clear that rebound ACh is formed under physiological conditions, other patterns of neuronal activity, which may occur *in vivo*, promote its formation; Birks (1978) reported that short repetitive bursts of high frequency pulses could increase ACh content even though the same mean frequency, when applied in evenly spaced pulses, did not. In addition, experimental hypoxia produced a *phenomenon similar* to rebound ACh (Birks, 1978); this effect resulted from an increased discharge rate of preganglionic neurons because it was not evident if the cervical sympathetic trunk was first transected.

The mechanism by which the synthesis of rebound ACh is initiated remains to be elucidated; transmitter release during the tetanic conditioning is necessary for its induction (Collier et al., 1983), but the phenomenon appears not to require ACh action on muscarinic (Bourdois et al., 1974) or nicotinic (Collier et al., 1983) receptors. These observations suggest the possibility that some releasable factor other than ACh might be involved in initiating the synthesis of rebound ACh. As mentioned above, adenosine can increase ganglionic ACh content, and, thus, suggested itself as a candidate for this putative factor that mediates the formation of the rebound ACh, and the aim of the present study was to test this idea. To do so, we tested if the drugs that prevented the adenosineinduced increase in transmitter content could also affect the accumulation of rebound ACh. If the two phenomena are related, they should have a similar pharmacological sensitivity to nucleoside transport inhibitors. A second related objective of this work was to examine the relationship between rebound ACh and the releasable ACh compartments in order to clarify the uncertainty mentioned above. Some of this work has been presented in abstract form (Tandon and Collier, 1991).

#### 6.3 Materials and Methods

Materials used:  $[\gamma$ -32P]ATF (10 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA); butyronitrile was obtained from Aldrich Chem. Co. (Milwaukee, WI, USA); OptiPhase HiSafe II from LKB; acetylcholine chloride was from Hoffmann-LaRoche (Basel, Switzerland); Meclonazepam and RO 11-3624 were generous gifts from Hoffmann-LaRoche (Mississauga, Canada); EGTA was obtained from Eastman Kodak (Rochester, NY, USA); cyclohexyladenosine and cyclopentyltheophylline were from Research Biochemicals Inc. (Natick, MA, USA); Amberlite CG-400 (chloride form), choline chloride, dipyridamole, nitrobenzylthioinosine, tetraphenylboron (sodium salt),  $\alpha$ chloralose, acetylcholinesterase (EC 3.1.1.7, ACh hydrolase, type V-S), choline kinase (EC 2.7.1.32, ATP:choline phosphotransferase), and physostigmine sulfate were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Vesamicol was a gift of Dr. S.M. Parsons (University of California at Santa Barbara) or was synthesized by Dr. T.H. Chan (McGill University) by reacting 4-phenylpiperidine (a gift of Dr. M. Peel, Glaxo) with cyclohexene oxide. All other chemicals and reagents were obtained from Fisher Scientific (Montreal, Que., Canada).

#### **6.3.1 Experimental procedures**

Anaesthesia was induced in cats of either sex (1.5-4.0 kg) with a 2:1 mixture of N2O/O2 with 2% halothane and maintained with an i.v. injection of  $\alpha$ -chloralose (80mg/kg). Superior cervical ganglia were prepared surgically for perfusion according to the method of Kibjakow (1933) as described by Collier and Kwok (1982). All animal use procedures were in strict accordance with the guidelines of the Medical Research Council of Canada and the Canadian Council on Animal Care and were approved by the local Animal Care Committee. One or both ganglia were perfused (0.2-0.4 ml/min) with a Krebs solution of the following composition (mM): NaCl 120, KCl 4.6, CaCl2 2.4, KH2PO4 1.2, MgSO4 1.2, NaHCO3 25, and dextrose 10. Choline chloride (10  $\mu$ M) was always added

before perfusion. The medium was filtered and subsequently equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> to maintain a pH of 7.4 at 37°C. In experiments that measured ACh release, the perfusion medium also contained physostigmine sulfate (15  $\mu$ M); other drugs were used as described in the appropriate text.

All drugs were added to the Krebs solution just before perfusion. After the perfusion, ganglia were excised and placed in 1 ml of 10% trichloroacetic acid (TCA) for 1 hour on ice. In an effort to shorten the period of rest following tetanic stimulation in some experiments, the ganglia were dissected from the distal end first so that preganglionic stimulation could be maintained until placement into ice-cold TCA. The TCA was removed by extraction with ether and the aqueous layer was assayed for ACh content.

Ganglia were stimulated via the preganglionic sympathetic trunk with square wave pulses (8-10V, 0.5ms, 5 or 15 Hz) using a platinum electrode. During periods of prolonged stimulation, the electrode was moved a few millimeters proximally along the nerve every 10 minutes. When measuring ACh release, the perfusate was collected in 2 or 5 minute periods into pre-chilled tubes and stored on ice for not more than one hour. Samples were stored overnight at -20°C for assay the following day.

#### 6.3.2 Measurement of ACh

ACh was obtained from the aqueous tissue extracts and the perfusate samples by the method of Fonnum (1969) as described Welner and Collier (1984). Briefly, the samples were extracted by 400  $\mu$ l of tetraphenylboron in butyronitrile (TPB/butyronitrile; 10 mg/ml). The choline esters were removed from the organic phase by 150  $\mu$ l of AgNO3 (20 mg/ml); excess silver was precipitated with 10  $\mu$ l of MgCl<sub>2</sub> (1 M). Samples were lyophilized to dryness.

The ACh content of the samples was determined according to the radioenzymatic method of Goldberg and McCaman (1973). Choline was phosphorylated by choline kinase

and ATP. ACh was hydrolyzed to choline with acetylcholinesterase and phosphorylated with  $[\gamma-32P]$ ATP. The phosphorylated choline was separated from the labelled ATP by anion-exchange chromatography and the radioactivity measured by liquid scintillation spectrometry. 32P was measured in 10 ml OptiPhase HiSafe II with an counting efficiency of 99%.

#### 6.3.3 Statistical analysis

Statistical comparisons were done using two-tailed Students t-test for paired samples. This analysis was appropriate for all comparisons between the two ganglia of each animal: one was used as the test preparation and its contralateral served as control. The values are presented as percent or amount of change; each such value was calculated for each pair of ganglia. To evaluate differences between unpaired conditions, ANOVA and Newman-Keuls for the posthoc comparison were used.

#### 6.4 Results

#### 6.4.1 Effect of nucleoside transport inhibitors on the accumulation of rebound ACh

Nucleoside transporters are classified into two groups on the basis of their sensitivity to inhibition by nitrobenzylthioinosine (NBTI), ie. NBTI-sensitive or NBTIresistant nucleoside transporters; both types are blocked by dipyridamole. The initial experiments tested whether the synthesis of rebound ACh is altered by the presence of nucleoside transport inhibitors. In these tests, the ACh content of the contralateral ganglion, neither stimulated nor exposed to the nucleoside transport inhibitors, allowed us to estimate the initial ACh content of the test ganglion since the amount of transmitter contained in both superior cervical ganglia is normally the same (Feldberg, 1943; Birks and MacIntosh, 1961). The test ganglia were subjected to preganglionic stimulation at 15 Hz for 45 min and then allowed to rest for 15 min. Conditioning of this nature has been previously shown to increase ganglionic ACh stores (see Introduction) and the present experiments yielded similar results; the ACh content of the conditioned ganglia was increased by  $588 \pm 113$  pmoles ( $45 \pm 11\%$ , n=7; p<0.01) when compared to the contralateral ganglia which contained  $1397 \pm 136$  pmoles (fig. 6.1). When ganglia were conditioned and then rested with 10 µM dipyridamole present throughout the experiment, the increase in ACh content was  $163 \pm 66$  pmoles ( $11 \pm 5\%$ , n=5; 0.1>p>0.05), significantly less than in dipyridamole's absence (p<0.05, ANOVA/Newman-Keuls). In contrast, when 100  $\mu$ M NBTI was present, the amount of extra ACh contained was increased to  $1289 \pm 212$  pmoles (88  $\pm$  10%, n=5; p<0.01), more than double the increase produced by conditioning alone (p<0.05, ANOVA/Newman-Keuls).

The two classes of transporters mentioned above exhibit stereoselectivity with respect to the two benzodiazepine stereoisomers, RO 11-3624 and meclonazepam (formerly called RO 11-3128). Both transporters are equally sensitive to RO 11-3624, but the NBTI-resistant carrier is 50-fold less sensitive to meclonazepam (Lee and Jarvis,

1988a). Thus, we tested these agents to determine if their effects were similar to those of dipyridamole and NBTI reported above. When ganglia were conditioned and rested with 100  $\mu$ M RO 11-3624 present throughout, the synthesis of rebound ACh was 418 ± 39 pmoles (30 ± 2% of the initial content), approximately 30% less than that produced by conditioning in the absence of drug. This change, however, represented a significant increase in ACh content compared to the contralateral ganglia (p<0.05). In contrast to RO 11-3624, exposure of ganglia to 100  $\mu$ M meclonazepam throughout the experiment increased the accumulation of extra ACh to 1445 ± 301 pmoles (102 ± 15%, p<0.01), significantly greater than that in the presence of its stereoisomer (p<0.01, ANOVA/Newman-Keuls) (fig. 6.1).



Figure 6.1: Effect of nucleoside transport inhibitors on the accumulation of rebound ACh. Ganglia were perfused with a Krebs solution (No drug) or one containing one or more of the following: dipyridamole (Dip; 10  $\mu$ M), nitrobenzylthioinosine (NBTI; 100  $\mu$ M), RO 11-3624 (11-3624; 100  $\mu$ M), or meclonazepam (meclonaz.: 100  $\mu$ M). The ganglia were stimulated at 15 Hz for 45 min and then allowed to rest for 15 min. Bars represent ACh content (means ± SEM of 3 to 7 experiments) of stimulated ganglia as a percentage of the contralateral ganglion's value (mean contralateral ACh content was 1450 ± 57 pmoles). \* p<0.05, increased ACh content compared to contralateral control ganglion.

To test whether dipyridamole could prevent the extra ACh induced by NBTI, ganglia were stimulated and rested in the presence of both dipyridamole and NBTI. In three such experiments, the ACh content of conditioned ganglia was increased by only 219  $\pm$  96 pmoles (14  $\pm$  6% compared to the contralateral ACh content of 1493  $\pm$  113 pmoles). This change was not statistically significant (0.2>p>0.1) and was not different from that observed in ganglia conditioned and rested in the presence of dipyridamole alone; it was clearly less than the change in the presence of NBTI alone (fig. 6.1).

#### 6.4.2 Effect of dipyridamole on ACh release and ACh stores without conditioning

Dipyridamole reduced the accumulation of rebound ACh in the tests above, just as it reduced the adenosine-induced increase in ACh synthesis (Tandon and Collier, 1993). Before concluding that this might indicate a role for endogenous adenosine in the formation of rebound ACh, it was necessary to assess the effects of dipyridamole on ACh turnover during stimulation; transmitter release during stimulation is necessary for the increased synthesis following conditioning (Collier et al., 1983). It has been suggested that dipyridamole interacts with the glucose transporter, and, although, in earlier tests dipyridamole did not affect basal ACh synthesis in resting ganglia (Tandon and Collier, 1993), it remained possible that dipyridamole might inhibit ACh release or reduce ACh synthesis during stimulation and, consequently, mask the appearance of rebound ACh.

To test the effect of dipyridamole on transmitter release, ACh output was measured from ganglia stimulated in the presence of dipyridamole. In these experiments, ACh release was measured from both ganglia of each cat before, during, and after the conditioning stimulation (45 min of 15 Hz stimulation preceding 15 min of rest); one ganglion was exposed to 10  $\mu$ M dipyridamole during this time. Physostigmine (15  $\mu$ M) was present throughout the experiment to prevent the loss of secreted ACh due to cholinesterase activity. As illustrated in fig. 6.2a, basal ACh output before stimulation was similar between the test and control ganglia (5.3 ± 0.7 vs 5.1 ± 0.7 pmol/min, respectively). Preganglionic stimulation clearly increased ACh release; indeed, it

appeared to augment initial transmitter output somewhat, but this difference did not reach statistical significance. The total evoked ACh output from dipyridamole-treated ganglia ( $3266 \pm 589$  pmoles) was not different from that released by the control ganglia ( $3006 \pm 220$  pmoles). Moreover, ACh efflux from both sets of ganglia returned to basal values following the stimulation period.



Figure 6.2: Effect of dipyridamole on ACh release. Both ganglia of each animal were perfused with a Krebs solution containing 15  $\mu$ M physostigmine for 70 min; the test ganglion was also exposed to 10  $\mu$ M dipyridamole. **a:** ACh release in the absence ( $\Box$ ) or presence (•) of dipyridamole from ganglia rested for 10 min, stimulated for 45 min at 15 Hz (indicated by horizontal bar), and then allowed to rest for 15 min. **b:** ACh content of ganglia following the stimulation and rest periods. Results shown are the mean ± SEM of 5 experiments. \*p<0.05.

The transmitter retained in these ganglia following the conditioning and rest was also measured (fig. 6.2b). These ACh levels were clearly greater than the normal ACh content (compare to mean of 29 control ganglia from experiments in the previous section:  $1450 \pm 57$  pmoles), as was expected due to the presence of physostigmine in the perfusion buffer which promotes the accumulation of "surplus ACh" resulting in a doubling of the transmitter content (Birks and MacIntosh, 1961). However, as in the experiments without

physostigmine, the tissues that had been perfused with dipyridamole contained some 30% less ACh than did those not exposed to that drug. Indeed, the amount of ACh ( $1097 \pm 320$  pmoles) by which the untreated and treated ganglia differed was greater in these experiments than it was in the ones without physostigmine (588 pmoles) as if the presence of physostigmine might increase the accumulation of rebound ACh.



Figure 6.3: Effect of dipyridamole on ACh content following stimulation. Pairs of ganglia were perfused with a Krebs solution and stimulated for 45 min at 15 Hz; one ganglion of each pair was also exposed to 10  $\mu$ M dipyridamole. Bars represent mean  $\pm$  SEM of ACh content of 5 experiments.

In other experiments, we tested the effect of dipyridamole on ACh synthesis during stimulation in the absence of physostigmine. Normally, the production of transmitter is augmented during neuronal activity to match ACh output such that tissue ACh stores are maintained relatively constant. We verified this in five experiments in which one ganglion of each cat was stimulated at 15 Hz for 45 min before its removal (stimulation of the preganglionic nerve was maintained during excision of these ganglia so that the amount of time between the end of stimulation and placement in ice-cold TCA was minimal). The ACh content of the stimulated ganglia was  $1692 \pm 129$  pmoles, comparable to the 1707  $\pm$  182 pmoles of the contralateral ganglia, indicating that ACh synthesis indeed maintained ACh content during stimulation. Since release was not affected by dipyridamole, as shown above, any decreased ACh synthesis caused by the drug would be manifest as a decrease

in ACh content at the end of stimulation. Both ganglia of each cat were perfused with Krebs buffer while one ganglion was also exposed to 10  $\mu$ M dipyridamole. The ganglia were stimulated for 45 min before being excised and assayed for tissue ACh. In five such experiments, the ACh content of the ganglia stimulated in the presence of dipyridamole was 93 ± 4% of the contralateral content, a mean reduction of 106 ± 55 pmoles. The difference did not reach statistical significance (0.2>p>0.1; fig. 6.3).

Thus, neither evoked ACh release nor ACh content was altered significantly during dipyridamole errosure indicating that net ACh synthesis during prolonged stimulation is not affected by the drug. Dipyridamole appears to inhibit specifically the post-tetanic potentiation of ACh stores without compromising the ability of nerve terminals to maintain their normal stores.

## 6.4.3 Effectiveness of dipyridamole to inhibit rebound ACh accumulation during stimulation or rest

To test whether the action of dipyridamole to inhibit the synthesis of rebound ACh requires its presence during the tetanic stimulation or during the period of rest following the stimulation, one ganglion of each cat was subjected to the conditioning as before, but instead of continuous exposure to dipyridamole throughout the experiment, the drug was present only for the 45 min stimulation period or for the subsequent 15 min rest period. Exposure to dipyridamole only during stimulation had no effect on the accumulation of rebound ACh; transmitter stores were increased by  $613 \pm 133$  pmoles ( $45 \pm 8\%$ , n=4; p<0.01) compared to the contralateral ACh content (fig. 6.4). However, in the presence of dipyridamole only during the rest period, only  $344 \pm 133$  pmoles ( $24 \pm 10\%$ , n=6) of extra ACh were formed. This increase failed to be statistically significant in comparison to the ACh content of contralateral control ganglion (0.1 ). Thus, the dipyridamole-sensitive signal involved in stimulating the synthesis of rebound ACh appears to be manifest mainly following the tetanic stimulation, not during it.



Figure 6.4: Effect of dipyridamole during stimulation or during rest. Ganglia were stimulated for 45 min at 15 Hz and then allowed to rest for 15 min. Dipyridamole was present either during the stimulation (n=4) or during period (n=6). the rest For comparison, the control (no drug) from fig. 6.1 is also shown. The bars represent ACh content of the test ganglion expressed as a percentage  $\pm$ SEM of that in the contralateral ganglion (mean contralateral ACh content:  $1349 \pm 65$  pmoles). \*p<0.05.

# 6.4.4 Effect of an adenosine receptor antagonist on ACh release during and following tetanic stimulation

The inhibitory effects of adenosine on neurotransmission are well characterized and the present experiments confirmed this using cyclohexyladenosine (CHA) as the agonist. CHA decreased ACh release evoked by 5 Hz stimulation in a concentration dependent manner (fig. 6.5).



Figure 6.5: Concentration dependent effect of cyclohexyladenosine (CHA) on ACh release. Ganglia were perfused with 15  $\mu$ M physostigmine and increasing concentrations of CHA during 5 Hz stimulation (n=3). ACh release is shown as a percentage  $\pm$  SEM of the control ACh release evoked by 5 Hz stimulation. Mean ACh output evoked by 5 Hz stimulation in the presence of 0, 1, 10, 100  $\mu$ M CHA was 58  $\pm$  5, 55  $\pm$  6, 48  $\pm$  3, 39  $\pm$  4 pmoles/min, respectively. \*p<0.05.

These data indicating that adenosine receptor activation during stimulation significantly lessens ACh output measured at 5 Hz suggested a way to test whether adenosine is released following tetanic stimulation, as was implied by the result of fig. 6.4. The prediction was that if adenosine is released immediately following the tetanic stimulation, its presence might have an inhibitory action on evoked ACh release during this period due interaction with inhibitory adenosine to an receptors. Cyclopentyltheophylline (CPT) was used as the adenosine antagonist. Both ganglia of six cats were perfused with Krebs buffer containing physostigmine (15  $\mu$ M); one ganglion of each pair was perfused with medium that also contained 100 µM CPT. Both ganglia were stimulated at 15 Hz for 45 min, allowed to rest for 2 min to allow for the washout of ACh released by the preceding stimuli, and then they were subjected to a test stimulation of 5 Hz. The effluent was collected in successive 5 min periods following the onset of each stimulation period. The pattern of ACh output from these experiments is shown in fig. 6.6. The basal release from ganglia perfused with CPT was similar to that from control ganglia indicating that the drug alone did not promote ACh release. The onset of 15 Hz stimulation was accompanied by rapid rise in ACh release in the first collection which subsequently stabilized at a lower output rate from both sets of ganglia; no difference in the pattern or the amount of ACh released was evident whether CPT was present or not. Initiation of the subsequent 5 Hz test stimulation increased ACh release from both sets of ganglia, expectedly lower than that observed in the first stimulation period because of the lower frequency used, and the ACh released from the CPT-treated ganglia was 40% greater (p<0.02) during the first collection than that from the contralateral controls. ACh output in the subsequent collections also tended to be greater but failed to be statistically significant. The rate of release was stable from the drug-treated ganglia, but from the control ganglia, it increased progessively during the entire stimulation period. These results are suggestive of an inhibitory action of endogenous adenosine present just following the end of the 15 Hz stimulation, and support the argument that adenosine is present in effective concentration within the synaptic cleft following prolonged high frequency stimulation.



Figure 6.6: Effect of cyclopentyltheophylline (CPT) on ACh release. Pairs of ganglia were perfused with Krebs medium containing 15  $\mu$ M physostigmine; one ganglion of each pair was also exposed to 100  $\mu$ M CPT. Ganglia were stimulated at 15 Hz for 45 min (hatched bar), followed by 2 min of rest to washout released ACh, and then stimulated at 5 Hz (crosshatched bar) for another 25 min. ACh release is shown from control ( $\Box$ ) and CPT-exposed ( $\blacksquare$ ) ganglia (means ± SEM of 6 experiments). \*p<0.02.

#### 6.4.5 Potentiation of ACh release following conditioning

Previous studies suggested that transmitter release is potentiated following the formation of rebound ACh, but the time course of potentiation v as somewhat equivocal; in one study, ACh release following the conditioning and rest protocol was potentiated, but only after 2000 impulses had been delivered (Bourdois et al., 1974). On the other hand, Birks (1977) found that ACh release was increased immediately upon stimulation in

proportion to the amount of extra ACh available. To examine this issue, both ganglia of six cats were prepared for perfusion. One ganglion was perfused with Krebs medium for 45 min during which time it was conditioned with 15 Hz preganglionic stimulation. The medium was then changed to one with physostigmine (15  $\mu$ M) and the ganglion was rested for 15 min, before being stimulated with a 5 Hz test stimulation for another 45 min The contralateral ganglion did not receive the conditioning stimulation, but was perfused with Krebs medium containing physostigmine and subjected only to the test train of 5 Hz stimulation.



Figure 6.7: Release of ACh from conditioned control ganglia. and The test ganglia had been conditioned for 45 min with 15 Hz stimulation, and then allowed a 15 min rest during which time the medium was changed to one with physostigmine (15 µM). Following the rest, the ganglia were stimulated at 5 Hz for 45 min and ACh release from these tissues is compared to that from the contralateral gan-

glia that had not been conditioned. ACh release during the 5 Hz test stimulation from the control (°) and conditioned (•) ganglia is shown as means  $\pm$  SEM of 6 experiments. Inset: total evoked release from control (open bar) and conditioned (closed bar). Evoked release was calculated as the total ACh release minus the mean basal output estimated from measures before stimulation.

As shown in fig. 6.7, basal ACh release was similar whether or not the ganglia had been subjected to the conditioning stimulation. However, ACh output evoked by the 5 Hz test stimulation (ie. total ACh output minus basal ACh output) was greater from the conditioned ganglia compared to the contralateral controls and remained so for the duration of the simulation period. Overall, the conditioned ganglia released  $44 \pm 15$  % (p<0.05) more ACh than did their contralateral controls (fig. 6.7, inset). The amount of ACh remaining in these ganglia was measured at the end of the experiment; the ACh content of the conditioned ganglia was similar to that of their unconditioned counterparts (2960 ± 331 and 2710 ± 366 pmoles, respectively) indicating that the conditioned ganglia do not retain their extra ACh during stimulation. Thus, initial ACh output is increased in proportion to the increase in transmitter content, although towards the end of the experiment this relationship does not hold as the rate of ACh output remains high while ACh content returns to control levels.

## 6.4.6 Relationship between rebound ACh and the readily releasable compartment of ACh

Preganglionic nerve stimulation in the presence of vesamicol (formerly known as AH5183), a vesicular ACh transport blocker, releases only approximately 14% of the total ACh stores that is considered to represent the readily-releasable pool of ACh (as defined by Birks and MacIntosh, 1961) by virtue of its similarity in both quantity and its temporal pattern of release (Collier et al., 1986). As shown above, rebound ACh is associated with a releasable pool following its synthesis resulting in increased ACh output, but it is not known whether the size of the readily-releasable pool or the mobilization of ACh is altered as a consequence of this extra ACh.

To test this point, both ganglia of each cat were perfused with Krebs buffer for one hour. One ganglion of each pair was perfused at rest for this period, while the second ganglion was conditioned with 45 min of 15 Hz stimulation followed by a 15 min rest. The medium was then changed to one containing vesamicol (10  $\mu$ M) and physostigmine (15  $\mu$ M). Following another 10 min during which time the effluent was collected for measurement of basal ACh release, the sympathetic trunk was stimulated at a rate of 5 Hz and the samples collected every 2 min for the first 10 min and subsequently every 5 min for another 20 min. The conditioning did not affect basal ACh release or the pattern of ACh release evoked by stimulation compared to the controls (fig. 6.8). Moreover, the amount of ACh released as a result of stimulation was unchanged by the conditioning (fig. 6.8, inset). Thus, the size of the readily-releasable pool is not modified by the rebound ACh, and the increased ACh release (fig. 6.7) presumably results from increased mobilization of transmitter.



Figure 6.8: Effect of vesamicol on the release of rebound ACh. Both ganglia had been perfused with Krebs medium for 1 h. One ganglion was stimulated for 45 min at 15 Hz and then allowed a 15 min rest while the other ganglion was rested. The medium was changed to one containing vesamicol (10  $\mu$ M) and physostigmine (15  $\mu$ M). Following another 10 min of rest, the preganglionic trunk was stimulated at 5 Hz and the ACh output was measured from the control (°) and conditioned (•) ganglia and is shown as means of 6 experiments. Error bars have been omitted for clarity and the variation (± SEM) is shown in the inset. Inset: total evoked ACh release from control (open bar) and conditioned (closed bar) ganglia in the presence of vesamicol. Evoked release was calculated as the total ACh release minus the mean basal output estimated from measures before stimulation.

#### 6.5 Discussion

#### 6.5.1 The role of endogenous adenosine in the accumulation of rebound ACh

The present experiments were initiated in an attempt to test whether adenosine plays a role in an adaptive response of cholinergic nerve terminals that is caused by a period of conditioning stimulation. The rebound phenomenon is fairly well described in the literature (see introduction); it is manifest as an increase in ACh stores as a result of increased synthesis following prolonged tetanic activity. An increase in ACh content is evident in the presence of adenosine also (Tandon and Collier, 1993), and thus, it appeared possible that this purine, if endogenously released, could mediate the synthesis of rebound ACh.

We have shown previously that the adenosine effect to increase ACh stores was prevented by dipyridamole, an inhibitor of nucleoside transport. In the present work, dipyridamole clearly reduced the accumulation of rebound ACh, supporting the notion that adenosine might be involved in activating rebound ACh synthesis. Dipyridamole's reported ability to inhibit glucose transport (Deuticke et al., 1964) is unlikely to have been a factor in this action because neither ACh synthesis nor ACh release was altered by dipyridamole during prolonged stimulation. As both synthesis and release are highly sensitive to glucose deprivation during neuronal activity and cannot be sustained for long periods without it (Kahlson and MacIntosh, 1939), and dipyridamole is about 100-fold more potent in its inhibition of nucleoside transport compared to glucose transport (Woffendin and Plagemann, 1987), the most plausible explanation for dipyridamole's action is its inhibition of adenosine uptake. Moreover, the ACh synthesis machinery was not impaired, *per se*, by dipyridamole, and its inhibition of ACh synthesis was limited to rebound ACh, suggesting a clear difference in at least one of the mechanisms required for the activation of synthesis during and following tetanic stimulation.

Dipyridamole's inability to prevent the appearance of the extra ACh when it was present only during the stimulation period further eliminates the possibility that dipyridamole interfered with evoked ACh release or ACh synthesis during stimulation. It was, however, partly effective if present only in the rest period following stimulation, suggesting that, if adenosine is responsible for rebound ACh, its appearance and action follows the period of stimulation rather than during it.

This notion of a post-stimulation appearance of adenosine is compatible with results obtained in the experiments measuring ACh release in the presence and absence of an adenosine antagonist. This test exploited the adenosine receptor mediated suppression of transmitter release that occurs in a variety of preparations (see review by White, 1988), including the sympathetic ganglion as evidenced by the present result showing 33% inhibition of evoked ACh release by CHA. Blockade of these receptors by CPT revealed a 40% increase in the rate of ACh release evoked by test stimulation following tetanic conditioning, as if endogenous adenosine present at such time was inhibitory to ACh release in the absence of CPT. This increased output coincided with both the activation of rebound ACh synthesis and the dipyridamole-sensitive stage. It is also notable that the variability in ACh output from successive samples during the test stimulation after high frequency activity increased in the absence of CPT but did not in its presence (see fig. 6.6), possibly indicating that the time course or amount of adenosine release was somewhat variable between individual preparations. A similar variability in evoked ACh discharge following a prolonged high frequency stimulation was reported by Birks (1977).

Overall, these pharmacological tests support the possibility that adenosine acts as a signal to initiate the synthesis of rebound ACh after high frequency stimulation. However, some of our results might not be entirely consistent with adenosine as the only mediator of this phenomenon. First, dipyridamole present following the stimulation was only partly effective, perhaps because its access to its target was delayed when the perfusion medium was changed following tetanic stimulation, but possibly indicating that adenosine was only partly responsible. Second, RO 11-3624, an agent pharmacologically similar to dipyridamole in its ability to block both the NBTI-sensitive and -resistant carriers with
equivalent potency (Lee and Jarvis, 1988a), only partly inhibited the accumulation of rebound ACh, although in previous tests, the same concentration as used here was as effective as dipyridamole to decrease the adenosine effect. This also could indicate a role for some mediator other than adenosine; however, it must be kept in mind that the efficacy of this compound to completely block movement of adenosine through the NBTI-resistant transporters has not been properly studied. Shank and Baldy (1990) have reported that at least two, possibly three, NBTI-resistant nucleoside transport systems can be distinguished in synaptosomes prepared from rat or guinea-pig cerebral cortex, and the possibility that inhibition by RO 11-3624 is incomplete in one or more of these systems cannot be excluded.

An interesting feature of the present results from the experiments that tested the effect of NBTI or of meclonazepam was their ability to increase considerably the amount of rebound ACh formed. It is possible that this effect is unrelated to their ability to inhibit nucleoside uptake. However, considering that these two drugs are structurally quite different and that their only known similarity is their inhibition of the NBTI-sensitive nucleoside transporter, it might be that their effect to increase rebound ACh is based on this characteristic. If so, the result suggests that blockade of the NBTI-sensitive transporters shunts adenosine toward the NBTI-resistant sites and, in this way, provides more adenosine to whatever mechanism is responsible for its ability to increase ACh synthesis. The present result showing dipyridamole to block the NBTI-induced increase of rebound ACh is compatible with this notion. There are two interesting corollaries to this: first, the endogenous adenosine available to the preganglionic nerve terminals for transport by the NBTI-resistant carriers must be sub-saturating; second, the two types of nucleoside transporters must be in close enough apposition to each other for the postulated shunting of adenosine to be functional. With respect to the last point, the two types of nucleoside transporters are considered to co-exist on certain cell types (Belt, 1983ab; Plagemann and Wohlheuter, 1985), including neurons (Lee and Jarvis, 1988ab; Shank and Baldy, 1990;

Jones and Hammond, 1992). If this co-existence were to be so for the preganglionic nerve endings, it implies that the intracellular accumulation of adenosine that increases ACh synthesis would have to be restricted to a site close to the NBTI-resistant carriers, otherwise transport through the NBTI-sensitive transporters would be expected to have the same effect as it is unlikely that any intracellular process could distinguish adenosine on the basis of its site of entry. Thus, it seems more likely that the NBTI-sensitive uptake is not associated with preganglionic nerve endings but on structures close enough to them to affect the synaptic concentration of adenosine. Furthermore, the presence of adenosine transporters not linked to the synthesis of rebound ACh may represent potential targets for regulatory mechanisms to determine synaptic adenosine concentration, and thus, govern the extent of potentiation of transmitter stores depending upon the strength and duration of the conditioning stimulation.

We have previously speculated that one possible source of synaptic adenosine in the ganglia could be from ATP co-stored with ACh in synaptic vesicles (Tandon and Collier 1993). However, the results discussed above suggest that adenosine efflux in the present study likely occured after, not during, the conditioning stimulation, that is when ACh output had returned to basal levels. Thus, the endogenous synaptic adenosine does not appear to originate from vesicular stores co-released with ACh. Doubtless there are other mechanisms which might allow adenosine into the synaptic cleft that can be considered, including the nucleoside transporters themselves which are able to operate in reverse direction (see review by Geiger and Nagy, 1990). However, the simplest interpretation of our previous results with exogenous adenosine application suggest that adenosine uptake into nerve terminals preceeded activation of ACh synthesis, so it would be unlikely that, in the context of the present experiments, the same presynaptic sites which import adenosine are involved first with its export into the synapse. Thus, a postsynaptic source of adenosine appears to be more likely and there is evidence for this in other systems (Wolinsky and Patterson, 1985), as well as sympathetic ganglia (Rubio et al., 1988). Adenosine might act as a diffusible retrograde signal to accelerate ACh synthesis; there is appreciable interest in the involvement of retrograde transmission in activity-induced synaptic plasticity (see review by Jessell and Kandel, 1993).

This notion leads to the question of what signals adenosine release if it is to act as a retrograde transmitter. The role of ACh, the principal neurotransmitter, as this signal is unlikely. First, neither muscarinic (Bourdois et al., 1974) nor nicotinic (Collier et al., 1983) receptor activation appears to be obligatory for the formation of rebound ACh. Second, the rate of ACh output evoked by 16 Hz stimulation is not different from that at 64 Hz (Birks and MacIntosh, 1961), yet the synthesis of rebound ACh is clearly frequency dependent (Bourdois et al., 1974; Birks, 1977). Alternate transmitters, such as the peptides present in synaptic boutons of sympathetic ganglia (for review see Elfvin et al., 1993) may be more likely candidates, especially when it is considered that high frequency stimulation is generally a prerequisite for peptide release (for reviews see Bartfai et al., 1988; Kupfermann, 1991), just as it is for the initiation of the rebound phenomenon.

Overall, these results support a role for endogenous adenosine acting as a signal to activate the synthesis of rebound ACh; whether it is the only potentiating factor involved in the post-tetanic phenomenon is not clear.

### 6.5.2 Compartmentalization and release of rebound ACh

Kinetic analysis of transmitter output from ganglia suggests that the ACh release behaves as if it is stored in at least two compartments: a smaller readily releasable pool, and a larger reserve pool (Birks and MacIntosh, 1961). The compartmentalization of rebound ACh in this scheme has remained somewhat ambiguous. Bourdois et al. (1974) suggested that the rebound ACh incorporated into the reserve pool because conditioned ganglia exhibited an increased output of ACh only after a significant lag period. However, Birks (1977) reported that evoked release following the synthesis of the extra ACh was proportional to the increase in total transmitter stores. Furthermore, Collier et al. (1983) radiolabel!ed rebound ACh by presenting conditioned ganglia with [<sup>3</sup>H]choline during the rest period; when ACh release was evoked by subsequent preganglionic stimulation, the specific activity of the released ACh matched that of the tissue ACh, indicating that rebound ACh had mixed with preexisting stores prior to its discharge.

We extended these studies by measuring evoked ACh release following conditioning in the absence or presence of vesamicol, a drug which prevents the accumulation of ACh by vesicles (for review see Parsons et al., 1993) and allows the release of only about 14% of the total ganglionic ACh stores upon stimulation, a fraction which is considered to represent the readily releasable pool of transmitter (Collier et al., 1986; Cabeza and Collier, 1988; Prado et al., 1992). In the absence of vesamicol, the initial ACh output from conditioned ganglia was increased in proportion to the amount of rebound ACh accumulated. However, ACh release remained potentiated for the duration of stimulation whereas the ACh stores had returned to control levels by the end of stimulation. Therefore, while rebound ACh may have been the source of ACh initially, some other mechanism maintained release at an elevated level. It is not clear whether this longer lasting potentiation of release is related to an effect of adenosine or to some other simultaneously occurring process. For instance, it may be related to a similar phenomenon observed by Briggs et al. (1985) after a short tetanus, which did not require increased transmitter content. Alternatively, adenosine has been reported to induce a persistent increase in glutamate release from hippocampal slices, an action that is not mimicked by A1 or A2 receptor agonists (Nishimura et al., 1990, 1992; Okada et al., 1992); it is possible that a similar effect of adenosine is apparent in ganglia following the conditioning paradigm of the present experiments.

In the presence of vesamicol, conditioned ganglia released the same amount of ACh as their unconditioned controls, suggesting that the less readily releasable compartment adapts to incorporate the extra ACh and the size of the readily releasable compartment remains the same. The increased rate of ACh output from conditioned ganglia in the absence of vesamicol is presumably the result of an increased rate of mobilization from the less readily releasable pool. As rebound ACh mixes with the preexisting stores (Collier et al., 1983), it would appear that, although only the size of the reserve pool is modified, rebound ACh takes part in the tonic exchange of ACh molecules between between the reserve and readily releasable compartments. Similarly, our results using exogenous adenosine suggested also that the extra ACh synthesized in adenosine's presence was incorporated into, and increased release from, the ACh pool that requires mobilization for release (Tandon and Collier, 1993).

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4

# Preface to Section 7

The results presented in Section 6 suggest that endogenous adenosine might act as a retrograde messenger to activate the synthesis of rebound ACh following tetanic stimulation. Thus, the following experiments examine the role of the postsynaptic structures in producing the rebound phenomenon. This was done by directly stimulating the postganglionic nerves of the ganglia and determing whether ACh content was affected.

# Increased Acetylcholine Content Induced by Antidromic Stimulation of a Sympathetic Ganglion: A Possible Retrograde Action of Adenosine

Submitted to Neuroscience

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Abbreviations used: ACh, acetylcholine; NBTI, nitrobenzylthioinosine; TCA, trichloroacetic acid; TPB, tetraphenylboron; ChAT, choline acetyltransferase.

### 7.1 Abstract

Prolonged high frequency orthodromic stimulation of superior cervical ganglia is known to result in increased ACh synthesis and ACh content after the period of stimulation. In a previous study, we provided evidence to suggest that adenosine acts as an extracellular signal to activate this increased ACh synthesis and we proposed that the source of that adenosine might be postsynaptic. Thus, the purpose of the present study was to test whether direct stimulation of the postganglionic nerves could affect ganglionic ACh content. Antidromic conditioning of ganglia (15 Hz, 45 min) did not affect their ACh content. However, if ganglia were allowed a 15 min rest period after this antidromic conditioning, their ACh stores were increased by 20%; a similar increase was induced by 4 Hz stimulation before the rest period. During the 15 Hz antidromic stimulation, ACh release was not increased above the basal level, indicating that preganglionic nerve endings were not stimulated. Orthodromic stimulation (5 Hz) of ganglia 15 min after they been subjected to antidromic conditioning (15 Hz, 45 min), showed increased ACh release in comparison to that from control unconditioned ganglia. Moreover, the extra ACh released by the conditioned ganglia was quantitatively similar to the increase in the ACh stores, as if most or all of the additional ACh was released by preganglionic stimulation. If the antidromic conditioning and the rest period were done during perfusion with Ca2+-free medium, the ganglia did not accumulate extra ACh. The ACh content was also not changed if ganglia were conditioned in the absence of Ca<sup>2+</sup>, but rested with normal Ca<sup>2+</sup>. However, ACh content was increased by 23% when the antidromic stimulation was done with normal Ca<sup>2+</sup> but the rest period was without Ca<sup>2+</sup>. To test the role of adenosine in this retrograde effect, the effect of nucleoside transport inhibitors was tested. Dipyridamole blocked the antidromic stimulation-induced increase, but NBTI did not. Overall, these results are consistent with the idea that a diffusible retrograde messenger activates ACh synthesis. The sensitivity to blockade by dipyridamole suggests that adenosine might be that signal.

### 7.2 Introduction

Activity dependent modulation of synaptic transmission is recognized as an important feature of neuronal information processing (see reviews by Madison et al., 1991; Bliss and Collingridge, 1993). In sympathetic ganglia, several forms of synaptic potentiation can be revealed following certain paradigms of stimuli (see review by Briggs, 1994). One particular form of synaptic potentiation in superior cervical ganglia is a long-lasting increase in acetylcholine (ACh) content that can be induced by prolonged tetanic stimulation of preganglionic axons. This extra ACh (known as 'rebound ACh') results from an increased rate of ACh synthesis by ganglia following the period of increased preganglionic activity (Rosenblueth et al., 1939; Friesen and Khatter, 1971; Bourdois et al., 1974; Birks and Fitch, 1974; O'Regan and Collier, 1981; Collier et al., 1983).

To date, this rebound ACh has been studied only as a consequence of orthodromic stimulation. The present experiments test whether antidromic activity is an effective stimulus. The question arose from a previous suggestion that, as a result of preganglionic tetanic stimulation, postsynaptic structures release a diffusible mediator that acts retrogradely upon presynaptic nerve endings to increase ACh synthesis and the consequential accumulation of rebound ACh (Tandon and Collier, 1994). In that study, the accumulation of rebound ACh was sensitive, at least in part, to nucleoside transport inhibitors, and the results were interpreted as suggesting that an endogenous adenosinelike agent was present at the time that rebound ACh was synthesized, that is following, not during, the tetanic conditioning. We argued that a postsynaptic origin of this adenosinelike substance was more likely than a presynaptic one. We were attracted to adenosine because the rebound phenomenon was sensitive to blockage by adenosine transport inhibition and because we had previously reported (Tandon and Collier, 1993a) that ganglia exposed to exogenous adenosine increase their rate of ACh synthesis, and, consequently, their store of releasable transmitter; this effect of adenosine was not blocked by an adenosine receptor antagonist, but was prevented by certain nucleoside transport inhibitors.

Thus, the purpose of the present work was to test whether postsynaptic cells of a sympathetic ganglion can produce a diffusible substance that increases presynaptic ACh content during or following stimulation of the postganglionic nerves, and, if so, whether the effect is sensitive to nucleoside transport inhibitors. Some of this work has been presented in abstract form (Tandon and Collier, 1993b).

# 7.3 Materials and Methods

Materials used:  $[\gamma^{-32}P]$ ATP (10 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA); Amberlite CG-400 (chloride form), choline chloride, dipyridamole, nitrobenzylthioinosine, tetraphenylboron (sodium salt),  $\alpha$ -chloralose, acetylcholinesterase (EC 3.1.1.7, ACh hydrolase, type V-S), choline kinase (EC 2.7.1.32, ATP:choline phosphotransferase), and physostigmine sulfate were all purchased from Sigma Chemical Co. (St. Louis, MO, USA); butyronitrile was obtained from Aldrich Chem. Co. (Milwaukee, WI, USA); OptiPhase HiSafe II from LKB; EGTA was obtained from Eastman Kodak (Rochester, NY, USA); acetylcholine chloride was from Hoffmann-LaRoche (Basel, Switzerland).

### 7.3.1 Experimental procedures

Cats of either sex (1.5-4.0 kg) were anaesthetized with a 2:1 mixture of N2O/O2 containing 2% halothane, and, afterwards, with an i.v. injection of  $\alpha$ -chloralose (80 mg/kg). Surgical preparation for perfusion of superior cervical ganglia was done according to the method of Kibjakow (1933) as described by Collier and Kwok (1982). For antidromic stimulation, the postganglionic nerves were carefully desheathed and gently lifted onto a hooked electrode. The tissue was bathed in mineral oil to prevent the conduction of current to the surrounding tissues. All animal use procedures were in strict accordance with the guidelines of the Medical Research Council of Canada and the Canadian Council on Animal Care and were approved by the local Animal Care Committee. One or both ganglia were perfused (0.2-0.4 ml/min) with a Krebs solution of the following composition (mM): NaCl 120, KCl 4.6, CaCl<sub>2</sub> 2.4, KH<sub>2</sub>PO4 1.2, MgSO4 1.2, NaHCO3 25, and dextrose 10. The medium was filtered and then equilibrated with 5% CO<sub>2</sub> in O<sub>2</sub> to maintain a pH of 7.4 at 37°C. Choline chloride (10  $\mu$ M) was always added prior to perfusion, as were other drugs specified in the text describing particular experiments.

Ganglia were removed following the perfusion and placed in 1 ml of ice-cold 10% trichloroacetic acid (TCA) for 1 hour. In some experiments, the period of rest following tetanic stimulation was minimized by maintaining the stimulation until placement of ganglia into ice-cold TCA. The TCA was removed by extraction with ether and the aqueous layer was assayed for ACh content.

Stimulation of ganglia was done with square wave pulses (10 V, 0.5 ms) using a platinum electrode applied to the post- or preganglionic sympathetic nerves. For measuring ACh release, the perfusion medium contained physostigmine (15  $\mu$ M) and the perfusate was collected in 5 minute periods into pre-chilled tubes and stored on ice for not more than one hour. Samples were stored overnight at -20°C for assay the following day.

# 7.3.2 Determination of ACh

ACh in aqueous tissue extracts and the perfusate samples was recovered by the method of Fonnum (1969) as described Welner and Collier (1984). In brief, the samples were extracted by 400  $\mu$ l of tetraphenylboron in butyronitrile (TPB/butyronitrile; 10 mg/ml). The choline esters were removed from the organic phase by 150  $\mu$ l of AgNO3 (20 mg/ml); precipitation of excess silver was done with 10  $\mu$ l of MgCl<sub>2</sub> (1 M). Samples were lyophilized to dryness.

The ACh content of the samples was assayed by the radioenzymatic method of Goldberg and McCaman (1973). Choline was phosphorylated by choline kinase and ATP. ACh was hydrolyzed to choline with acetylcholinesterase and phosphorylated with  $[\gamma$ -32P]ATP. Anion-exchange chromatography was used to separate the phosphorylated choline from the labelled ATP and the radioactivity was determined by liquid scintillation spectrometry. 32P was measured in 10 ml OptiPhase HiSafe II with an counting efficiency of 99%.

# 7.3.3 Statistical analysis

Students t-test for paired samples was used for statistical comparisons. In all experiments, one ganglion was used as the test preparation and its contralateral served as control. The results, shown as percent or amount of change, were calculated for each pair of ganglia.

# 7.4 Results

#### 7.4.1 Effect of antidromic stimulation on ACh content

The initial experiments tested the effect of prolonged high frequency stimulation of the postganglionic nerves on ganglionic ACh content. As both superior cervical ganglia of each cat normally contain similar amounts of ACh (Feldberg, 1943; Birks and MacIntosh, 1961), the contralateral ganglion served as the control to provide an estimate of the initial content of the stimulated ganglion. In these experiments, the postganglionic fibers were stimulated at 15 Hz for 45 min. Stimulation of the nerves was maintained during removal of the ganglia so that the time between the end of stimulation and placement in cold trichloroacetic acid was minimal. Eight such experiments (fig. 7.1) indicated that the ACh content was not significantly (p>0.05) changed by the antidromic stimulation; the test ganglia contained  $8 \pm 4\%$  more than their contralateral controls (mean contralateral ACh content was  $1283 \pm 91$  pmoles). In seven other experiments, the test ganglia were stimulated antidromically for 45 min at 15 Hz as above, and were allowed a 15 min rest period before removal. As shown in fig. 7.1, ganglia conditioned in this manner increased their ACh content by  $20 \pm 5\%$  (p<0.02) over that of the contralateral ganglia (which contained  $1383 \pm 102$  pmoles). Thus, high frequency antidromic stimulation of the ganglion increased its ACh content; the effect was delayed and only apparent after the end of stimulation. These characteristics are similar to those associated to orthodromic stimulation (see Introduction for references).

We tested also whether antidromic stimulation at a lower frequency could elicit an increase in ACh content. As shown in fig. 7.1, six ganglia subjected to 4 Hz antidromic stimulation for 45 min followed by a 15 min rest contained an extra  $23 \pm 9\%$  (p<0.05; mean contralateral ACh content was  $1240 \pm 102$  pmoles). If the conditioning stimulation was 0.5 Hz, the extra ACh accumulated was  $15 \pm 7\%$  (n=6) more than the contralateral ACh content (1311 ± 93 pmoles); this difference was not statistically significant (p>0.05).



Figure 7.1: Effect of antidromic stimulation on ACh content. Ganglia were perfused with a Krebs solution and stimulated at the indicated frequency for 45 min; tissue was removed immediately or after a period of rest as indicated. Columns represent ACh stores of the stimulated ganglion as a percent of the untreated contralateral ganglion's value. The mean ACh content of 27 contralateral ganglia was  $1306 \pm 47$  pmoles. Each column shows the mean  $\pm$  SEM of 6-8 experiments, \*p<0.05.

# 7.4.2 Effect of antidromic stimulation on ACh output

High frequency stimulation of the ganglion's preganglionic input results in rebound ACh that, too, is only apparent during a rest period following the tetanic activity. Thus, it was necessary to test the possibility that the increased ACh levels in the experiments above (fig. 7.1) could have resulted from stimulation of preganglionic nerve terminals if the depolarizing current was conducted inadvertently over the surface of the ganglia to activate presynaptic nerve endings, or indirectly by stimulation of through-fibers that form synaptic contacts with preganglionic neurons. If presynaptic nerve terminals were being excited by the antidromic stimulation, an increased output of ACh would be expected to accompany such stimulation. To test this point, the effect of antidromic stimulation on ACh release was measured. Ganglia were perfused with a Krebs medium containing 15  $\mu$ M physostigmine to prevent the hydrolysis of released ACh by cholinesterase and stimulated antidromically for 45 min at 15 Hz. Table 7.1 shows that ACh release before and after stimulation was not clearly different from that during stimulation; the basal ACh release varied throughout the experiment with no clear change at the onset or offset of the stimulation. Thus, these results eliminate the possibility that the change in ACh content induced by antidromic stimulation was due to altered presynaptic activity and are consistent with the notion that a diffusible postsynaptic messenger can activate ACh synthesis. The results also indicate that the extra ACh that accumulates during the rest period following antidromic stimulation is the result of increased net synthesis and not reduced ACh release.

_	Collection	ACh output (pmol/min ± SEM)
Basal release before	1	$6.2 \pm 1.0$
conditioning	2	$4.5 \pm 0.9$
	3	3.5 ± 0.9
Release during antidromic stimulation	4	$6.3 \pm 1.5$
	5	$4.8 \pm 0.8$
	6	$5.4 \pm 1.0$
	7	$8.1 \pm 2.1$
	8	$6.8 \pm 1.7$
	9	$6.9 \pm 1.2$
	10	$6.2 \pm 1.3$
	11	$6.0 \pm 1.2$
	12	$4.5 \pm 0.8$
Basal release after	13	$3.3 \pm 0.8$
conditioning	14	$6.2 \pm 1.0$
	15	$4.1 \pm 1.6$

Table 7.1 Effect of antidromic stimulation on ACh release

Ganglia were perfused with a Krebs solution containing 15  $\mu$ M physostigmine and ACh release was measured before (collections 1-3), during (collections 4-12) and after (collections 13-15) a 45 min period of 15 Hz antidromic stimulation. ACh output (mean  $\pm$  SEM) measured in 5 min intervals from 8 individual experiments is presented.

### 7.4.3 Releasability of the extra ACh

These experiments tested whether the extra ACh synthesized following the antidromic conditioning is mobilized for release by subsequent preganglionic stimulation. To do this, ganglia were subjected to antidromic stimulation (45 min, 15 Hz), rested for 15 min, and then the preganglionic nerves were stimulated at 5 Hz for 30 min in the presence of physostigmine (15  $\mu$ M) during which time ACh release was measured. For the comparison, the ACh output from the contralateral ganglia, which had not been subjected to the antidromic conditioning, was measured also during 5 Hz stimulation of their preganglionic input.



Figure 7.2: Releasability of the extra ACh. Pairs of ganglia were perfused with Krebs solution containing 15  $\mu$ M physostigmine. The postganglionic trunk of the test ganglion had been stimulated for 45 min at 15 Hz and then allowed to rest for 15 min (the antidromic stimulation ended at time = 0 min); the control ganglia had not been stimulated antidromically. The preganglionic trunk of both ganglia was stimulated at 5 Hz for 30 min and ACh release was measured. The mean ± SEM ACh output from conditioned (•) and control (°) ganglia is shown. n=6, \*p<0.05.

Figure 7.2 shows the pattern of ACh release from the test and the control ganglia. Basal ACh release from the test ganglion (antidromically conditioned) was not different from

that from the control ganglia, but the initiation of the orthodromic 5 Hz test stimulation evoked the release of almost 50% more ACh from the conditioned ganglia than from the controls. The potentiation of release was significant (p<0.05) in the first 10 min of preganglionic stimulation and declined thereafter so that, by the end of the experiment, the test and the control ganglia did not differ in their rate of release. Overall, the extra ACh released in the first 20 min of preganglionic stimulation was  $291 \pm 100$  pmoles (p<0.05), which is similar in amount to the increase in ACh content induced by the antidromic conditioning, indicating that most, if not all, of the extra transmitter was mobilized into a releasable compartment.

### 7.4.4 Effect of Ca2+-free medium on synthesis of the extra ACh

To examine whether extracellular  $Ca^{2+}$  was required for the conditioning stimuli to induce ACh synthesis, the antidromic stimulation was done during perfusion of ganglia with Krebs medium in which the  $Ca^{2+}$  had been omitted and 0.1 mM EGTA added. Perfusion of three ganglia with this medium during 45 min of 15 Hz stimulation of the postganglionic nerves and 15 min of rest prevented the accumulation of extra ACh; the difference in ACh content between test and control ganglia was  $6 \pm 43$  pmoles (fig. 7.3; mean contralateral ACh content was  $1217 \pm 97$  pmoles). Thus, extracellular Ca<sup>2+</sup> appears to be required for the accumulation of the extra ACh.

Further tests were done to determine whether  $Ca^{2+}$  was necessary during the conditioning stimulation or during the subsequent rest period when the extra ACh is synthesized. In three experiments, the antidromic conditioning (15 Hz for 45 min) was done in the absence of Ca<sup>2+</sup> and the following 15 min rest period was with normal Ca<sup>2+</sup> containing medium. These ganglia contained 1600 ± 120 pmoles of ACh, not significantly greater (p>0.05) than the 1495 ± 89 pmoles of ACh in the contralateral ganglia (fig. 7.3). In contrast, five ganglia conditioned in the presence of Ca<sup>2+</sup>, but rested in its absence contained an extra 316 ± 90 pmoles (p<0.05) of ACh compared to the contralateral ACh

content of 1430  $\pm$  106 pmoles. Thus, the mechanism that triggers the formation of the extra ACh appears to be more dependent upon the presence of extracellular Ca<sup>2+</sup> for whatever occurs during stimulation than it is for whatever occurs following stimulation.



Figure 7.3: Effect of Ca<sup>2+</sup>-free medium on the accumulation of extra ACh. Ganglia were stimulated antidromically for 45 min at 15 Hz and then allowed a 15 min rest period. The Krebs medium was Ca<sup>2+</sup>-free for the entire experiment, during the stimulation period only, or during the rest period only. Columns represent ACh stores of the stimulated ganglion as a percent  $\pm$  SEM of the ACh contained in the untreated contralateral ganglion. The mean ACh content of 11 contralateral ganglia was 1390  $\pm$  64 pmoles. n= 3 to 5 experiments, \*p<0.05.

### 7.4.5 Effects of nucleoside transport inhibitors on the synthesis of extra ACh

Nucleoside transporters are classified into two groups, those blocked potently by nitrobenzylthioinosine (NBTI-sensitive) and those insensitive to high concentrations of NBTI (NBTI-resistant); both are inhibited by dipyridamole. In our previous tests, the effect of exogenous adenosine and of orthodromic stimulation to increase ACh content

was blocked by dipyridamole but not by NBTI. Thus, the final objective of the present study was to test whether the activation of ACh synthesis after antidromic conditioning shows similar pharmacological sensitivity with respect to nucleoside transport inhibitors. In these experiments, ganglia were conditioned as before (15 Hz antidromic stimulation for 45 min followed by a 15 min rest period) in the presence of nucleoside transport inhibitors. Figure 7.4 shows that dipyridamole (10  $\mu$ M) prevented the accumulation of the extra ACh induced by stimulation; ganglia conditioned and rested in its presence contained 71 ± 58 pmoles of ACh more than the contralateral ganglia (1439 ± 138 pmoles), not a significant change (p>0.2; n=6). In contrast, the presence of NBTI (100  $\mu$ M) during conditioning and rest did not block the stimulation-induced increase in ACh content. The transmitter store of the test ganglia (1580 ± 179 pmoles) was significantly greater than of the controls (1242 ± 91 pmoles; p<0.05; n=4). The increase in transmitter content of 26 ± 7% was comparable to that measured for ganglia which were stimulated in the absence of a nucleoside transport inhibitor.



Figure 7.4: Effect of nucleoside transport inhibitors on the sythesis of the extra ACh. Ganglia were conditioned (45 min of 15 Hz antidromic stimulation followed by 15 min of rest) in the presence of either dipyridamole (Dip; 10 µM) or nitrobenzylthioinosine (NBTI; 100 μM). n=6 4 ог experiments, respectively. Columns represent ACh stores of the stimulated ganglion as a percent  $\pm$  SEM of the untreated contralateral ganglion's value. The mean ACh content of 10 contralateral ganglia was  $1360 \pm 92$ pmoles. \*p<0.05.

### 7.5 Discussion

The traditional principle that synaptic transfer of information is unidirectional from the presynaptic nerve terminal to its target cell is no longer tenable in that now it is evident that neuronal communication can occur in a bidirectional fashion. Retrograde signalling across synapses is a characteristic that appears to be important in many facets of neuronal function, from developmental regulation of growth and transmitter phenotype to short- or long-term modification of synaptic transmission (for reviews see Jessell and Kandel, 1993; Davis and Murphy, 1994). The present work examined the possibility that the changes observed in ganglionic ACh synthesis following prolonged preganglionic tetanic stimulation might involve a retrograde signal. In order to do this, we tested whether antidromic stimulation could activate ACh synthesis in sympathetic ganglia. The answer was positive: direct stimulation of the postganglionic nerves increased ganglionic stores of ACh. The effect was not manifest immediately after the antidromic stimulation, but appeared several minutes later. Moreover, the effect of the conditioning did not depend on activation of preganglionic nerve endings as the ACh output rate remained at basal levels during and following the antidromic conditioning.

It is generally accepted that ganglionic choline acetyltransferase (ChAT) activity and ACh are uniquely present in the preganglionic fibers, particularly concentrated at presynaptic boutons. The basis for this notion is the disappearance of both these markers with degeneration following transection of the cervical sympathetic trunk (Feldberg, 1943; Banister and Scrase, 1950) and immunocytochemical localization of ChAT to preganglionic axons and terminals (Kasa et al., 1991). Furthermore, chronically decentralized ganglia lose the ability to synthesize ACh (Collier and Katz, 1971; Collier and Katz, 1974). Thus, the most plausible location of the extra ACh measured in the present work is the presynaptic nerve endings. The measurements of ACh release are compatible with this notion: soon after its formation, transmitter release evoked by preganglionic stimulation of conditioned ganglia was potentiated compared to that from

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the contralateral control ganglia. In these tests, the amount of extra ACh released from the conditioned ganglia was similar in quantity to the size of the stimulation-induced increase in ACh content, suggesting that most, or all, of the additional transmitter can be mobilized for release.

Overall, these results are in accord with the idea that antidromic stimulation promotes the secretion of a diffusible compound which acts upon presynaptic cells to initiate ACh synthesis. It is tempting to suggest that this mechanism is also involved in the effect of prolonged preganglionic stimulation to result in the production of rebound ACh. However, in the present experiments, the synthesis of extra ACh showed little dependence upon the rate of antidromic stimulation; ACh content was increased to the same extent by low-(4 Hz) or high-(15 Hz) frequency antidromic stimulation. This feature distinguishes the antidromic-induction of the rebound effect from that following orthodromic stimulation, which is clearly frequency dependent, requiring preganglionic stimulation in the range of 10-60 Hz (Friesen and Khatter, 1971; Bourdois et al., 1974; Birks and Fitch, 1974; Birks, 1977). If the two phenomena are related, then the requirement for high frequency activity is presumably presynaptic. We have previously speculated that, to induce rebound ACh, preganglionic high frequency stimulation may be necessary to effect the release of a non-cholinergic transmitter, possibly stored in dense-cored vesicles, the contents of which might activate the postsynaptic mechanisms to produce the necessary signal (Tandon and Collier, 1994); such vesicles are mobilized by high, but not low frequency stimulation (see reviews by Bartfai et al., 1988; Kupfermann, 1991; see also Weldon et al., 1993 for information on sympathetic ganglia). According to this scheme, antidromic stimulation would circumvent the requirement for high frequency presynaptic activation by directly exciting postganglionic cells.

The second objective of the present work was to test the possibility that the soluble messenger arising from postsynaptic sites is endogenous adenosine or an adenosine-like agent. We have recently reported two experimental conditions that increased ACh content, exposure of ganglia to exogenous adenosine (Tandon and Collier, 1993a) and tetanic stimulation of preganglionic axons (Tandon and Collier, 1994), where the increased rate of ACh synthesis was reduced by dipyridamole, a drug that blocks nucleoside transport. The action of adenosine to increase ACh content was not prevented by NBTI, as if particular adenosine transporters were involved, and the rebound phenomenon in response to preganglionic stimulation showed a similar selectivity. In the present experiments, the extra ACh formed as a result of antidromic stimulation was prevented if the conditioning was done in the presence of dipyridamole, but it was unaffected by the presence of NBTI, as if the postsynaptic signal that appears to initiate the presynaptic change of ACh synthesis required transport through dipyridamolesensitive, NBTI-resistant carriers. The possibility that dipyridamole depressed ACh synthesis in a nonspecific manner, unrelated to nucleoside transporters, is unlikely because it has been shown not to impair the ability of ganglia to synthesize and secrete transmitter during rest or stimulation and its actions, in previous tests, were limited to antagonizing the effect of exogenous adenosine or preventing the induction of rebound ACh (Tandon and Collier, 1993a; Tandon and Collier, 1994).

Thus, the most parsimonious interpretation of the action of dipyridamole to prevent the increased ACh synthesis that resulted from antidromic stimulation is that it indicates the involvement of an adenosine-like mediator as the retrograde messenger. The dipyridamole could act to prevent the uptake of released adenosine to the site at which it increases ACh synthesis or it could act to inhibit the release of adenosine. The inhibition by dipyridamole of adenosine efflux from a variety of neuronal preparations is wellreported (Daval and Barberis, 1981; Fredholm et al., 1982; Jonzon and Fredholm, 1985). It is possible that the effect of dipyridamole in the present results is two-fold: a decrease in the amount of adenosine released from postsynaptic sites and an inhibition of presynaptic nucleoside uptake.

The omission of Ca2+ from the perfusion medium prevented the action of

antidromic conditioning to increase tissue ACh. This effect was clearly not due to an inhibition of ACh production because the synthesis of the extra ACh, which occurred mainly after the stimulation, was independent of the presence of extracellular Ca2+ at the time of its synthesis. Therefore, the Ca2+ requirement most likely reflects decreased production or efflux of the retrograde messenger. With respect to a !enosine formation, it is known that increased metabolic activity for excended periods increases the demand for ATP hydrolysis (see reviews by Newby, 1984; Stone et al., 1990). If utilization of the nucleotide exceeds its synthesis, the local adenosine concentration is increased, which, consequently, increases its efflux. Adenosine production as a result of stimulation is dependent upon the presence of extracellular Ca<sup>2+</sup> (Kuroda and McIlwain, 1974; Hollins and Stone, 1980; Jonzon and Fredholm, 1985; Wolinsky and Patterson, 1985), suggesting that the nucleoside is likely derived from Ca2+-activated processes, possibly ones that involve ATP hydrolysis. For instance, increased adenosine concentration might result from enhanced use of ATP following activation of Ca<sup>2+</sup>-dependent protein kinases during stimulation (see reviews by Walaas and Greengard, 1991; Otani and Ben-Ari, 1993). The present results are compatible with this notion; a Ca2+-dependent increase in metabolic activity, which, after a prolonged period of ATP hydrolysis, outruns the ability of the cells to regenerate adequate nucleotide, leading to an increased adenosine concentration at postsynaptic sites and increased efflux as a consequence.

Relatively few studies have investigated whether adenosine released in sympathetic ganglia might originate from the postganglionic neurons. Stimulation-evoked release of [<sup>3</sup>H]adenosine has been shown to occur from cultured sympathetic neurons pre-loaded with [<sup>3</sup>H]adenosine (Wolinsky and Patterson, 1985), but because these neurons develop processes in culture, the contribution of the cell bodies to the adenosine efflux is difficult to estimate. McCaman and McAfee (1986) reported that rat sympathetic ganglia pre-exposed to radiolabelled adenosine subsequently released radiolabelled adenosine during preganglionic stimulation or K<sup>+</sup>-induced depolarization, but the source of the radiolabel

was not determined. However, Rubio et al. (1988) showed that antidromic stimulation of sympathetic ganglia which had been pre-exposed to [<sup>3</sup>H]adenosine elicits the release of the radiolabel, thus, providing direct support for the notion that postganglionic cells are capable of releasing adenosine in an activity-dependent manner. There is some evidence that suggests that exocytotic transmitter release can occur from dendrites in sympathetic ganglia (Zaidi and Matthews, 1991) and in other systems (Geffen et al., 1976, Pow and Morris, 1989). Thus, adenosine may be derived from postsynaptic vesicles. Alternatively, adenosine's transport across plasma membranes may be mediated by facilitated nucleoside carriers which operate in either direction and, thus, support the influx and the efflux of adenosine (see review by Geiger and Nagy, 1990). The direction of transport is dependent upon the concentration gradient, which is presumably inwards under most conditions due to the low levels of intracellular adenosine, but outwards when intracellular adenosine concentration is sufficiently elevated. Such carriers, if present on both pre- and postsynaptic surfaces could account for the postulated transneuronal movement of adenosine without a requirement for exocytotic release.

If adenosine is indeed the retrograde message that serves to increase ganglionic ACh content induced by conditioning stimulation, its consequence appears to be manifest following that stimulation, not during it. With preganglionic (Friesen and Khatter, 1971; Bourdois et al., 1974; Birks and Fitch, 1974; O'Regan and Collier, 1981; Collier et al., 1983) and postganglionic (present results) stimulation, this rebound ACh forms after, not during, activity, as if the adenosine release might be a post-stimulation phenomenon. Indeed, in many studies of stimulation-induced adenosine release, this feature is evident; most of the adenosine output occurred after the end of the stimulus (Hollins and Stone, 1980; Daval and Barberis, 1981; Pedata et al., 1990; Jonzon and Fredholm, 1985; Lloyd et al., 1993). This delay was not always attributed to slow diffusion out of the tissue, because the appearance of other transmitters was rapid and coincident with the period of stimulation (Jonzon and Fredholm, 1985). A particularly striking example was reported by

Hollins and Stone (1980); regardless of the duration of the stimulation period, adenosine was released from hippocampal slices only after stimulation. The formation of adenosine by increased metabolic activity and its subsequent passage through nucleoside transporters can account for the apparent lag in the appearance of adenosine. It has been suggested that nucleoside transporters are inhibited by depolarizing stimuli, secretagogues, and protein kinases A and C (Shank and Baldy, 1990; Sen et al., 1990; Delicado et al., 1991; Sen et al., 1993). Thus, an interesting consequence of this might be that efflux of adenosine is curtailed during stimulation that activates these kinases and only upon cessation of stimulation are these transporters disinhibited to allow the residual adenosine flow outwards. In the context of the present experiments, this could explain the dependence of the antidromic stimulation on  $Ca^{2+}$  during stimulation but not during the rest period, as facilitated adenosine transport is not particularly sensitive to low extracellular  $Ca^{2+}$  (Bender et al., 1980; Banay-Schwartz et al., 1980). Moreover, in our tests with preganglionic tetanic stimulation, the presence of adenosine appeared to occur following the stimulation period, but not during it (Tandon and Collier, 1994).

Altogether, the results reported in this work suggest that antidromic stimulation elicits adenosine efflux, which, following its transport into presynaptic nerve endings, activates ACh synthesis. Furthermore, the results are consistent with the possibility adenosine efflux from postsynaptic sites contributes to adaptive changes in ACh synthesis following preganglionic tetanic stimulation.

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**III. General Discussion** 

#### 8. Adenosine Increases Ganglionic ACh Content

In the last two decades, a number of studies have reported various effects of adenosine and activation of adenosine receptors on neuronal transmission. Most of these reports have characterized and established this purine as an inhibitory neuromodulator (reviewed by Stone, 1981; White, 1988; Dunwiddie, 1990; Greene and Haas, 1991), although there are a few reports (Brown et al., 1990; Correia-de Sá et al., 1992; Sebastião and Ribeiro, 1992; Kirk and Richardson, 1994) of facilitatory effects; both inhibition and augmentation of transmitter release in these studies were blocked by adenosine receptor antagonists. The present work also found evidence of adenosine's inhibitory action on neurotransmitter release, but, in addition, uncovered a role for adenosine in promoting ACh synthesis in a sympathetic ganglion that seems rather different from the previously described facilitatory effects. The first study of this thesis (section 5) showed that exogenous adenosine induced an increase in the rate of ACh synthesis, and, consequently, increased the tissue ACh content. This action of adenosine was somewhat unusual in that it did not result from the activation of classical extracellular adenosine receptors, as the effect was not blocked by a receptor antagonist. Furthermore, agents such as dipyridamole that inhibit adenosine's removal from the extracellular space by preventing its cellular uptake, blocked the stimulation of transmitter synthesis induced by exposure to adenosine. This pharmacological distinction suggested an important difference between the mechanism of action of adenosine observed in these studies and its effects typically mediated through the aforementioned extracellular receptors. Since dipyridamole did not by itself inhibit ACh synthesis, the simplest interpretation of these results was that by inhibiting the entry of adenosine into nerve endings, it prevented adenosine's access to and action at an intracellular site(s). In addition, it seemed possible that, if endogenous adenosine is released as a result of preganglionic stimulation, it might play a role in regulating ganglionic ACh content.

The second study (section 6) attempted to test this notion that endogenous adenosine might have a role in modulating ganglionic ACh synthesis by seeking a dipyridamole-sensitive component. Of the various aspects examined, the one that showed sensitivity to dipyridamole was the phenomenon whereby repetitive preganglionic stimulation produces a long-lasting increase in ganglionic ACh content, referred to as 'rebound ACh'. It was found that the formation of this rebound ACh showed some sensitivity to nucleoside transport inhibitors, suggesting that adenosine might function as a trigger for the post-stimulation phenomenon. Surprisingly, dipyridamole reduced the formation of rebound ACh if it was present only after the stimulation, but failed to do so if ganglia were exposed to it only during stimulation, indicating that the dipyridamolesensitive event was evident after the presynaptic conditioning. The role of adenosine as this post-conditioning signal was strengthened by the finding that the presence of an adenosine receptor antagonist following the tetanus appeared to facilitate ACh release, as if endogenous adenosine was present at that time at least in an amount sufficient to activate inhibitory presynaptic receptors to suppress transmitter output. The presence of synaptic adenosine after stimulation, but not during it, suggested a role for postsynaptic cells as the source of adenosine.

The final study (section 7) presented in this thesis tested this idea that a postsynaptic message might alter presynaptic function by determining whether stimulation of postsynaptic cells could influence presynaptic ACh synthesis. Indeed, antidromic stimulation was found to increase ACh synthesis, an effect that was inhibited by dipyridamole. These results are consistent with the notion that adenosine acts as a retrograde messenger.

### 8.1 A Model of Adenosine's Retrograde Action to Induce Rebound ACh

Since the specific aspects of the results have been discussed in the previous sections (5, 6 and 7), this 'general discussion' will attempt to link those three studies into a

single model. This model, illustrated in fig 8.1, is presented as speculation and is described in the context of orthodromic stimulation: repetitive preganglionic activity leads to the postsynaptic formation of adenosine, which, acting retrogradely, induces presynaptic ACh synthesis.

## 8.1.1 The orthograde signal: do neuropeptides modulate synaptic efficacy?

Because one of the main proposals of this thesis is that adenosine acts as a retrograde transmitter following high frequency presynaptic activity, it is important to consider what presynaptic factor(s) induces the postsynaptic cells to produce adenosine and release it. Two lines of evidence suggest that it is doubtful that the ACh released by the conditioning stimulation represents this orthograde signal: (i) the extent of rebound ACh formation is proportional to the rate of preganglionic stimulation in the range of 10-60 Hz (Bourdois et al., 1974; Birks and Fitch, 1974), whereas evoked ACh release is only frequency-dependent between 0-16 Hz (Birks and MacIntosh, 1961), and (ii) the accumulation of the extra transmitter is not dependent upon muscarinic (Bourdois et al., 1974) or nicotinic (Collier et al., 1983) cholinoceptor activation.

Thus, an alternate source of this signal must exist and is presumably noncholinergic. The non-cholinergic mediators are usually considered to be associated with the large dense-cored vesicles (LDCVs), which can be found in the synaptic boutons of the cat SCG (Elfvin, 1963). Thus, it is postulated that the release of the contents of LDCVs represent the initiating signal for the rebound phenomenon (fig. 8.1, step 1). Like the induction of rebound ACh, LDCVs generally appear to be mobilized for exocytosis more effectively by high frequency stimulation than low (reviewed by Hökfelt, 1991), a feature that is evident in the cat SCG (Weldon et al., 1993). The contents of these LDCVs have also been implicated as key players in another form of synaptic enhancement at sympathetic ganglia, a long-term potentiation (LTP) of synaptic transmission following brief (5 s) preganglionic tetanic stimulation (Bachoo and Polosa, 1991; Bachoo et al.,

1992). These studies showed that the ability of a tetanus to produce LTP was correlated with the presence of presynaptic LDCVs; the depletion of these organelles by overstimulation rendered the ganglia unable to generate subsequent LTP. Furthermore, the progressive recovery of the ability to produce LTP after over-stimulation followed a time course that matched the delivery of LDCVs to nerve endings from the cell soma. This recovery could be blocked by the disruption of fast axonal transport by colchicine, as if the LTP inducer was not synthesized locally, but needed to be replenished by axonal transport after synthesis in preganglionic cell bodies. Other studies have implicated neuropeptides, which are contained in LDCVs (reviewed by Fried, 1982; Elfvin et al., 1993), as inducers of synaptic plasticity at synapses in the central nervous system. Xie et al. (1991) reported that tetanic stimulation of cortical tissue released several substances, of which one was tentatively identified as a 69 kDa peptide, that induced LTP in hippocampal slices. There is also some evidence suggesting that opioid peptides might be involved in modulating hippocampal synaptic plasticity. This notion is based upon blockade by an opioid receptor antagonist, naloxone, of certain forms of hippocampal LTP and the potentiation of subthreshold tetanus by exogenous application of opioid agonists (reviewed by Bramham, 1992).

There is now extensive documentation supporting the notion that the release of neuropeptides at various synapses occurs in a frequency-dependent manner, requiring patterns of activity that involve rapid firing of nerve endings (e.g., Dutton and Dyball, 1979; Andersson et al., 1982; Lundberg et al., 1986; Whim and Lloyd, 1989). This has been shown for sympathetic ganglia, where high frequency preganglionic excitation increases peptide release and reduces the ganglionic content of neuropeptide immunoreactivity (Bachoo et al., 1987; Maher et al., 1991; Peng and Horn, 1991). The differential pattern of activity that is required for neuropeptide release as compared to the mohilization of classical transmitters has important implications with respect to the control of synaptic transmission; frequency-coding of preganglionic impulses is translated into

chemical coding across synaptic junctions. In effect, the input pattern, and, to some extent, the quantity of impulses, underlies the fine tuning of transmission. In the context of this thesis, presynaptic neuropeptides represent potential candidates for the presynaptic signal that initiates the sequence of events leading to the synthesis of rebound ACh.

The released peptide presumably acts postsynaptically (Fig 8.1, step 2) on its particular receptor or target and somehow produces the next postulated step, adenosine production (Fig. 8.1, step 3). Thus the next section will explore possible candidates for this step in the scheme.

## 8.1.2 Postsynaptic action of neuropeptides

A variety of actions of neuropeptides in sympathetic ganglia have been reported (reviewed by Dun, 1983; Elfvin et al., 1993). For instance, preganglionic stimulation in the presence of cholinergic antagonists has been shown to elicit a late, slow postsynaptic depolarization thought to represent a peptidergic component of transmission (Ashe and Libet, 1981). The length of this depolarization is prolonged by increasing the frequency or the duration of stimulation. In amphibian sympathetic ganglia, this non-cholinergic effect appears to be induced by a luteinizing hormone-releasing hormone-like factor (Jan et al., 1979; Jan et al., 1980; Jan and Jan, 1982). In mammalian sympathetic ganglia, however, the effector is not known with certainty, although several possible candidates exist based on their effects following exogenous application: substance P (Beleslin et al., 1960; Dun and Karczmar, 1979), neurotensin (Bachoo and Polosa, 1988; Maher et al., 1991) or vasoactive intestinal peptide (Machovà, 1987). In addition, peptides promote biochemical changes in postsynaptic neurons that may last for many minutes after the removal of the peptide. For example, vasoactive intestinal peptide application increases the phosphorylation state of tyrosine hydroxylase in the rat SCG through a cyclic AMPdependent step and results in increased enzyme activity (Ip and Zigmond, 1984; Ip et al., 1982; Ip et al., 1985).

These effects of exogenous neuropeptides, coupled with their presence in presynaptic boutons of sympathetic ganglia (reviewed by Elfvin et al, 1993), suggests that ganglionic neuropeptides exert some modulatory influence upon synaptic transmission. This regulation could be determined by the frequency and duration of the invading impulses, and also which particular preganglionic fibers fire, because the presynaptic transmitter complement may differ between individual preganglionic cells. In the case of the formation of rebound ACh, which is induced experimentally by stimulation of the cervical sympathetic trunk, it is likely that most, or all, preganglionic axons are stimulated. Thus, the possibility exists that more than one neuropeptide could be involved in initiating the postsynaptic events that lead to the formation of adenosine, which, subsequently, activates presynaptic ACh synthesis.

The opioid peptides, however, are of particular interest to this discussion of possible peptide-induced adenosine release. The presence of immunoreactivity to enkephalins has been demonstrated in cat preganglionic neurons located in the spinal cord (Krukoff et al., 1985; Krukoff, 1987) and in ganglia (Araujo and Collier, 1987; Bachoo et al., 1987). In addition, preganglionic stimulation reduces peptide immunoreactivity in ganglia, suggesting that their release is coupled to presynaptic activity (Bachoo et al., 1987). In addition, opioids have been shown to depress ACh release and transmission in the cat SCG by a mechanism that shows some sensitivity to naloxone (Araujo and Collier, 1987; Zhang et al., 1991). Thus, ganglionic opioids appear to be inhibitory modulators of synaptic transmission, but it is possible that, in addition, other actions are manifest. The presence of endogenous opioids in ganglia is intriguing in the context of adenosine release because the opiate, morphine, has been shown to induce adenosine release in vivo and in vitro from the spinal cord (Sweeney et al., 1987; Sweeney et al., 1989). Morphine or metenkephalin also increase electrically-evoked purine release from the cerebral cortex (Fredholm and Vernet, 1978; Phillis et al., 1979; Stone, 1981a). In addition, methylxanthines block morphine's inhibitory effect on electrically-induced contractions of

the guinea-pig ileum, suggesting that effect of the opiate is mediated by adenosine release and action (Sawynok and Jhamandas, 1976; Sawynok and Jhamandas, 1979).

Although, the intracellular events following morphine action at its receptors are not clearly understood, the morphine-induced adenosine efflux is dependent upon the presence of extracellular  $Ca^{2+}$  (Cahill et al., 1993a) and is sensitive to pertussis toxin, suggesting that a G-protein-activated second messenger system is involved (Sawynok et al., 1990). Moreover, this adenosine release is inhibited by dipyridamole and NBTI (dipyridamole is ten-fold more potent than NBTI in this action), suggesting that it occurs through facilitated nucleoside transporters (Sweeney et al., 1993). It is conceivable that one of the actions of ganglionic opioids might be to increase postsynaptic production of adenosine (fig. 8.1, step 3).

Whatever the nature of the stimulus, the mechanisms by which adenosine is produced and released from the postsynaptic cells must be considered, and that is discussed in the following section.

#### 8.1.3 Postsynaptic events: adenosine formation

Since the intracellular free adenosine concentration is normally maintained quite low by specific enzymes, such as adenosine kinase and adenosine deaminase (Kuroda and McIlwain, 1973; Barberis et al., 1981; Richardson and Brown, 1987), the nucleoside is presumably generated locally as a result of increased ATP usage following activation of neuropeptide receptors. The exact mechanisms are not known. However, one aspect of repetitive stimulation is the resulting increase in the activity of various kinases and the enhanced state of phosphorylation of many proteins (reviewed by Walaas and Greengard, 1991). Indeed, this increased level of phosphorylation is considered to contribute to the generation and maintenance of various forms of synaptic plasticity (reviewed by Otani and Ben-Ari, 1993). Increased phosphorylation. could also follow from receptor mediated events, so it is possible that enhanced kinase activity as a result of neuropeptide action, or that of another mediator, might be an important source of adenosine.

If the resulting increase of the local nucleoside concentration was to exceed the capacity of the removal systems, the concentration gradient would, therefore, direct the outward movement of adenosine through facilitated nucleoside carriers (fig. 8.1, step 4). Because adenosine's presence appeared to follow the crthodromic conditioning rather than accompany it (section 6.4.4), it is possible that adenosine efflux is delayed initially by a regulated decrease in transport activity or capacity by the inhibitory action of kinases on nucleoside transport (Sen et al., 1990; Delicado et al., 1991; Sen et al., 1993). Depolarizing stimuli also reduce nucleoside transport activity (Shank and Baldy, 1990). Consequently, adenosine release may occur only after the period of stimulation when the inhibitory influence on the nucleoside carriers is lessened. It is likely that dipyridamole's effect on the synthesis of rebound ACh by orthodromic or antidromic stimulation is, in part, due to an inhibition of step 4 (fig. 8.1), the release of endogenous adenosine.

#### 8.1.4 Presynaptic activation of ACh synthesis by adenosine

Once released, the extracellular adenosine is presumably subject to removal mechanisms that exist in the synaptic cleft, including simple diffusion away from the synaptic cleft to be hydrolyzed by ecto-adenosine deaminase or taken up by surrounding glial cells. This uptake might be sensitive to NBTI and meclonazepam, since the synthesis of rebound ACh was increased when ganglia were conditioned and rested in the presence of these agents (section 6.4.1), as if more adenosine was directed towards the putative presynaptic NBTI-resistant transporters. These dipyridamole-sensitive, NBTI-resistant carriers appear to mediate its passage into nerve endings (fig. 8.1, step 5) for delivery to the mechanism that increases transmitter synthesis. Inhibition of this uptake process is probably the primary action of dipyridamole to prevent the effect of exogenous adenosine (section 5.4.2). Likewise, dipyridamole's action to inhibit the increased transmitter content

induced by orthodromic (section 6.4.1) or antidromic (section 7.4.5) conditioning might also be, in part, related to inhibition of this uptake; presumably, a combination of inhibition of adenosine release (step 4) and adenosine uptake (step 5) reduces the movement of adenosine from post- to presynaptic sites, where it activates ACh synthesis.

The fact that extracellular choline was used for the increased ACh synthesis during perfusion of ganglia with exogenous adenosine (section 5.4.1) suggests that the rate choline uptake was either enhanced or that transport was more efficiently coupled to the acetylation mechanism (fig. 8.1, step 6). Increased choline transport seems to be associated with the synthesis of rebound ACh as the accumulation of choline analogs by conditioned ganglia is increased when compared to unstimulated controls (O'Regan and Collier, 1981; Collier et al., 1983). The mechanism by which the change in choline transport is effected, or the intracellular site through which adenosine promotes its effects on ACh synthesis, has not been investigated in the present work. The fact that adenosine has been shown to inhibit choline kinase in vitro (Wecker and Reinhardt, 1988), suggested the possibility that adenosine might be increasing ACh formation at the expense of phosphorylcholine or phospholipid synthesis. This, however, seems unlikely as the incorporation of radiolabelled choline into either of these phosphorylated choline pools was not reduced in the presence of adenosine. In fact, isotopic labelling of phosphorylated choline metabolites appeared to be increased somewhat in adenosine's presence, suggesting that some of the extra choline transported as a result of adenosine's action escaped acetylation and was phosphorylated, or, alternatively, that the activity of the low affinity choline transporter was increased. Chatteriee and Bhatnagar (1990) have proposed that the activity of the high affinity choline transporter can be modified by an ATPdependent phosphorylation, but whether intracellular adenosine affects this process is not known. Also, an intracellular adenosine receptor (the P-site) which inhibits cAMP formation has been identified on the catalytic subunit of adenylyl cyclase (Florio and Ross, 1983; Yeager et al., 1986; Johnson et al., 1989; Marone et al., 1990). However, it is difficult to envisage how a decrease in cAMP formation might increase choline transport or ACh synthesis, because increased cAMP production is correlated with increased choline transport and hemicholinium-3 binding (Breer and Knipper, 1990; Knipper et al., 1992). It cannot be excluded that adenosine affects this second messenger system, or others, through some alternate, as yet unidentified, intracellular site.

If one considers the view that choline transport and acetylation are dissociable processes, both requiring activation for optimal synthesis, it might be that the role of adenosine extends to modification of the acetylation rate. The synthesis of rebound ACh differs from transmitter synthesis during stimulation not only in its sensitivity to nucleoside transport inhibitors, but also in its supply of precursors. It is clear that, for both conditions, the supply of choline is enhanced. However, the provision of acetyl-CoA does not appear to be uniform in these conditions of increased ACh synthesis. This was shown by Kwok and Collier (1982) who measured isotope incorporation into ACh during perfusion of ganglia with tritiated acetate. In this experimental paradigm, where the conversion of acetate into acetyl-CoA is presumably catalyzed by a cytosolic acetyl-CoA synthetase, less radiolabelled ACh was accumulated if ganglia were stimulated than if they were rested, as if the supply of endogenous acetyl-CoA was increased during activity so as to compete with that derived from the exogenous tritiated acetate. When the incorporation of radiolabel into rebound ACh was measured, it was found to be increased compared to that of rested ganglia, as if the supply of endogenous acetate precursor was not augmented for rebound ACh synthesis. Thus, the argument that the provision of precursors is the only determinant for the rate of ACh synthesis does not hold, and suggests that the rate of acetylation might also be under regulatory control. Whether this regulation is mediated by a change in ChAT activity or some other event is unclear. Collier et al. (1983) reported that the total ganglionic ChAT activity was unaltered by tetanic conditioning. Furthermore, no measurable changes were observed in the activities of cytosolic, ionically membrane bound and the non-ionically membrane bound fractions of ChAT after tetanic

conditioning (Tandon and Collier, unpublished observations). However, these measures were made after the enzyme was solubilized, and the possibility that regulatory changes in enzyme activity are lost upon exposure to detergents cannot be excluded.

## 8.1.5 Compartmentalization of the extra transmitter

Once formed, the extra ACh could be stored in one of several compartments (fig. 8.1, step 7). It can become part of the releasable pool of transmitter (depot), which includes two fractions, or it can associate with transmitter that is not mobilized for release by nerve impulses (stationary ACh). Because the extra transmitter induced by adenosine or that which accumulated after ortho- or antidromic conditioning was releasable during preganglionic stimulation, it is concluded that most, or all, of the newly-synthesized ACh incorporates into the depot pool. Also, some of the rebound ACh might be subject to destruction by intracellular cholinesterases, since the presence of eserine augments the accumulation of extra ACh by preganglionic conditioning (section 6.4.2).

The depot pool is composed of a readily releasable fraction, and a less readily releasable fraction (Birks and MacIntosh, 1961). Access into the readily releasable compartment, presumably those vesicles closely associated with their release sites, appears to be gained at the expense of the pre-existing ACh molecules, since the amount of ACh in the readily releasable fraction, as determined by preganglionic stimulation during vesamicol exposure, seems unaltered by conditions that form rebound ACh (section 6.4.6); thus, a newly-synthesized ACh molecule enters if an older molecule is lost. Such exchange between the vesicular and extravesicular compartments is likely ongoing, probably associated with replacement of transmitter leaked out of vesicles.

The other fraction of the releasable store, the reserve pool, is considered to be comprised of extravesicular ACh and transmitter contained in vesicles away from release sites. The size of this fraction is presumably expanded to meet the demands of the extra transmitter (fig 8.1, step 8), though it remains unclear whether the extravesicular portion is enlarged or whether the vesicles accommodate more ACh. However, since basal ACh release is thought to be supported by the cytoplasmic ACh stores (see section 3.3.4) and basal efflux of transmitter was not modified by adenosine treatment, or by the ortho- or antidromic conditioning, it might be that most of the extra ACh entered a vesicular compartment. Indeed, the specific radioactivity of the spontaneous ACh output following exposure of ganglia to adenosine and radiolabelled choline was significantly lower than that of the subsequent evoked release, and appeared to contain almost no radiolabelled ACh that was synthesized in the presence of adenosine (fig. 5.3c).



Fig 8.1 The retrograde action of adenosine in the formation of rebound ACh. The drawing illustrates a schematic of the pre- and postsynaptic structures. High frequency stimulation mobilizes LDCVs to exocytose (1). The contents of these vesicles activate postsynaptic receptors (2), which result in increased ATP hydrolysis (3), thereby increasing the levels of free adenosine in the postsynaptic cells. The subsequent passage of adenosine down its concentration gradient into the synaptic cleft is mediated by facilitated nucleoside carriers (4). Synaptic adenosine transported by presynaptic NBTI-resistant nucleoside carriers to its intracellular targets (5). Choline transport and ACh synthesis are increased (6). The extra ACh formed is available to mix with the existing transmitter stores (7). The size of the 'depot' pool is consequently increased, but this change is accommodated by the less readily releasable ACh fraction (8), not the readily releasable one.

#### 8.2 Potentiation of ACh Release

As a result of the ACh exchange between the releasable fractions, the additional transmitter induced by exogenous adenosine (section 5.4.3) or orthodromic stimulation (Collier et al., 1983) appears to be mixed with the pre-existing stores shortly after its formation, and is given no preferential treatment for subsequent release. The total evoked ACh release, however, is significantly potentiated following either treatment that increased content. Since the amount of transmitter in the readily releasable compartment following adenosine treatment or orthodromic conditioning was not different from that in the control ganglia, at least as measured by evoked ACh release in vesamicol's presence (sections 5.4.4 and 6.4.6), the initial component of the enhanced release might be due to increased vesicle fusion per impulse. The results do not distinguish whether this potentiation follows from an increased number of release sites or an increased probability of release per site. The latter prospect is favored because it is generally argued that the readily releasable pool consists of docked vesicles. However, if one considers the possibility that the vesicles are somehow positioned near their release sites rather than at them, perhaps in a 'pre-docked' arrangement, either possibility can be envisioned. Recently, Neher and Zucker (1993) and Parsons et al., (1993) have proposed a model whereby a readily releasable compartment of transmitter is composed of vesicles associated with their release sites ('immediately available' for release) as well as a second group of vesicles that need to be mobilized to those release sites. Thus, if additional release sites were to be made accessible by adenosine action or by high frequency conditioning, those vesicles of the readily releasable fraction that normally require mobilization might become part of the 'immediately available' pool. Thus, evoked ACh could be increased without a change in the overall transmitter content of the readily releasable pool.

Once the readily releasable fraction is discharged, the subsequent release of transmitter presumably depends upon increased synthesis and increased mobilization of transmitter from the reserve pool. In those tests that measured the releasability of the extra

ACh following exposure to exogenous adenosine (section 5.4.3), enhanced transmitter synthesis was not a factor because the release was measured in the presence of HC-3, which prevents ACh synthesis (MacIntosh et al., 1956; Birks and MacIntosh, 1961). Since transmitter release was, nevertheless, enhanced, adenosine may have some direct or indirect effect on transmitter mobilization. It is difficult to speculate upon adenosine's mechanism of action because the factors that control mobilization are not well understood. However, in the last several years, a number of reports have appeared concerning synaptic vesicle movement and fusion, painting an increasingly complex picture of how exocytosis might be coordinated (reviewed by Kelly, 1993; Südhof et al., 1993; Jaha and Südhoff, 1994; O'Conner et al., 1994). These studies have provided insight into the molecular properties of vesicle proteins and their modulation by various biophysical and biochemical processes, in particular, calcium fluxes and kinase activity. It is conceivable that adenosine may have some effect on one or more of the processes that affect vesicle trafficking.

The potentiation of ACh release after preganglionic conditioning was long lasting, and continued for at least 45 min, even though the tissue ACh content had returned to the control value (section 6.4.5). This was not so when ACh release was measured from ganglia subjected to antidromic conditioning; in that case, the extra ACh released matched the amount by which the transmitter stores were increased and the enhanced output lasted for the first 10-15 min of stimulation. Thus, the long-term enhancement of transmitter release requires presynaptic activity, implying that the presynaptic biochemical events that follow the two forms of conditioning are different, at least in this respect. One aspect of long-term potentiation of transmitter release in sympathetic ganglia appears to be a strict requirement that presynaptic depolarization be accompanied by Ca<sup>2+</sup>-influx (Koyano et al., 1985; Briggs et al., 1985a; Briggs et al., 1985b). It is reasonable to assume that the ionic fluxes in nerve endings as a result of antidromic stimulation will not be the same, or as robust, as those induced by orthodromic stimulation. Thus, the increased ACh content and potentiation of ACh release following antidromic stimulation appear to be the result of the retrograde signal, whereas the increase in ACh release after orthodromic stimulation appears to represent both the retrograde signal and a separate component of potentiation, probably dependent upon presynaptic activity.

### 8.3 Maintenance of the Readily Releasable ACh Fraction

An interesting characteristic of the readily releasable fraction apparent in these studies is its relatively inflexible size despite the increased tissue ACh. This property appears to hold even when the total ACh content is reduced (Tandon, Bachoo, Weldon, Polosa, and Collier, submitted). In that study, ganglionic ACh content was reduced by 50% four days following blockade of fast axonal transport by exposure of the cervical sympathetic trunk to colchicine. In spite of this reduction of transmitter, the amount of ACh in the readily releasable pool appeared to be unchanged compared to that from control ganglia. The functional aspects of this ACh fraction also appeared to be relatively unaltered; ACh release induced by a low rate of stimulation (2 Hz) was similar to that from the controls, as if the transmitter pool that contributed to this release was not affected by the overall reduction in transmitter stores. Only during high rates of stimulation was the release impaired, suggesting the transmitter synthesis and mobilization could not keep pace. Thus, the size of the readily releasable pool appears to be preferentially maintained during periods of increased or decreased ACh content, while the reserve fraction accommodates these fluctuations. Since it is the readily releasable compartment that is considered to supply the initial transmitter for release during stimulation, this feature might serve as a homeostatic mechanism to allow nerve terminals to release a relatively uniform amount of transmitter, independently of the total transmitter content or nerve terminals.

It is not known how the ACh content of vesicles is controlled, although one governing factor is likely to be the activity of the vesicular ACh transporter. If ACh uptake into the vesicles of this fraction is always fully active, it could afford a simple mechanism

by which the ACh complement of the readily releasable fraction could be maintained, regardless of the tissue ACh content. Hence, the ACh content of this pool would be limited only by the summed capacity of the vesicles belonging to this compartment. In contrast, the amount of ACh stored in the reserve pool would be proportional to the total tissue ACh. However, for the readily releasable pool to be given priority over the reserve fraction in this manner would also imply that the regulation of ACh transporter activity of the two compartments differs. Some evidence that supports this notion has been provided by Gracz et al. (1988). They isolated VP1 and VP2 vesicle fractions from the electric organ of Torpedo and compared the ratio of [3H]ACh transport to [3H]vesamicol binding. It was found that this ratio was 4-7 times greater in the VP2 fraction as compared to that of the VP1 fraction. This suggests that the ACh transporters of the smaller, more metabolically active pool are more active that those in the larger, reserve pool. If the assumption is valid that the VP1 and VP2 fractions from Torpedo are homologous to the reserve and readily releasable fractions in mammalian nerve terminals, respectively, then it offers some explanation for the observed control of these pools in the sympathetic ganglion: the higher ACh transport activity of the readily releasable fraction might serve to keep its complement of transmitter at its maximum limit, while the reserve fraction, its uptake less brisk, could be more susceptible to changes in the total transmitter content of nerve terminals.

## **8.6 Retrograde Transmission as a Means of Synaptic Communication**

The most distinctive feature of neurons is clearly their ability to transmit electrical impulses over distances to specialized zones where the signal is translated into a chemical message and released across a brief span to an apposing cell. The target cell, equipped with appropriate receptor sites on its surface, responds to this chemical signal by triggering a change in its own electrical properties or by activating various intracellular biochemical pathways. This conventional view that synaptic communication between neurons occurs from pre- to postsynaptic cells only, has required some modification as a result of experimental evidence that suggests that dendrites can release transmitter (Geffen et al., 1976). It is now apparent that not only do the postsynaptic cells act as recipients of chemical information, but release substances into the synaptic cleft to transfer information retrogradely to the presynaptic cells. These retrograde signals participate in the early stages of neuronal development to define the physical and biochemical characteristics of presynaptic terminals, and during later stages to alter presynaptic excitability and orthograde transmission (reviewed by Jessel and Kandel, 1993).

The search for retrograde synaptic transmitters involved in presynaptic potentiation has intensified in recent years because of the properties associated with the induction of long-term potentiation (LTP), particularly that studied in the hippocampus. Brief, high frequency stimulation can produce a long-lasting increase in the efficacy of synaptic transmission (reviewed by Madison et al., 1991; Stevens, 1993). This activitydependent change observed in hippocampal synapses is widely considered to be related to learning and memory storage, whereby the strengthening of particular synapses in a usedependent manner leads to the consolidation and storage of experiences within an organized framework of neuronal connections. An important aspect of LTP induction is that both pre- and postsynaptic structures must undergo concurrent stimulation for transmission to be strengthened (Barrineuvo and Brown, 1983; Gustafsson and Wigström, 1986). The induction phase, however, appears to occur postsynaptically, because LTP is not induced if the postsynaptic action of glutamate is blocked by N-methyl-D-aspartate (NMDA) receptor antagonists (Collingridge et al., 1983; Collingridge and Bliss, 1987), if the postsynaptic cell is experimentally hyperpolarized during presynaptic tetanization (Malinow and Miller, 1984), or if postsynaptic kinases are inhibited (Malinow et al., 1989). Since part of the increase in synaptic efficacy is likely due to an enhancement of transmitter release from presynaptic terminals following LTP induction (Bekkers and Stevens, 1990; Malinow and Tsien, 1990), some form of communication must occur in a retrograde fashion to inform the stimulated nerve endings that LTP has been initiated. This signal appears diffusible because adjacent cells appear to be affected; weak presynaptic stimulation coupled with postsynaptic depolarization produces LTP in the paired synapse, but, in addition, enhances transmission of the same afferents that synapse onto adjacent cells (Bonhoeffer et al., 1989; Schuman and Madison, 1994b). This effect is limited by distance from the stimulated cell and to those cells whose dendritic field is intertwined with that of the paired cell, suggesting that the retrograde messenger is short-lived and unable to diffuse far from its release site.

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There are several studies that provide evidence of transmitter release from postsynaptic structures, involving the conventional organelles for transmitter storage and release (Geffen et al., 1976; Pow and Morris, 1989; Zaidi and Matthews, 1991). However, it appears that the chemical agents involved in hippocampal LTP used by postsynaptic cells for retrograde communication and the mechanisms to deliver them into the synaptic cleft are distinct from those used by presynaptic cells. Consequently, it is assumed that retrograde messengers are either readily available in the cytoplasm or produced locally just before release. The identity of the retrograde messenger(s) is not known with certainty, but several candidates have been proposed, based on their effects on synaptic transmission after exogenous application or on blockade of LTP by drugs that inhibit their synthesis: arachidonic acid (Linden et al., 1987; Lynch et al., 1989; Williams et al., 1989), nitric oxide (O'Dell et al., 1991; Schuman and Madison, 1991; Schuman and Madison, 1994b), carbon monoxide (Zhuo et al., 1993), and platelet-activating factor (Kato et al., 1994). The common feature of these compounds is that they are small, short-lived by virtue of their sensitivity to degradation, and pass through lipid bilayers with relative ease. Thus, their chemical properties fit the requirement for a short-lived retrograde messenger: diffusible, but for short distances.

To date, nitric oxide (NO) has received the most attention and the most experimental support for its role in LTP (reviewed by Jesell and Kandel, 1993; Schuman and Madison, 1994). Its intracellular targets are reported to be the soluble guanylyl cyclase and ADP-ribosyltransferase (Furchgott and Zawalski, 1980; Garthwaite et al., 1988), the latter of which might initiate the long-lasting features of LTP by covalent modification of presynaptic proteins. However, NO does not appear to play a role in all forms of hippocampal LTP. Lum-Ragan and Gribkoff (1993) recently showed that only LTP induced by certain trains of stimuli could be prevented by inhibitors NO synthase, the enzyme responsible for the generation of NO. This suggests the possibility that different patterns of stimulation might effect LTP using different retrograde messengers or different the nature of potentiation.

Several different forms of long-lasting synaptic potentiation can be induced experimentally in sympathetic ganglia, of which some may involve diffusible mediators (Briggs, 1994). However, the role of retrograde transmission in the induction of sympathetic LTP is not yet established. The present work (section 7) showing antidromic stimulation to alter presynaptic function provides support for this notion that retrograde transmission can effect activity-dependent changes in sympathetic ganglia. The sensitivity of this effect to dipyridamole is consistent with the possibility that the identity of this retrograde signal is adenosine. In general, the characteristics of the adenosine molecule fit some of the 'requirements' for diffusible retrograde transmitters. Adenosine is a relatively small compound whose entry into and out of cells is mediated by facilitated nucleoside carriers. If these transporters are present on both sides of the synapse, adenosine may easily pass into presynaptic terminals after being produced in postsynaptic structures as a result of activity or receptor-mediated events.

### 8.7 Summary

The three studies presented in this thesis have examined the role of adenosine in modulating ACh synthesis and release in a sympathetic ganglion. By using agents that reduced the effect of exogenous adenosine on these processes, evidence supporting a role for endogenous adenosine in mediating a component of use-dependent synaptic potentiation was uncovered. Overall, these results were interpreted as implicating adenosine as a retrograde transmitter to bring about this change in ganglionic ACh synthesis and release. In addition, these studies have provided some evidence that adenosine action is not necessarily associated with activation of its classical extracellular receptors, but that direct interaction of adenosine at unidentified intracellular targets might be involved.

## Contributions to Original Knowledge

The present work investigated the role of adenosine in modulating ACh synthesis in a sympathetic ganglion. In doing so, a role for adenosine in the in<sup>-</sup>tiation of a form of synaptic potentiation was uncovered. ACh synthesis, and consequently, ACh content, were increased by exogenous adenosine. The increased ACh content induced by high frequency stimulation showed properties compatible with a role for adenosine in this phenomenon and a retrograde messenger with properties like adenosine is implicated. As far as I am aware, these findings are original. Thus, the following are the my contributions to cholinergic neurobiology:

- 1. The effect of various purines on ACh synthesis in sympathetic ganglia was examined. Exposure to ATP, ADP, AMP and adenosine increased the ganglionic ACh content. Neither inosine nor a nonhydrolyzable analog of ATP changed the transmitter content. It was concluded that the active component inducing the change in synthesis was adenosine, as it is one of the degradation products of the phosphorylated purines, but not of inosine, or of the nonhydrolyzable ATP analog. This increased ACh content induced by adenosine was associated with an increased incorporation of radiolabelled choline into ACh as if the entry of choline from an extracellular source and its subsequent acetylation were increased.
- 2. Adenosine's action to increase ACh synthesis was not blocked by aminophylline, an inhibitor of adenosine receptors, but it showed sensitivity to inhibitors of nucleoside transport. The increase in ACh content induced by adenosine was blocked by dipyridamole and RO 11-3624, but not by nitrobenzylthioinosine (NBTT) or meclonazepam. This pharmacology was inconsistent with a role for extracellular adenosine receptors, but suggested an involvement of nucleoside transporters. These results are interpreted as suggesting that the action of adenosine was mediated through

an interaction at an intracellular site, following its uptake into nerve terminals through dipyridamole-sensitive, NBTI-resistant nucleoside transporters.

- 3. Preganglionic stimulation of ganglia pre-treated with adenosine and radiolabelled choline, released more ACh and more radiolabelled ACh than did control ganglia. The quantity of the extra ACh released matched the increase in ACh stores induced by adenosine, suggesting that most, or all, of the additional transmitter entered a releasable pool after synthesis. Furthermore, because the specific radioactivity of the ACh released from the test ganglion was not different from that from the control ganglia, it was concluded that the extra ACh was as releasable as the pre-existing transmitter stores.
- 4. At least two distinct subcellular pools of releasable ACh can be distinguished by their kinetics of release during preganglionic stimulation (Birks and MacIntosh, 1961): a smaller, readily releasable pool, and a larger, less readily releasable pool. The former, but not the latter, appears be released by preganglionic stimulation in the presence of vesamicol, a vesicular ACh uptake inhibitor (Collier et al., 1986). Stimulation of preganglionic input in vesamicol's presence of adenosine pre-treated ganglia released a similar amount of ACh as did the control ganglia. Thus, the size of the readily releasable pool was not modified by the adenosine-induced transmitter and the less readily releasable pool must therefore accommodate the extra ACh after its formation.
- 5. The role of endogenous adenosine in a post-stimulation increase in ACh content ('rebound ACh') was explored. The increase in ACh content following tetanic orthodromic stimulation was sensitive to nucleoside transport inhibitors: dipyridamole blocked the accumulation of rebound ACh, RO 11-3624 partly reduced it, and both, NBTI and meclonazepam doubled it. These changes were interpreted as suggesting a

role of endogenous adenosine in activating the synthesis of rebound ACh. The increase in rebound ACh by NBTI and meclonazepam was ascribed to an inhibition of NBTIsensitive nucleoside transport by adjacent cells, thus increasing the adenosine available for transport through NBTI-resistant transport on nerve terminals. The finding that dipyridamole blocked the increase induced by NBTI was consistent with this notion.

- 6. Dipyridamole reduced the accumulation of rebound ACh if it was present only during the rest period after stimulation, but did not affect the synthesis of the extra ACh if it was present only during stimulation. Thus, the dipyridamole-sensitive signal occurred after the period of stimulation. The identity of the signal was investigated by using an adenosine receptor antagonist: ACh release was measured in the presence of cyclopentyltheophylline (CPT) during 5 Hz test stimulation applied to the preganglionic trunk 2 min after the conditioning stimulation was completed. ACh release in the presence of CPT was transiently greater than that it its absence, as if CPT was preventing the inhibitory action of endogenous adenosine on ACh release just after the tetanic stimulation. These results were interpreted as suggesting that endogenous adenosine is present in the synaptic cleft after tetanic stimulation. One consequence of this adenosine might be that it triggers the formation of rebound ACh. These results also raised the possibility that adenosine was released from postganglionic cells, acting as a retrograde messenger.
- 7. Previous studies have shown that rebound ACh is releasable (Bourdois et al., 1974; Birks, 1977; Collier et al., 1983). This characteristic was confirmed in the present experiments also. However, it was not known which releasable fraction of ACh incorporates the extra transmitter. This question was addressed with vesamicol. Preganglionic stimulation of conditioned ganglia in the presence of vesamicol released a similar amount of ACh as did unconditioned controls. This suggested that the less

readily releasable pool of ACh is modified to accept the rebound ACh, whereas the size of the readily releasable pool is unchanged compared to that from the control ganglia which were not conditioned.

- 8. To test the possibility that postganglionic cells produce a signal that induces the rebound phenomenon, the effect of antidromic stimulation on ganglionic ACh content was tested. Stimulation of the postganglionic trunk increased presynaptic ACh content as a result of increased ACh synthesis after the period of conditioning. Since ACh release during antidromic stimulation was not increased, it was concluded that preganglionic nerve terminals were not stimulated. Thus, the increased synthesis was attributed to a diffusible retrograde signal released from postganglionic cells.
- 9. Preganglionic stimulation of ganglia that had been antidromically conditioned released more ACh than the control unconditioned ganglia. The extra ACh released was similar in quantity to the increase in ACh content suggesting that most or all of the extra ACh could be released by preganglionic stimulation.
- 10. The increased ACh content induced by antidromic stimulation was prevented by dipyridamole, but not NBTI. Thus, the action of the retrograde messenger shows pharmacological similarity to the adenosine-induced increase in ACh content.
- 11. These results suggest that endogenous adenosine released from postganglionic cells may act as a retrograde signal to activate preganglionic ACh synthesis following prolonged high frequency preganglionic stimulation.

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for General Introduction and General Discussion

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APPENDIX

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## Pharmacology and Thorepoutics

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