PACEMAKING IN EMBRYONIC CHICK HEART

by

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ABSTRACT

Experiments were conducted to determine the currents involved in the pacemaker activity of aggregates and single cells from the embryonic chick heart. Two microelectrode voltage clamp studies of embryonic chick ventricular heart cell aggregates revealed two time-dependent current components in the pacemaker range of potentials (-60 to -120 mV). Barium (Ba, 5 mM) blocked the more negatively activated time-dependent component unmasking a component which remained inwardly directed for hyperpolarizing steps beyond the potassium equilibrium potential (E_K). This demonstrates that the pacemaker current in embryonic chick ventricular heart cells is not a potassium (K) current which deactivates upon membrane hyperpolarization (the I_{k2} model) as proposed by Shrier and Clay (1980) and Clay and Shrier (1981a,b). This component, which was blocked by cesium (Cs, 2 mM), is consistent with an inward current which activates upon hyperpolarization (the I_f model) as proposed by DiFrancesco (1981a,b), for Purkinje fibers. The activation range of I_f suggests that this current could play an important role in the pacemaking of embryonic ventricular heart cell aggregates. Experiments conducted on embryonic chick atrial heart cell aggregates provide evidence for a current similar to the ventricular aggregate I_f current, but activated at more negative potentials (-90 to -120 mV). The more negative activation range suggests that I_f plays an insignificant role in the pacemaking of embryonic atrial heart cell aggregates.

In order to minimize the problems associated with the accumulation/depletion of ions in the extracellular space during voltage clamp experiments, studies were carried out on single ventricular cells or small clusters of ventricular cells. The currents measured in the single cells with

the whole cell patch clamp technique were similar to those observed in the ventricular aggregates except that I_f was activated at more negative potentials (-90 to -120 mV); a similar activation range was also found in single atrial cells. This current was blocked by cesium and its magnitude was greater when K_o was increased.

The time-dependent background current, which was blocked by Ba, displayed properties similar to the background component found in aggregates. In the pacemaker range of potentials, with 10 mM $[K]_o$, this current exhibited time-dependent inactivation. Moreover, the current versus voltage (I/V) plot of the instantaneous current showed inward-going rectification for steps positive to -70 mV and a linear voltage dependence for hyperpolarizing steps. This current is similar to the inward rectifier K current, I_{k1}, found in a variety of other cardiac cells and does not appear to be due to an accumulation/depletion atrifact, as proposed by DiFrancesco and Noble (1985). In the embryonic heart cells the kinetics of inactivation of I_{k1} are slower than that reported for other cardiac preparations. Although I_{k1} was present in embryonic atrial heart cell aggregates and in single atrial cells, its magnitude and time-dependent inactivation were less prevalent than in embryonic ventricular cells.

RESUME

Des recherches on été menées afin de déterminer les courants électriques impliqués dans l'activité rhytmogéne d'agrégats et de cellules uniques obtenus du coeur d'embryons de poussins. Des expériences utulisant la technique du potentiel imposé par double microélectrodes dans des agrégats de cellules ventriculaires embryonniques de poussins ont révélées la présence de deux courants électriques à dépendance temporelle dans la gamme de potentiel rhythmogénique (-60 à -120 mV). En bloquant le courant qui est activé le plus négativement par l'application de 5 mM de barium cela dévoile l'exisitence du deuxième courant qui demeure dirigé vers l'intérieur de la cellule de l'agrégat et ce pour tous les paliers hyperpolarizants au-delà du potentiel d'équilibre pour le potassium (E_K). Ce résultat démontre que le courant rhytmogène des cellules ventriculaires embryoniques des poussins n'est pas un courant potassique qui se désactive lors d'hyperpolarization comme c'est le cas dans le modèle I_{k2} proposé par Shrier et Clay (1980) et Clay et Shrier (1981a,b). Ce courant qui est bloqué par 2 mM de césium est compatible avec un courant dirigé vers l'intérieur de la cellule est activé lors d'hyperpolarization (le modèle I_f) proposé par DiFrancesco (1981a,b), pours les fibres de Purkinje. La zone d'activation de I_f suggére que le courant pourrait jouer un rôle important dans l'activité rhytmogène des cellules ventriculaire. Des expériences menées sur des aggrégats de cellules auriculaires d'embryons de poussins fournissent des preuves pour un courant similaire à I_f des aggrégats ventriculaires, mais activée a des potentiels plus négatif (-90 à -120 mV). L'activation de I_f à des potentiels plus négatif suggére que I_f ne pourait pas jouer un rôle important dans l'activité rhytmogène des cellules auriculaire.

Afin d'éviter des problèmes dus a l'accumulation ou la diminution d'ions souvent observés dans le cas de préparation multicellulaire, une autre série d'expériences ont été menées sur des cellules ventriculaire uniques où

sur de petits groupes de cellules ventriculaires. Les courants mesurés dans les cellules uniques par la méthode de "patch clamp" sont similaires à ceux observés dans les agrégats à l'exception que I_f est activé à des potentiels plus négatifs (-90 à -120 mV); une activation semblabe à été trouvé pour les cellules auriculaires uniques. Ce courant à été bloqué par le césium et son ampleur était plus grand avec une augmentation de K_o

Le courant de fond à dépendance temporelle qui est bloqué par l'application de barium, a des propriétés similaires au courant observé dans les agrégats. Dans la zone de potentiel rhytmogénique en présence de 10 mM de potassium extracellulaire, ce courant présente une inactivation à dépendance temporelle. De plus, le graphique du courant contre le voltage (I/V) pour le courant instantané montre une rectification vers l'intérieur pour des paliers positifs à -70 mV et une dépendance linéaire pour le voltage pour des paliers hyperpolarizants. Ce courant est similaire au courant potassium rectificateur vers l'intérieur (I_{k1}) retrouvé dans d'autres préparation cardiaques et ne semble pas être un artéfact produit par l'accumulation ou l'élimination d'ions comme proposé par DiFranceso et Noble (1985). Dans les cellules cardiaques embryonique les cinétiques du procédé d'inactivation de I_{k1} sont plus lentes que celles rapportées dans d'autres preparations cardiaques. Memes ci I_{k1} était présent dans les agrégats auriculaires du coeur d'embryons et dans les cellules auriculaires uniques son inactivation était moins apparent que dans les cellules uniques d'embryons ventriculaire.

TO MY FAMILY

JEAN-RENE, ROSE, RENE, DIANE, MICHAEL,
and TRACEY

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encouragement, and support
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TABLE OF CONTENTS

ABBREVIATIONS

Ag:AgCl	*silver: silver chloride*
AP	*action potential*
APD	*action potential duration*
AV node	*atrioventricular node*
Ba	*barium*
°C	*degrees Celsius*
Ca	*calcium*
Ca_i	*intracellular calcium*
Cs	*cesium*
D	*diameter*
E_i	*equilibrium potential (I_i)*
E_k	*potassium equilibrium potential*
E_{k2}	*equilibrium potential (I_{k2})*
E_{rev}	*reversal potential*
I_{bg}	*background current*
IBI	*inter-beat interval*
$I_{b,Na}$	*background sodium current ($I_{Na,bg}$)*
I_f	*pacemaker current (Na/K selective)*
I_h	*pacemaker current (SA node)*
I_k	*potassium current, delayed rectifier current*
I_{k1}	*inward rectifier current*
I_{k2}	*pacemaker current (K-selective)*
I_l	*leakage current (non-selective)*
I_{Na}	*sodium current, fast inward current*
I/V	*current versus voltage relationship*
I_x	*delayed rectifier*
I_{x1}	*delayed rectifier (1st component)*
I_{x2}	*delayed rectifier (2nd component)*
K	*potassium*
KCl	*potassium chloride*

K_i	*intracellular potassium*
K_o	*external potassium*
M	*molar*
MDP	*maximum diastolic potential*
ml	*milliliter*
M Ohms	*mega Ohms*
mM	*millimolar*
mV	*millivolt*
ms	*millisecond*
n	*number of preparations sampled*
nA	*nanoamps*
Na	*sodium*
Na_o	*external sodium concentration*
Na/K	*sodium/potassium*
pA	*picoamps*
P_{Na}	*sodium permeability*
s	*second*
SA node	*sinoatrial node*
TTX	*tetrodotoxin*
uM	*micron*
um	*micromolar*

CHAPTER I

CHAPTER I:
GENERAL INTRODUCTION

I.1 INTRODUCTION:

The normal heartbeat is initiated by a wave of electrical excitation arising from the sinoatrial (*SA*) node. This wave spreads through the cells of the right atrium and converges on a region of specialized cells, the atrioventricular (*AV*) node, which acts as an anatomical and functional link between the atria and the ventricles. Due to the special properties of the *AV node* the impulse is delayed allowing the ventricles time to fill with blood. From the *AV node* the electrical impulse then travels along the interventricular septum down specialized bundles which ramify into a latticework of fibres that cover the endocardial surface of the ventricles. Rapid spread of the impulse along these specialized conducting pathways ensures the appropriate coordination of ventricular excitation essential for the efficient pumping of blood.

The electrical impulse reflects a transient change in the transmembrane potential of the cardiac cell, referred to as the action potential (AP). The AP is a consequence of the movement of charged particles (ions) across the cell membrane. Ions flow across the membrane through small pores (channels) which span the membrane. The rate of change of membrane potential is determined by the magnitude of the flux of ions through these channels.

Some regions of the heart, such as the *SA* node, are spontaneously active because of a gradual diastolic depolarization of the membrane between *APs*. This slow diastolic depolarization has also been referred to as the pacemaker depolarization. The rate of change of the pacemaker depolarization is relatively slow when compared to the rate of voltage change during other phases of the cardiac cycle. This slow rate of

depolarization is a reflection of the small amplitude and slow rate of change of the currents flowing during this phase of the cardiac cycle.

The rate at which the *SA node* reaches threshold for the initiation of an action potential is largely determined by the ionic currents which underly the pacemaker depolarization. Therefore, the rate of change in conductance of the currents in the pacemaker range of potentials influences the rate at which the heart beats. Most regions of the adult heart, including the atria and ventricles, typically do not exhibit spontaneous activity. However, atrial or ventricular cells isolated from embryonic chick heart will generate spontaneous electrical activity when incubated in low $[K]_o$ solutions. This thesis will present experiments designed in an attempt to understand the ionic currents underlying the pacemaker mechanism of embryonic chick atrial and ventricular heart cells. Studies of the pacemaker mechanism will help to elucidate changes in the spontaneous activity of the heart which occur during embryonic differentiation, and also provides a basis for studies of the ionic mechanisms underlying the response of these cells to electrical stimulation and pharmacology. This information is essential for an understanding of the ionic nature of complex rhythms (dynamics) generated during the periodic stimulation of these cells, which may serve as a model for other spontaneously active cardiac cells.

I.2 THE VOLTAGE CLAMP TECHNIQUE:

The method used to study ionic currents is known as the voltage clamp technique. This technique of controlling membrane potential was first developed by Marmont (1949) and Cole (1949), and then gained popularity through a series of papers presented by Hodgkin and Huxley (1952,a-d) describing the currents underlying the *AP* of the squid giant axon. The general arrangement of the voltage clamp consists of the measurement of transmembrane voltage, a feedback amplifier which compares the recorded

voltage and the desired voltage, and the injection of current from the output of the feedback amplifier to reduce any error signal. A number of different voltage clamp methods have been developed including: (i) the axial wire (squid giant axon) technique, (ii) the two microelectrode technique, (iii) the sucrose gap technique, and (iv) the patch clamp technique, as well as many variations or combinations of the above. In the present study the two microelectrode voltage clamp and the whole cell patch clamp (Hamill et al., 1981) methods were employed.

I.3 THE K-DECAY THEORY:

An understanding of the ionic fluxes which generate the pacemaker depolarization is of fundamental importance for understanding the genesis of the normal cardiac cycle. Weidmann (1951) studied the conductance changes associated with the slow diastolic depolarization of the membrane potential in Purkinje fibers by injecting small current pulses. He noted a decrease in membrane slope conductance during the pacemaker depolarization and proposed that a decrease in *K* conductance, which had been activated during the cardiac *AP*, would allow a background *Na* permeability (P_{Na}) to drive the membrane potential away from E_k and towards threshold (*the K-decay theory*).

The earliest ionic model used to reproduce spontaneous activity evolved from voltage clamp experiments performed on the squid giant axon by Hodgkin and Huxley (1952a,b). Three ionic currents were described in these nerve fibers, a sodium current (I_{Na}), a potassium current (I_k), and a leakage current (I_l). Huxley (1959) modeled the repetitive firing of nerve axons observed in calcium (*Ca*) deficient solutions using the Hodgkin and Huxley (1952d) equations. Following the description of the ionic currents generating the *AP* in squid axons by Hodgkin and Huxley, efforts were made to measure and model the currents of cardiac preparations. Noble (1960, 1962a) modeled the pacemaker activity of Purkinje fibers according to the *K*-

decay theory. However, it soon became evident, after the introduction of the voltage clamp technique to cardiac tissue by Deck, Kern, and Trautwein (1964), that more currents were involved in the generation of the cardiac *AP*. The leakage current in cardiac preparations had been separated into a background *Na* current ($I_{b,Na}$), and a background *K*-current (I_{k1}) which showed inward-going rectification (Hutter and Noble, 1960). The presence of this inward rectifier current in Purkinje fibers helped to explain the higher membrane resistance found during the plateau of the action potential and the decrease in slope conduction during pacemaker depolarization described by Weidmann (1955).

Stronger evidence for a *K-decay theory* was provided by Deck and Trautwein (1964) from voltage clamp experiments performed on Purkinje fibers. They showed records of a pacemaker current which reversed at E_k, but failed to make a distinction between the pacemaker current and the delayed rectifier current (I_k) activated in the plateau range (-50 to 0 *mV*). Noble and Tsien (1968, 1969a,b) made this distinction. They provided evidence for a *K*-current with a slow activation upon depolarization and deactivation upon hyperpolarization within the plateau range. Since E_{rev} was found to be positive to E_k this current was not thought to be purely *K*-selective and was called I_x. Later, this current was separated into two components (I_{x1} and I_{x2}) due to a difference in kinetics and activation range of two *K* currents found in the plateau range (Noble and Tsien, 1969a). These plateau currents were believed to be responsible for the initial repolarization of the *AP*. The plateau currents were distinct from the current activated in the pacemaker range (-60 to -100 *mV*) which was thought to deactivate upon membrane hyperpolarization (I_{k2} *model*). The E_{rev} for this current varied by 60 mV with a 10-fold change in K_o in a manner expected for a pure *K*-current (Noble and Tsien, 1968; Peper and Trautwein, 1969). Vassale (1966) further strengthened the *K-decay theory* by reporting a decrease in membrane conductance in the pacemaker range.

Shrier and Clay (1980) paralleled the loss of automaticity in chick embryonic ventricular cells with a decrease in conductance of a voltage and time-dependent potassium current. This current was very similar to the I_{k2} current described by Noble and Tsien (1968) for Purkinje fibers. The current they described obeyed Hodgkin and Huxley (1952b) first order kinetics and had a steady state activation range between -60 and -90 *mV*. The kinetics of this channel were unaffected by K_o and had a peak time constant of 1 *s* at -75 *mV*. The fully activated *I/V* relationship displayed inward rectification with negative slope conductance at potentials 15 *mV* positive to its reversal potential. They also described a time-independent background current (I_{bg}) which inwardly rectified and had negative slope conductance for voltages positive to -80 *mV*. The decay of the I_{k2} component coupled with a net inward current supplied by a background *Na* current ($I_{Na,bg}$) brought the potential to the threshold of the *TTX*-sensitive Na current. The dominant factor for a loss of automaticity was attributed to I_{bg} becoming less inward with development.

I.4 THE REINTERPRETATION OF THE PACEMAKER CURRENT:

Several discrepancies in the behaviour of I_{k2} did not fit the *model*. The current was found to disappear in *Na*-free solutions (McAllister and Noble, 1966; DiFrancesco and McNaughton, 1979). The explanation for such a result was that I_{k2} was sensitive to internal calcium concentrations Ca_i which was known to increase in *Na*-free solutions. Another inconsistency involved the reversal potential for I_{k2} which was found to be 5 to 15 *mV* more negative than the expected reversal potential (Noble and Tsien, 1968; Peper and Trautwien, 1969; Cohen *et al.*, 1976). One factor which complicates the interpretation of the above results is the fact that changes in the concentration of ions in the extracellular cleft may vary with time, changing the equilibrium potential of a particular ion. This can result in time-dependent

changes in an ion current which are not an accurate reflection of changes in channel conductance with voltage. Cohen et al., (1976) suggested that E_{k2} might be more negative because of an increase in the activity of the Na/K pump causing a depletion of *K*-ions in the extracellular clefts. Moreover, Baumgarten *et al.*, (1977) suggested that the decaying time-dependent component of I_{k2} in Purkinje fibers may be overlapped by a depletion component involving I_{k1}.

The need to reinvestigate the pacemaker current arose from the observations of an inward pacemaker current present in the *SA node*, which contradicted the I_{k2} model proposed for Purkinje fibers. Noma *et al.* (1977) described a pacemaker current in the rabbit *SA node* (which they termed I_h) as having a very similar activation range to I_{k2}. Similarly, a time-dependent current was seen in the frog sinus venosus by Brown *et al.* (1977). Brown *et al.* (1979a) also noted that the acceleratory enhancement of adrenaline was mediated by its effect on the pacemaker current (which they termed I_f because of its *funny* nature). Several similarities existed between the I_f current in the *SA node* and the I_{k2} current in Purkinje fibers. DiFrancesco and Ojeda (1980) noted that I_f in the rabbit *SA node* was blocked by *Cs* in a manner similar to I_{k2}, and was decreased upon lowering $[Na]_o$. They also showed that I_f in these preparations was an inwardly-activating current which did not reverse at E_k. Soon after, DiFrancesco (1981a,b) reinterpreted the pacemaker current in Purkinje fibers as being an inward-going component which is activated upon membrane hyperpolarization (the I_f *model*). The effects of *Cs* were shown to be compatible with the blockade of an inward current, not an outward current and *Ba* was shown to block the apparent current reversal around E_k. DiFrancesco suggested that *Ba* blocked I_{k1} thereby removing a K-depletion process which was obscuring the true nature of the pacemaker current (DiFrancesco and Noble, 1980, DiFrancesco, 1981). The reversal potential for I_f was found to be around -30 *mV* and was dependent upon K_o and Na_o, suggesting that it is *Na/K* selective.

In cardiac Purkinje fibers the evidence for ion accumulation was the presence of a biphasic time-dependent current in response to hyperpolarizing voltage clamp pulses in the vicinity of E_k (Baumgarten and Isenberg, 1977; DiFrancesco and Noble, 1980). Contrary to the results in Purkinje fibers, Clay and Shrier (1981a) observed a flat response around E_k and ruled out the problem of ion accumulation during voltage clamp steps in the pacemaker range of embryonic chick ventricular heart cell aggregates. Clay and Shrier (1981a) attributed the lack of ion accumulation with a favorable geometry of heart cell aggregates and the small amplitude of I_{k2} relative to the currents activated in the plateau region.

DiFrancesco and Noble (1985) attempted to model the experimental results of Clay and Shrier (1981a,b). They made a number of changes to their equations to model a spherical aggregate including: (i) equations were modified for *K* diffusion within a spherical space instead of cylindrical equations, (ii) the sphere was assumed to have a radius of 100 *um* and an extracellular space volume of 4%, and (iii) the ionic currents were scaled down by a factor of 10 to give absolute values similar to those described by Clay and Shrier (1981a,b). DiFrancesco and Noble (1985) estimated that a 4% extracellular space would lead to a depletion of about 0.5 *mM* at the reversal potential. They argued that a flat current observed around E_k required another process such as a slow activation of an inward current. Their conclusion was that it is quantitatively improbable that depletion would be negligible in a 100 *um* aggregate for the magnitude of currents measured by Clay and Shrier.

In this thesis the pacemaker mechanism in embryonic heart cells is reanalyzed under conditions where an attempt is made to minimize the problems associated with the accumulation/depletion of ions by using small aggregates of embryonic heart cells (100 - 150 *um*), as well as single cells or small clusters of cells.

CHAPTER II

CHAPTER II:
THE PACEMAKER CURRENT IN EMBRYONIC CHICK HEART CELL AGGREGATES

II.1 SUMMARY:

The two microelectrode voltage clamp technique was used to investigate the pacemaker current components of embryonic chick ventricular and atrial heart cell aggregates. Voltage clamp steps in the pacemaker range of ventricular aggregates revealed a pacemaker current with an activation range between -70 and -90 mV, and an apparent reversal around E_k. The presence of an inwardly directed pacemaker current (I_f) followed by the decay of a time-dependent background current (I_{k1}), which was blocked by Ba (0.5 to 5 *mM*), could account for this reversal. The addition of *Cs* (2 *mM*) blocked the time dependence of the I_f component. In contrast, atrial preparations demonstrate a pacemaker-like component at more negative potentials (starting at -90 mV), with no apparent reversal. The lack of a time-dependent inactivation of the background component in atrial preparations and the presence of an I_f-like component in these cells could account for these observations.

In summary, the results presented in this study suggest that the pacemaker current in chick embryonic ventricular heart cells suggested by (Shrier and Clay, 1980; Clay and Shrier, 1981a,b) to be a pure *K*-current which deactivates upon membrane hyperpolarization (the I_{k2} model), should be reinterpreted as an inward current which activates upon hyperpolarization (the I_f model) (DiFrancesco, 1981a,b). In addition, evidence is also provided for the presence of an I_f-like component at more negative potentials in atrial heart cell aggregates.

II.2 INTRODUCTION:

Developmental Changes:

The embryonic chick heart undergoes developmental changes in automaticity. At early stages of embryonic development all regions of the embryonic heart beat spontaneously with a gradient of pulsation rates. If the heart at this stage is cut into fragments, the piece from the sinoatrial region beats at the intrinsic rate of the heart in situ, whereas pieces from the ventricular region and conoventricular end beat about 25 % and 75% slower, respectively (Barry,1942). Moreover, the beating rate is an inherent property of the individual cardiac cells (Cavanaugh, 1955) and the rate at which reaggregates of dissociated cells beat is a reflection of the region of the heart from which the cells were derived (DeHaan, 1970). Atrial preparations tend to beat faster than ventricular preparations, however there is considerable variability. Factors leading to this variability include the number of cells within a given aggregate (larger preparations tend to have a slower rate than smaller ones) (Sachs and DeHaan, 1973: Clay and DeHaan, 1979) and a dependence of spontaneous activity on the developmental stage (DeHaan, 1970; Shrier and Clay, 1980; Clay and Shrier, 1981b).

Ventricular and atrial aggregates prepared from cells isolated from embryos incubated for 2-7 days beat spontaneously. However, aggregates prepared from cells dissociated from the heart at later developmental stages are not spontaneously active and rest at more negative potentials (DeHaan, 1970; McDonald and DeHaan, 1973; Sachs and DeHaan, 1973). The chick embryonic heart at day 3 of development has a mean resting potential of -50 *mV*, whereas around day 12 of development the resting potential is around -80 *mV*. Sperelakis and Shigenobu (1972) measured K_o at different stages in development and estimated the intracellular potassium concentration (K_i). They determined that older preparations rest at more negative potentials

due to an increase in potassium permeability (P_k) and not due to an increase in E_k. Shrier and Clay (1980) paralleled this loss of automaticity in chick embryonic ventricular cells with a decrease in conductance of a voltage and time-dependent potassium current. This current was very similar to the I_{k2} current described by Noble and Tsien (1968) for Purkinje fibers. The dominant factor for a loss of automaticity was attributed to I_{bg} becoming less inward with development. The need to reinvestigate the pacemaker mechanism in embryonic chick heart arose from the experiments performed on calf Purkinje fibers by DiFrancesco (1981a,b) around the same time as the I_{k2} interpretation by Clay and Shrier (1981a). In this chapter results are presented of two microelectrode voltage clamp experiments performed on chick embryonic heart cell aggregates to re-examine the properties of the pacemaker currents.

A Comparison of Ventricular and Atrial Pacemaking:

Shrier and Clay (1982) investigated and compared the pacemaker properties of atrial and ventricular chick embryonic heart cells. They noted a significant difference in the shape of the pacemaker depolarization which was characteristic of the cell type. Atrial preparations rapidly depolarize from maximum diastolic potential (-90 *mV*) to a relatively stable range of pacemaker potentials (-80 to -75 *mV*) followed by a more gradual depolarization to threshold (-65 *mV*). In contrast, ventricular preparations slowly hyperpolarize to a maximum diastolic potential (-100 to -95 *mV*) before gradually depolarizing to threshold. They attributed the difference in pacemaker characteristics to differences in the underlying ionic current components regulating this depolarization in the two tissue types. Atrial preparations have a linear background current (I_{bg}) in the pacemaker range and lack a time-dependent pacemaker current. Computer simulations revealed that the initial phase of pacemaker depolarization in atrial cells was determined by the membrane time constant, which is the product of membrane capacitance and the slope resistance of I_{bg}. In ventricular

preparations, the hyperpolarization after maximum diastolic potential was assumed to be caused by I_{k2}. The final phase of depolarization in both cell types was attributed to the steady-state amplitude (window current) of the fast inward sodium current (I_{Na}) (Shrier and Clay, 1982).

II.3 MATERIALS AND METHODS:

Tissue culture techniques

Fertilized White Leghorn *(Gallus gallus)* chicken eggs were incubated at 37 *°C* and 95 % humidity for a period of 5-7 days. The hearts of the chicken embryos were extracted following decapitation under aseptic conditions. The ventricular or atrial portions of chick embryonic hearts were then dissected and pooled separately under a dissection microscope. The tissue was diced into small fragments and then dissociated into its component cells by a multiple-cycle trypsinization process described below (DeHaan, 1967, 1970).

First the cells were treated with 10 *ml* of 0.5 % trypsin (Sigma) for a period of 10 minutes in a 25 *ml* Erlenmeyer flask placed on a gyrator in a 37 *°C* incubator. The enzyme was then discarded (since it contained mostly red blood cells and few heart cells) leaving the heart tissue behind. Fresh trypsin (3.3 *ml*) was added for three cycles of 7 minutes each. After the first two cycles, the 3.3 *ml* of enzymatically dispersed cells was collected in a 50 *ml* Erlenmeyer flask. This flask contained 20 *ml* of trypsin inhibiting media (*TI*), which differs from the normal culture medium (*818A*) (DeHaan, 1970) by having a higher concentration (20 %) of heat-inactivated horse serum (Gibco) which inactives the enzyme. After the third and final treatment with enzyme the remainind tissue was mechanically disrupted with a 5 *ml* pipette and added to the *TI* containing flask. The *TI* medium was then filtered through a 12 *uM* membrane filter (*Nucleopore*) into a 50 *cc* centrifuge tube (*Falcon*).

The cells were then centrifuged for 10 to 15 min at 1000 *rpm* on a table top clinical centrifuge. The *TI* was then poured off and the remaining pellet of cells was resuspended in 1 ml of *818A*. This results in a suspension of 85-95 % single cells and small clusters. An aliquot was taken and the number of cells was counted with a hemacytometer.

An inoculum of approximately $5x10^5$ cells was added to 3 *ml* of *818A* to each 25 *ml* Erlenmeyer flask. The flasks were gassed with 5 % CO_2, 10 % O_2, 85 % N_2, sealed with a silicone stopper, and placed on a gyrator at 65-70 rev/min at 37 *°C* for a period of 48-72 *hr.* This allowed the single cells to collide and stick to one another forming aggregates of heart cells having diameters ranging from 50 to 300 *uM*, depending upon the rate of gyration.

Medium *818A* was comprised of 25 % M199 (Gibco), 2 % heat inactivated horse serum (Gibco), 4 % heat inactivated fetal bovine serum (Gibco), and 0.5 % gentamicin (Schering) in a balanced salt solution made from concentrated stock solutions consisting of in (*mM*): *NaCl*, 116; $MgSO_4$, 0.8; $NaHPO_4$, 0.9; $CaCl_2$, 1.8; $NaHCO_3$ 26.2; *glucose*, 5.5; and *KCl* 1.3. During experiments the cells were perfused with medium *818P*, which was similar to *818A*, except that it lacked serum. The *K* concentration of the extracellular media for ventricular aggregates was varied between 1.3 and 4 *mM*, with or without the additions of *Ba* (0.5 to 5 *mM*) and/or *Cs* (2 *mM*). The *K* concentration was maintained at 1.3 *mM* for the atrial preparations and the perfusate contained 3 *uM* tetrodotoxin (*TTX*, Sigma) to suppress excitability. All solutions were adjusted to a pH of 7.4.

An aliquot of aggregates was transferred to a 35 *mm* tissue culture dish (Corning No. 25000) which was placed onto the heated stage of a dissection microscope the day of the experiment. The aggregates adhered firmly to the bottom of the dish within approximately 1 *hr.* The temperature was maintained at 37 +/- 0.5 °C (continuously monitored) and perfused at a

rate of 0.75 *ml/min* with medium 818P (gassed with the mixture described above). The volume of the 35 *mm* culture dish was minimized to 1 ml or less by the use of a stainless steel insert.

Electrophysiological Techniques

Spontaneous electrical activity and membrane currents were measured using the two microelectrode voltage clamp technique, and standard voltage clamp circuitry. Two glass microelectrodes filled with 3 *M KCl* were impaled within different cells of aggregates which ranged in diameter from 100 to 150 *uM*. The electrode resistance varied from 50 to 80 *M Ohms*. The bathing medium was grounded via an agar salt bridge inserted into a syringe containing 3 *M KCl* and a *Ag:AgCl* pellet.

Data Analysis

An electronic stimulator (*Frederic Haer*, Pulsar 6-i) was used to deliver rectangular voltage steps. Both membrane current and transmembrane potential were recorded onto FM magnetic tape a with a Hewlett-Packard (3946A) tape recorder which had a 3-dB frequency response at 3.75 in/sec (DC-1250 Hz). All experimental results were played back on a digital oscilloscope (*Nicolet*, model 206) and then reconverted to an analogue signal and plotted on an x-y plotter (*Hewlett-Packard*, model 7015B).

Suitability of heart cell aggregates for voltage clamping:

The heart cell aggregate is well suited for voltage clamp experiments. The cells within an aggregate are well coupled to each other due to the presence of gap junctions between cells (DeHaan and Fozzard, 1975; DeFilice and DeHaan, 1977). Intracellular recordings from two widely separated cells of a spontaneously beating aggregate show very brief delays

(Clapham, 1979) between the upstrokes of their action potentials (<40 usec). Moreover, Clay *et al.* (1979) demonstrated that the input capacitance of an aggregate was directly proportional to D^3 , where D is the aggregate diameter, indicating that changes in membrane resistance reflect all cell membranes within an aggregate. Voltage clamp steps to depolarized potentials in the plateau range for varying periods of time, where one might expect to get variations in the accumulation of K, caused no change in E_k (personal observation). All these results indicate that the heart cell aggregate can be treated as having uniformity in potential with minimal ion accumulation or depletion.

II.4 RESULTS

Spontaneous activity

The majority of aggregates beat spontaneously in 1.3 *mM* $[K]_o$ at a fixed rate for extended periods of time (days) with inter-beat intervals (IBI) in the range of 0.5 to 2 *s*, the rate of which diminishes with increasing aggregate size (Sachs and DeHaan, 1973). Both the duration of the action potential (*AP*) and shape of the pacemaker depolarization are different in atrial and ventricular preparations. Atrial preparations have a short *AP* duration (*APD*) in the range of 85 *ms* (measured between the upstroke of the *AP* and the maximum diastolic potential *MDP*) with a *MDP* typically of -85 *mV* and an overshoot of approximately +25 *mV* (Shrier and Clay, 1986), whereas ventricular preparations have a longer *APD* in the order of 180 *ms* a *MDP* of -90 *mV* and an overshoot of +30 *mV* (Clay and Shrier, 1981a). The shape of the pacemaker depolarization for the two preparations differs and this has been attributed to a lack of the pacemaker current in atrial aggregates (Shrier and Clay, 1982) (see Fig. 2.1).

Fig 2.2 shows superimposed traces of the spontaneous activity recorded from two microelectrodes impaled within widely separated cells in a 7 day atrial aggregate. The amplitude of the two action potentials are similar and no apparent delay can be measured between the upstrokes of the two action potentials indicating a successful impalement and good electrical coupling between cells. Typically these two parameters were used as markers for optimal conditions before attempting to voltage clamp a particular preparation.

Voltage clamp analysis

I. Ventricular Aggregates:

Results from a 7 day old ventricular heart cell aggregate ($[K]_o$ = 4 *mM*; aggregate diameter = 100 *uM*) is shown in the left hand panel of Fig. 2.3. The holding potential was -60 *mV* and the step potentials were to -78, -87, -95, and -102 *mV*, respectively. The steps reveal a relatively small time-dependent current at -78 and -87 *mV* followed by an apparent reversal at -95 *mV*. These results, taken aione, are consistent with Clay and Shrier (1981a) and the I_{k2} interpretation of the pacemaker current (Noble and Tsien, 1968); except for the small amplitude and slow apparent reversal of this component beyond -95 *mV*. The time course of the decaying component is much slower than what was reported for Purkinje fibers (DiFrancesco, 1981a) and ventricular aggregates (Clay and Shrier, 1981a). DiFrancesco and Noble (1985) modeled this decaying component as a process of depletion for aggregates. The right hand panel of Fig. 2.3 represents the effects of 5 *mM Ba* on the same preparation. The holding potential is -60 *mV* and the steps are to -80, -88, -95, and -100 mV. The apparent reversal is no longer present and the current continues to be inwardly directed for steps beyond -100 *mV* (not shown). The background component is clearly decreased by *Ba* as evidenced by a decrease in the instantaneous jumps in current during hyperpolarizing steps. These results suggest that the effects of *Ba* are to

block the background current I_{k1}, which has a time-dependent relaxation. Similar results were observed in four other preparations with varying concentrations of *Ba* (0.5 to 5 *mM*). One of these results is shown in Fig. 2.4 for a 7 day ventricular aggregate (K_o = 4 *mM;* diameter = 150 *um*). The left hand panel of Fig. 2.4 differs from the left hand panel of Fig. 2.3 because of the biphasic appearance of the control record. The right hand panel represents the effects of *Ba* (0.5 *mM*) which removes both the biphasic appearance and apparent reversal. These results are similar to those seen for cardiac Purkinje fibers (DiFrancesco, 1981a) suggesting that the pacemaker current in these cells is indeed an inwardly activated component (the I_f interpretation).

Voltage clamp records from a holding potential of -67 *mV* for a 5-day old ventricular preparation ($[K]_o$ = 4 *mM;* diameter = 125 *um*) are shown in Fig. 2.5. These records exhibit a time-dependent current which increases in amplitude up to a potential of -94 *mV* followed by a clear biphasic time dependence between -102 and -109 *mV*, and a decaying current at -112 and -117 *mV*. This result clearly differs from the observations of Clay and Shrier (1981a) who never observed a biphasic response under identical conditions. The appearance of this biphasic nature of the pacemaker current is suggestive of two components interacting with one another around E_k. The saturation of current tails for this particular preparation occurred around -90 *mV* giving the pacemaker current an activation range of -70 to -90 *mV*. This activation range of the pacemaker current is consistent with the results of Clay and Shrier (1981a), however, the amplitude of the pacemaker current at day 7 of incubation on average was reduced. In order to attain a more robust pacemaker current younger developmental stages were also used in this study. The lack of a large time-dependent *Ba* insensitive current at 7 days suggests that there may be variability in the developmental loss of this time-dependent current.

The effect of *Cs* (2 *mM*) is illustrated in Fig. 2.6 for a 5 day old

ventricular aggregate (K_o = 1.3 *mM; Ba* = 5 *mM*; diameter = 150 *uM*). The holding potential was -65 *mV* and the control step was to -105 mV, and to -100 *mV* in the presence of *Cs*. Cesium blocked most of the pacemaker current at this concentration. A similar blocking effect of *Cs* was seen in two other preparations. These results are also comparable to the results shown for cardiac Purkinje fibers (DiFrancesco, 1981a,b).

II. Atrial Aggregates:

The results shown in Fig. 2.7 are taken from a 7 day old atrial aggregate (K_o = 1.3 *mM*, *TTX* = 3 *um*; *diameter* = 200 *um*). The holding potential is -60 *mV* and the voltage steps are to -70, -80, -90, -100, and -110 *mV*, respectively. The steps to -70 and -80 mV display a time-independent component, whereas, steps beginning at, and more negative than, -90 mV display a time-dependent component which increases in amplitude with no apparent current reversal. These results are consistent with the observations of Clay and Shrier (1986) who found no time-dependent current over the pacemaker range of potentials. However, they suggest the presence of an additional I_f-like component at potentials negative to -90 *mV*. Similar results were observed in four other preparations (K_o = 1.3 *mM*; developmental stage = 7 days) all of which displayed a time-dependent component of small amplitude at negative potentials, and no apparent reversal of this component. The relative sizes of the atrial aggregates used in these experiments was equivalent to that used for the ventricular aggregates described above. The fact that no apparent reversal was seen for equivalent sized atrial preparations where one may expect to observe a depletion process as described by DiFrancesco and Noble (1985) argues against such a process contributing significantly to ionic currents in these preparations. The lack of an apparent reversal is associated with the lack of a time-dependent background current (I_{k1}) which is present within ventricular aggregates.

The steady-state current voltage (*I/V*) relationship for the atrial

aggregate described in Fig 2.7 is illustrated in Fig. 2.8. The measurements were taken at the end of 5 *s* voltage clamp steps when the current had reached a *steady-state.* The *I/V* relationship crosses the zero current level around -60 mV, the potential at which most of these preparations rest when exposed to TTX (3 *um*). The current increases at potentials positive to -55 mV which is the potential range where the plateau currents (I_{x1}, I_{x2}) are activated (Clay and Shrier, 1986). A flat portion of the *I/V* relationship occurs between the potentials of -60 to -85 *mV,* where the amplitudes of the currents flowing are small when compared to those found in the plateau range. At potentials negative to -85 *mV* the I/V curve increases inwardly due to the activation of an I_f-like component in this range and perhaps an increase in I_{k1} conductance. Due to the flat nature of the *I/V* relationship between -60 and -85 *mV* very little inward current would be necessary to depolarize the membrane towards the threshold of the fast inward sodium current (I_{Na}).

II.5 DISCUSSION:

The results presented in this study suggest that the pacemaker current in chick embryonic ventricular heart cell aggregates should be interpreted as an inward current which activates upon hyperpolarization (the I_f model). Until more recently, the background component in cardiac preparations was believed to be time-independent. However, experiments performed on guinea pig heart cells by Sakmann and Trube (1984) clearly identify a time-dependent inactivation of I_{k1} based on single channel analysis. Furthermore, they demonstrated that *Ba* (0.5 *mM*) blocked the conductance of I_{k1}. DiFrancesco and Noble (1985) suggested that the effects of *Ba* were attributed to a blockade of a potassium ion accumulation/depletion effect produced by I_{k1}. They modeled the effects of this accumulation/depletion process for ventricular aggregates according to the conditions described by Clay and Shrier (1981a,b). From the experiments described above, on aggregates, we cannot exclude this possibility; however, experiments

described below, using single cells, suggest that, if present, accumulation/depletion is not a major factor. Although, DiFrancesco and Noble (1985) were successful in reproducing theoretically the Clay and Shrier (1981a,b) experimental results, it is more likely that they overlooked the time-dependent inactivation of I_{k1} which was not established at that time. A two component model of time-dependent currents incorporating an inward current (I_f) which activates and a background potassium current (I_{k1}) which inactivates upon membrane hyperpolarization is consistent with these results.

The activation range of the pacemaker component in ventricular aggregates suggests that it could play a role in the pacemaking of these preparations. The developmental loss of this component remains as one element which could contribute to the loss of automaticity in embryonic chick ventricular cells (Clay and Shrier, 1981b). However, I_{k1} is another attractive candidate which may be involved in the developmental decrease in automaticity and the more negative resting potential of these preparations. An increase selectivity of I_{k1} to K could lead to a more negative reversal for this component and consequently the preparation would rest at potentials closer to E_K. It is quite clear that embryonic chick heart cells rest at more negative potentials with development not solely because of a loss of I_f, but because the sum of the background currents becomes less inward with development.

An I_f-like component was seen in atrial preparations at potentials negative to -90 *mV*. The presence of such a component negative to the voltage range for spontaneous activity of these cells suggests that this component may not play a significant role in pacemaker depolarization. However, this component may play a more prominent role under conditions that hyperpolarize the membrane to a voltage range where I_f is activated. Such a condition exists when these preparations are exposed to acetylcholine, which activates a K conductance and hyperpolarizes the cell membrane (Shrier and Brochu, 1984: Shrier, Brochu, and Clay, 1985). The

lack of an apparent current reversal for these preparations may be due to a less prominent time-dependent inactivation of I_{K1} in these cells.

FIGURES

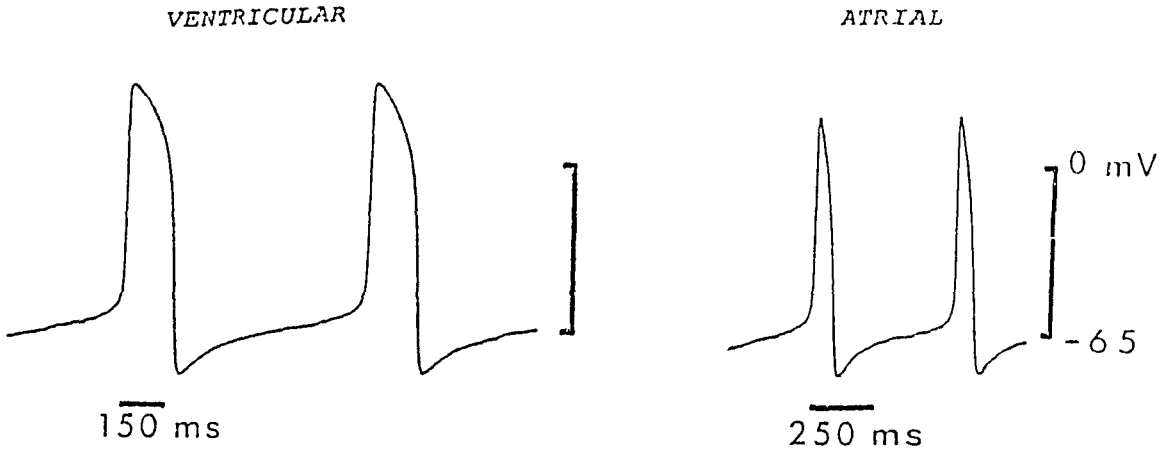

Fig. 2.1 Left hand panel: Spontaneous activity recorded from a 7 day old ventricular aggregate (K_o = 1.3 mM). Right hand panel: Spontaneous activity recorded from a 7 day old atrial aggregate (K_o = 1.3 mM).

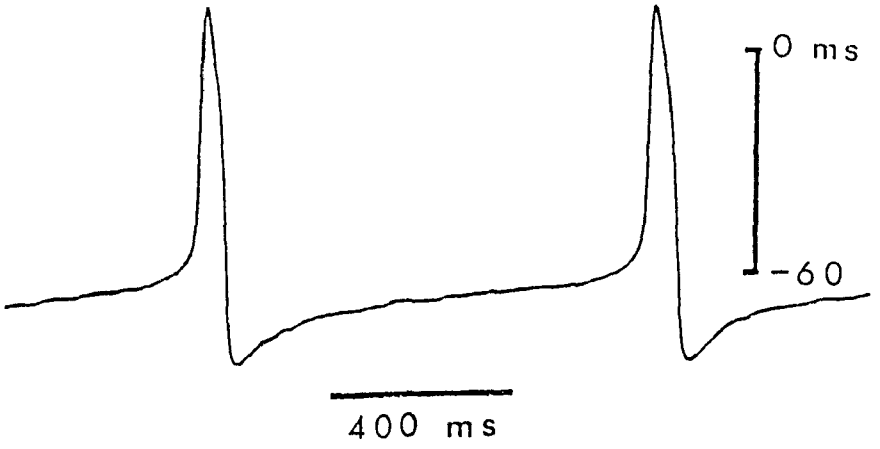

Fig. 2.2 Spontaneous activity induced by injury recorded from a 7 day old atrial aggregate following impalement by two microelectrodes within widely separated cells of the preparation (K_o = 1.3 mM; 3 um TTX).

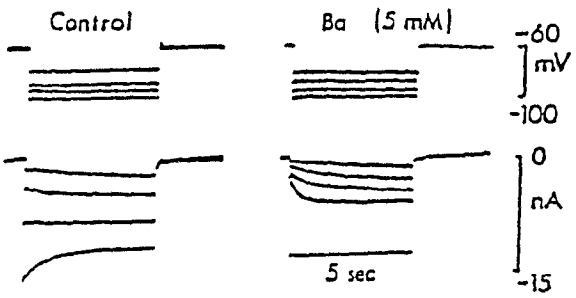

Fig. 2.3 Left hand panel: Voltage clamp results from a 7 day old ventricular aggregate (K_o = 4 mM; diameter = 100 um). Holding potential -60 mV, step potentials to -78, -87, -95, and -102 mV. Right hand panel: Effects of Ba (5 mM) on same preparation: Step potentials to -80, -88, -95, and -100 mV.

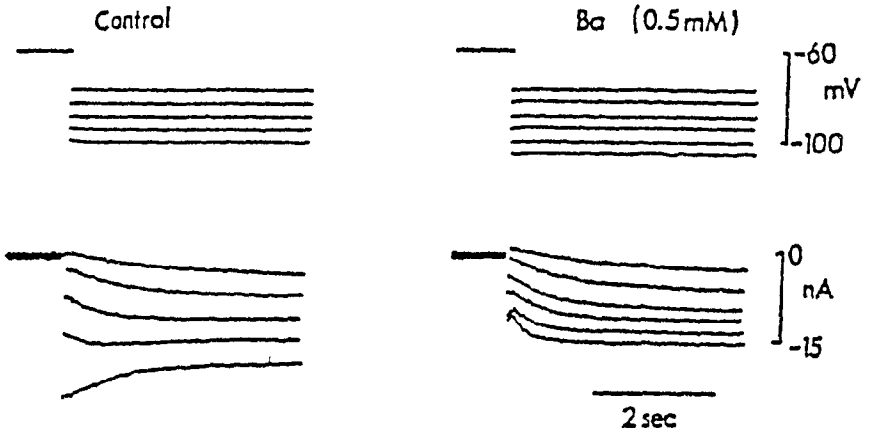

Fig. 2.4 Left hand panel: Voltage clamp results from a 7 day old ventricular aggregate (K_o = 4 mM; diameter = 150 um). Right hand panel: Effects of Ba (0.5 mM) on same preparation.

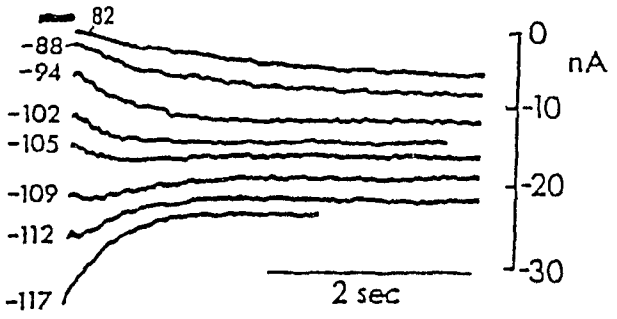

Fig. 2.5 Voltage clamp steps from a 5 day old ventricular aggregate (K_o = 4 mM; diameter = 125 um). Holding potential was -67 mV, step potentials are shown to the left.

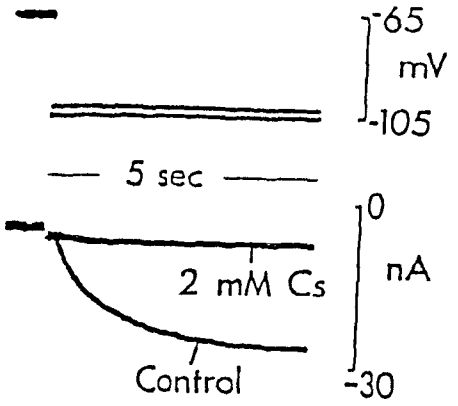

Fig. 2.6 Effects of Cs (2 mM) on a 5 day old ventricular aggregate (K_o = 1.3 mM; diameter = 150 um) Control step is to -105 mV, Cs step is to -100 mV.

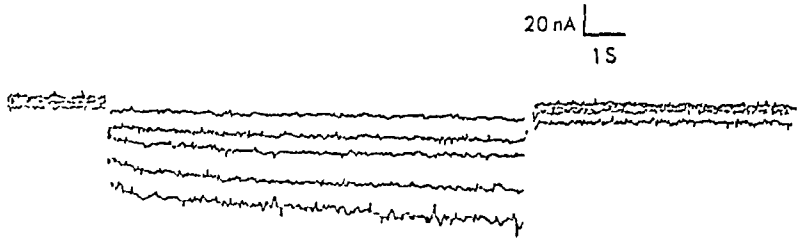

Fig 2.7 Voltage clamp steps for a 7 day old atrial aggregate (K_o = 1.3 mM, TTX = 3 um, diameter = 200 um). Holding potential = -60 mV, step potentials to -70, -80, -90, -100, and -110 mV.

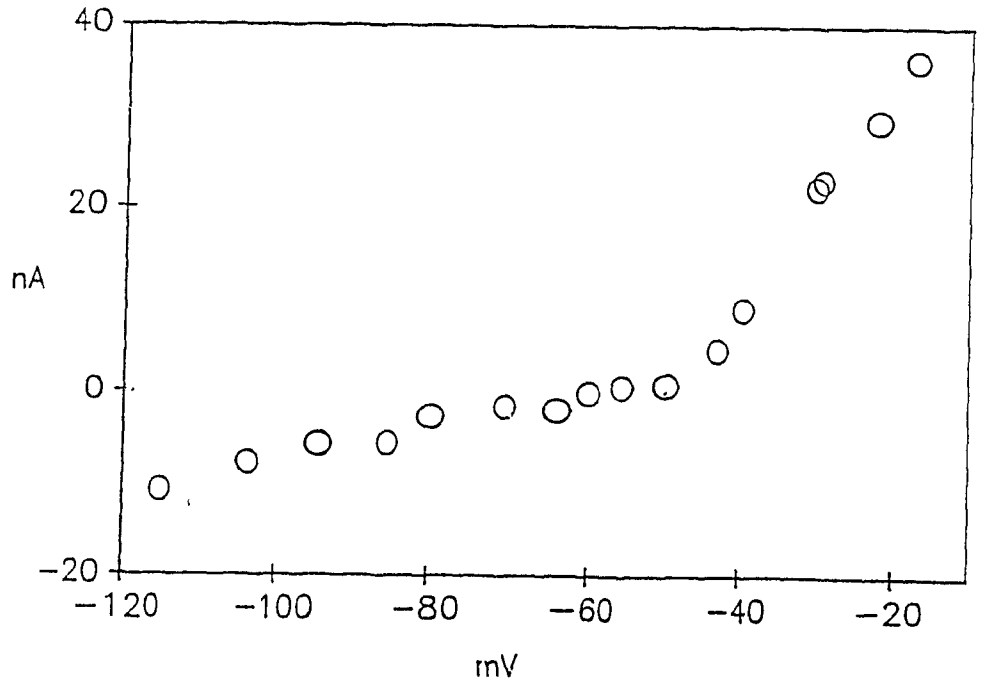

Fig. 2.8 Steady state I/V relationship for the preparation represented in Fig. 2.7. Measurements of current taken at the end of 5 s voltage clamp steps.

CHAPTER III

CHAPTER III:
I_f IN SINGLE CELLS
FROM EMBRYONIC CHICK HEART

III.1 SUMMARY:

The pacemaker current was studied in enzymatically dispersed ventricular and atrial cells from embryonic chick heart. The major goal of these experiments was to determine the nature of the pacemaker current in single cells and to compare these results with those from aggregates. The results presented in this study confirmed the presence of an I_f with a reversal potential (E_f) of approximately -30 *mV* for both cell types. The single cell results were consistent with the aggregate results except for a negative shift in the activation range of I_f in single ventricular cells; a similar activation range was found for single atrial cells. The biphasic nature of the pacemaker current in ventricular cells appears to be associated with the inward rectifier (I_{k1}) and not an accumulation/depletion process.

III.2 INTRODUCTION:

The mechanism first believed to be responsible for the genesis of the pacemaker potential in mammalian cardiac muscle was thought to be the decline of a *K* current (I_{k2}) (Noble and Tsien, 1968). Similarly, in embryonic chick ventricular heart cell aggregates an I_{k2} current was described and paralleled with a developmental loss of automaticity (Clay and Shrier, 1981a,b). However, more recent voltage clamp evidence suggests that the pacemaker depolarization in cardiac Purkinje fibers is not caused by the deactivation of an outward *K* current, but instead results from the activation of an inward current (I_f) (DiFrancesco, 1981a,b).

A number of ionic components have been measured in embryonic chick heart cells which include: (i) the fast inward sodium current (I_{Na}) (Ebihara et al., 1980), (ii) the slow inward calcium current (I_{si}) (Josephson and Sperelakis, 1982), (iii) the delayed rectifier (I_{x1}, I_{x2}, or I_K) (Clay and Shrier, 1986; Clay *et al.*, 1988; Clapham and Logothetis, 1988), (iv) the pacemaker current (I_{k2}) (Clay and Shrier, 1981a), (v) and the *K* and *Na* background components (I_{k1} and $I_{b,Na}$) (Clay and Shrier, 1981a). A large number of these investigations were carried out on aggregates which are multicellular preparations. More recently, the whole cell configuration of the patch clamp technique (Hamill *et al.*, 1981) has allowed the voltage clamping of single dissociated cells, thereby reducing the problems associated with the study of multicellular preparations such as accumulation/depletion. The aim of the experiments presented in this chapter is to clarify the nature of the pacemaker current components described in the previous chapter for embryonic chick ventricular heart cell aggregates, and to verify the presence of an *I_f-like* component in atrial heart cells.

III.3 MATERIALS AND METHODS:

Tissue culture techniques

Fertilized White Leghorn *(Gallus gallus)* chicken eggs were incubated at 37 *°C* and 95 % humidity for a period of 4 to 14 days for ventricular preparations, and 6 to 7 days for atrial preparations. The hearts of the chicken embryos were extracted following decapitation under aseptic conditions. The ventricular or atrial portions of the chick embryonic hearts were then dissected and pooled separately under a dissection microscope. The tissue was then diced into small fragments and then dissociated into its component cells using a multiple-cycle trypsinization process (DeHaan, 1967, 1970). An inoculum of cells was then added to a 35 *mm* tissue culture dish (*Corning*, 25000) in a region contained by a removable glass ring, to increase cell density, which surrounded a well that had been prepared in the floor of

the dish. The floor of the well was a glass coverslip which was attached to the dish with *Sylagard* (184). The glass coverslip was used to improve optics. The cells were allowed to settle down and attach themselves to the glass surface in an incubator for 1-14 days at 37 *°C*, in an atmosphere consisting of 5 % CO_2, 10 % O_2, and 85 % N_2. This procedure resulted in the formation of isolated single cells and small clusters of cells which were amenable to study with the patch clamp method. The incubation media *818A* (DeHaan, 1970) consisted by volume of 25 % *M199* (*Gibco*), 2 % heat-inactivated horse serum (*Gibco*), 4 % heat-inactivated fetal calf serum (*Gibco*), and 0.5 % Gentamicin (*Schering*) in a balanced salt solution which contained (in *mM*): *NaCl*, 116.0; $MgSO_4$, 0.8; NaH_2PO_4, 0.9; $CaCl_2$, 1.8; $NaHCO_3$, 26.6; *glucose*, 5.5, and *KCl*, 1.3.

Electrophysiology

The whole cell configuration of the patch clamp technique (Hamill et al., 1981) was used at room temperature (T = 20 to 24 *°C*) for voltage clamping single dissociated cells, or small clusters of cells. The patch electrodes were filled with intracellular medium composed of (in *mM*): *KCl*, 119.8; K_2EGTA, 5; $MgCl_2$, 4; Na_2CP, 5; Na_2ATP, 3.1; Na_2GTP, 0.42; $CaCl_2$, 0.062; *HEPES buffer* (*N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid*), 10. The *pH* of this solution was adjusted to 7.1 with KOH, and all solutions were filtered (0.22 *um* pore size). The extracellular medium contained (in *mM*): *NaCl*, 140; $MgSO_4$, 1; NaH_2PO_4, 0.2; $CaCl_2$, 1.8; *HEPES*, 10; *glucose*, 5; *KCl*, 1 to 50. In some experiments $BaCl_2$ (5 *mM*) and/or $CsCl_2$ (2 *mM*) were added, and in most cases tetrodotoxin (*TTX*, 3 *um*) was used to block the fast inward sodium current. Patch electrodes were pulled from Kimax-51 (*Kimble*) glass using a Narishige (*PP 83*) electrode puller. It was not necessary to fire polish the electrode tips. Electrode resistances were typically in the range of 1-5 *M ohms*. The liquid junction potential between the intracellular medium of the patch electrode and the extracellular medium was compensated. The microelectrodes were mounted in a plexiglass holder

with a suction port on the side and a chlorided silver wire was used to make contact with the electrode solution. The formation of a giga-ohm seal was monitored by the voltage response to 10 *mV* pulses. After initial seal formation the whole-cell recording configuration was established by applying a burst of suction to the electrode, and confirmed by monitoring an increase in capacitance and a negative membrane potential in the current-clamp mode after patch disruption.

Data recording and analysis

Both membrane currents and voltages were filtered at 10 kHz and recorded onto a video cassette recorder (*Sony*) by means of a pulse code modulation (*PCM, Sony*) digitizing unit. A List EPC-7 amplifier was used to measure membrane currents. An electronic stimulator (*Winston*) was used to deliver a protocol of rectangular voltage steps. All experimental results were played back on a digital oscilloscope (*Nicolet*, model 206) and then reconverted to an analogue signal and plotted on an x-y plotter (*Hewlett-Packard*, model 7015B).

Suitability of embryonic chick heart cells for the whole-cell recording technique

Embryonic chick heart cells are well suited for the whole-cell recording technique. Single cells dissociated from embryonic chick heart have a spherical appearance with a diameter typically in the 10-20 *um* range (Clay and DeHaan,1979; Veenstra and DeHaan, 1986). The membrane capacitance for single cells (Clay *et al*, 1988) and heart cell aggregates (Clay *et al.*, 1979) has been estimated to be approximately 1 $uF\ cm^{2}$. This suggests that these cells have relatively little infolding of their surface membrane which helps to minimize the problems associated with series resistance and/or the accumulation/depletion of ions.

III.4 RESULTS:

Electrical activity

Most ventricular and atrial single cells or small clusters beat spontaneously at 37 *°C*. However, there was considerable variability in the rate of spontaneous activity and many preparations were quiescent at room temperature. This may reflect the sensitivity of certain ionic current components to temperature. The addition of *TTX* (3 *um*) arrested almost all spontaneous activity. The average resting potential for ventricular cells with *TTX* (3 um) and varying K_o were; -39 *mV* (K_o = 1 *mM*; n = 10), -37 *mV* (K_o = 5 *mM*; n = 6), and -34 *mV* (K_o = 10 *mM*; n = 3). The average resting potential for atrial cells was -30 *mV* (K_o = 5 *mM*; n = 7). The resting potential for both types of single cells was more depolarized than equivalent aggregate preparations which rested typically around -60 *mV* (K_o = 1.3 *mM*: *TTX* = 3 *um*) It is unclear why single cells tended to rest at more depolarized potentials. Part of the cause may be due to a flat portion of the current voltage relationship seen in most preparations between about -30 and -50 *mV* allowing the membrane potential to drift between these two values (Clay *et al.*, 1988) The resting potential of these preparations is largely determined by the background current (I_{k1} and $I_{b,Na}$) which seems to have a reversal potential more positive than E_k. This suggests that the background current is not a pure *K* current.

Figure 3.1 represents a typical stimulated action potential recorded from a 7 day old embryonic chick ventricular heart cell at room temperature (K_o = 1 *mM*; *TTX* = 3 *um*). The overshoot of the action potential reached a value of +30 *mV* with a maximum diastolic potential (*MDP*) reaching -60 *mV*. The value for the *MDP* is less negative than the *MDP* obtained for equivalent aggregate preparations (see chapter 2). Similar results were obtained for atrial single cells (Clay *et al.*, 1988). This difference in repolarization between aggregates and single cells has been attributed to a lack of I_{x1} in

single cells (Clay *et al.*, 1988). The lack of I_{x1} in single cells may in turn be due to a wash-out of intracellular factors during whole-cell recordings, or an interaction between myocytes and fibroblasts present in aggregates which may regulate I_{x1} (Clay *et al.*, 1988). In some small clusters of cells (three or more cells) an I_{x1}-like component was recorded supporting this hypothesis. The lack of full repolarization would rule out the contribution of I_f in pacemaking which does not activate until -90 *mV*. This differs with ventricular aggregate preparations which fully repolarize to -90 *mV*, and where I_f has a more positive activation range (-70 to -90 *mV*) permitting this component to contribute in pacemaking.

Voltage clamp analysis

I. Ventricular cells

The upper left hand panel of figure 3.2 represents the membrane currents recorded from a 4 day old ventricular preparation (K_o = 1 *mM*; *TTX* = 3 *um*). The holding potential was -60 *mV* and the depolarizing pulse is to 0 *mV*, the hyperpolarizing pulses are in 10 *mV* steps from -70 to -120 *mV*. Steps in the depolarizing direction elicited a time-dependent outward current corresponding to the I_{x2} component seen in atrial and ventricular aggregates (Shrier and Clay, 1986) and I_k in ventricular and atrial single cells (Clay *et al.*, 1988; Clapham and Logothetis, 1988) Steps in the hyperpolarizing direction were time-independent between -70 and -80 mV, whereas steps beginning and more negative than -90 mV showed a small inwardly directed time-dependent current. These results differ from those obtained for ventricular aggregates by Clay and Shrier (1981a) who observed an activation range of the pacemaker component between -70 and -90 *mV* followed by an apparent reversal around E_k The lack of a current reversal at E_k suggests that the pacemaker depolarization results from the activation of an inward current (I_f) (DiFrancesco, 1981a,b). The upper right hand panel of figure 3.2 represents the effect of increasing K_o to 5 *mM* on the same

preparation with an identical voltage clamp step protocol. An increase in current was observed at all potentials in the pacemaker range due to the stimulatory effect of an increase in K_o on the inward rectifier (I_{k1}). This increase in the inward rectifier masked the development of the pacemaker current which still can be distinguished as an increase in inward current towards the end of the 10 *s* voltage clamp steps, and by the tail currents produced by this component upon returning to -60 mV. The lower panel of figure 3.2 represents the steady state current-voltage (*I/V*) relationship obtained by measuring the current amplitude at the end of 10 *s* voltage clamp steps. The closed circles represents the steady state *I/V* relationship for (K_o = 1 *mM*; *TTX* = 3 *um*), which crosses the axis (zero current level) at the resting potential which in this case was -50 *mV*. At potentials positive to -30 *mV* the *I/V* relationship increases outwardly due to the activation of the delayed rectifier (I_k). The closed triangles represent the steady state *I/V* relationship for (K_o = 5 *mM*; *TTX* = 3 *um*). At potentials negative to -80 *mV* there is an increase in inward current due to an increase in the conductances of I_{k1} and I_r. A negative slope region is visible between -60 and -30 *mV*, most likely due to the rectification of I_{k1} at more depolarized potentials. These results are consistent with the behavior of the inward rectifier which has been termed "*anomalous rectifier*", where the conductance of this current increases upon hyperpolarization and decreases upon depolarization (Katz, 1949; Adrian 1969; Hagiwara and Jaffe 1979). The sensitivity of I_{k1} to external *K* concentrations is another characteristic feature of this channel (Leech and Stanfield, 1981).

The upper left hand panel of figure 3.3 (K_o = 5 *mM*; *TTX* = 3 *um*) is taken from figure 3.2 for comparison with the right hand panel which was obtained from the same preparation following the addition of 5 *mM Ba* (K_o = 5 *mM*; *TTX* = 3 *um*; *Ba* = 5 *mM*). Steps in the pacemaker range with *Ba* (5 *mM)* now reveal an inward current with an activation range between -90 and -120 *mV*. Full activation of this component was seen at -120 *mV* as can be measured by the saturation of the tail currents of this component upon

returning to -60 *mV*. The lower panel of figure 3.3 represents the steady state *I/V* relationship for these two conditions. The closed triangles represent the *I/V* for (K_o = 5 *mM*, *TTX* = 3 *um*) and the open triangles represent the *I/V* for (K_o = 5 *mM*; *TTX* = 3 *um*; *Ba* = 5 *mM*). The addition of *Ba* (5 *mM*) caused the preparation to rest at more depolarized potentials (-15 *mV*) as is evidenced by a more positive crossing of the *I/V* at the zero current level. Ba (5 *mM*) blocks the conductance of I_{k1}, a component largely responsible for the negative resting potential. The negative slope region is no longer present and there is a decrease in the delayed rectifier component These results are consistent with the effects of *Ba* which has been shown to be effective in blocking the inward rectifier (Sakmann and Trube, 1984), and the delayed rectifier (Armstrong *et al.*, 1982). The left hand panel of figure 3.4 is taken from figure 3.3 (K_o = 5 *mM*; *TTX* = 3 *uM*; *Ba* = 5 *mM*) for comparison with the right hand panel which is obtained from the same preparation to which K_o had been elevated to 50 *mM* (K_o = 50 *mM*, *TTX* = 3 *um*; *Ba* = 5 *mM*) An elevation of K_o (50 *mM*) increased the conductance of I_f which is confirmed by the amplitude of the tail currents upon returning to -60 *mV*. A positive shift of approximately 20 *mV* in the activation of I_f is also apparent The increased conductance of I_f with an increase in K_o has also been shown in Purkinje fibers by DiFrancesco (1981b). Similar results to those described above were obtained for 7 other preparations which varied in developmental stage (4 day old, n = 3; 7 day old, n = 3; and 14 day old, n = 1). These results are consistent with the observations for ventricular aggregates (see chapter II) except for a more negative activation range of I_f The more negative activation range of I_f may be due to the washout by the patch pipette of substances modulating its gating, or the effects of surface charge shifting the activation range to more negative potentials.

Figure 3.5 represents a two step protocol for determining the reversal potential of I_f (K_o = 5 mM, *TTX* = 3 *um*; *Ba* = 5 *mM*) for a 14 day old ventricular preparation. The holding potential was -60 *mV* and the initial conditioning hyperpolarizing step was to -130 *mV*, a region where a robust I_f

current is recorded. The second step was to potentials ranging from -40 to +30 mV, in 10 *mV* increments. The reversal potential for I_f (E_{rev}) was determined to be around -30 *mV*, which is consistent with the results demonstrated in cardiac Purkinje fibers by DiFrancesco (1981b) suggesting that I_f is permeable to both *Na* and *K* ions. Under similar conditions (K_o = 1 *mM*; *TTX* = 3 *uM*; *n* = 4) the range of measurements of E_f varied from -30 to -10 *mV*, however, the dependence of E_f upon ionic conditions was not investigated.

II. Atrial cells

Figure 3.6A illustrates the membrane currents recorded from a 7 day old atrial aggregate (K_o = 1.3 *mM*; *TTX* = 3 *um*; *diameter* = 200 *um*; *T* = 37 *°C*) (taken from chapter II) using the two microelectrode voltage clamp technique. Figure 3.6 b represents the membrane currents recorded from a small cluster of 7 day old atrial cells (K_o = 3 *um*; *TTX* = 3 *um*; *T* = 24 *°C*) using the whole cell patch clamp technique. The holding potentials for both preparations was -60 *mV*, and the step potentials were in 10 mV increments from -70 to -110 *mV*. The steps to -70 and -80 mV are time-independent, whereas, steps beginning at -90 mV and more negative are time-dependent. Previously Shrier and Clay (1986), and Clay *et al.* (1988) had reported the absence of a pacemaker current in the -60 to -90 *mV* range for atrial aggregates and single cells However, extending the analysis to more negative potentials has revealed the presence of an I_f component in aggregates and single cells with an activation range beginning at -90 *mV*. Results similar to fig 6 were seen in 5 out of 5 aggregates and in 4 out of 8 single cells or small clusters. In the cells where an I_f current was not observed the currents in the pacemaker range were either time-independent, or in a few cases had an I_{k1} inactivating component negative to -90 *mV* (K_o = 5 *mM*; *TTX* = 3 *uM*). The E_f for atrial single cell preparations was determined to be around -30 *mV* (K_o = 5 *mM*; *TTX* = 3 *uM*; *n* = 2). The range of activation of I_f for atrial aggregates and atrial or ventricular single

cells or small clusters was the same.

III.5 DISCUSSION:

The results presented in this study confirm the presence of an I_f in embryonic chick heart similar to the pacemaker current described in Purkinje fibers (DiFrancesco, 1981a,b). In cardiac Purkinje fibers I_f provides an inward current which depolarizes the membrane towards the threshold of the fast inward sodium current (I_{Na}). Similarly in embryonic chick ventricular aggregates I_f plays an important role in pacemaking. The effect of I_f opposes the action of the inward rectifier current (I_{K1}) which tends to stabilize the preparation at its resting potential. The voltage-dependent inactivation of the inward rectifier has been described in the guinea-pig heart by Sakmann and Trube (1984b), and is dealt with in more detail in chapter IV. The present results obtained with single cells suggest that it is the kinetics of I_{K1}, and not an accumulation/depletion process as suggested by DiFrancesco and Noble (1985), modulates the pacemaker depolarization In fact, the *pacemaker current* in embryonic chick ventricular aggregates is actually a mixture of two components; I_f which tends to depolarize the membrane, and I_{K1} the time-dependent background current which tends to secure the membrane potential at rest. These results contrast with *primary pacemaker tissue* such as the bullfrog sinus venosus (Shibata and Giles, 1984) or the rabbit sino-atrial node (Noma *et al.*, 1984) which lack an I_{K1} current and in which I_f alone may play a dominant role in pacemaking. The pacemaker depolarization in embryonic chick atrial aggregates and ventricular or atrial single cells occurs without the presence of I_f. This is due to the negative activation range of I_f in these cells which begins at potentials negative to -90 *mV* In fact, at least in atrial and ventricular single cells, the *MDP* does not exceed -75 *mV* due to the lack of I_{x1} in single cells (Clay *et al.*, 1988). Therefore, the spontaneous beating in these cardiac cells requires the presence of a background inward sodium current ($I_{b,Na}$) which would cause the membrane to depolarize as the plateau *K* current decays. Part of the contribution to $I_{b,Na}$ may involve the

window sodium current due to the cross over phenomena of the activation and inactivation curves for I_{Na} (Attwell *et al.*, 1979).

The inherent rhythm of chick embryonic heart diminishes with development until day 21 when the chick hatches (Shrier and Clay, 1980; DeHaan, 1970). Shrier and Clay (1980), paralleled this loss of automaticity with a decrease in a *K* pacemaker current (I_{K2}) in ventricular cells over the developmental stage of 7 to 17 days. However, the loss of automaticity in these cells cannot be explained by a decrease in the pacemaker current, but more importantly must involve a developmental change in the background components. Clay and Shrier (1981b) lumped the background time-independent pacemaker currents into a component they referred to as I_{bg}, which they thought may be carried by *Na*, *K*, and/or possibly some other ion. They concluded that the loss of automaticity was due to an overall tendency of the background current becoming less inward with development. The change in I_{bg} could be attributed to either a change of an inward current ($I_{b,Na}$), or an inwardly rectifying outward current found in these cells. Judging from the similarities in shape of the inward rectification over the 7 to 17 days of development, Clay and Shrier (1981b) concluded that the outward component did not change much with development. Instead, they attributed the changes in I_{bg} to a decrease in $I_{b,Na}$. It is interesting to note that a decrease in an outward *K* pacemaker current (I_{K2}) would allow ventricular aggregates to beat more quickly by displaying a more steep diastolic depolarization unless paralleled by the background current becoming less inward with development. On the other hand, a decrease in an inwardly activated *Na/K* pacemaker current (I_f), without a change in I_{bg} would allow the ventricular aggregates to beat more slowly However, these preparations eventually lose their automaticity and rest at more negative potentials with development (DeHaan, 1970; McDonald and DeHaan, 1973; Sachs and DeHaan, 1973). Therefore, the key player in the developmental loss of automaticity involves I_{bg}, and perhaps a decrease in $I_{b,Na}$. If $I_{b,Na}$ does decrease with development it would then allow the inward rectifier (I_{K1}) to

dominate and hold the membrane potential closer to E_k eventually leading to a cessation of spontaneous activity.

FIGURES

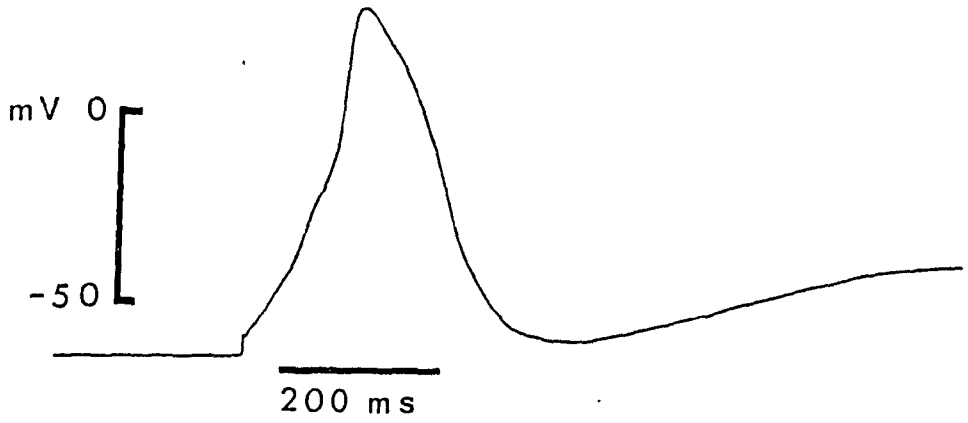

Fig. 3.1 Action potential stimulated from a 7 day old embryonic chick ventricular heart cell at room temperature (K_o = 1 mM; TTX = 3 um; T = 24 °C). Overshoot = +30 mV; maximum diastolic potential = -60 mV.

Fig.3.2 *Upper left hand panel:* Voltage clamp results from a small cluster of 4 day old ventricular cells (K_o = 1 *mM*; *TTX* = 3 *um*). Holding potential = -60 *mV*, depolarizing step is to 0 *mV*, hyperpolarizing steps are in 10 *mV* increments from -70 to -120 *mV*. Steps were on for 10 *s*. *Upper right hand panel:* The effect of increasing K_o to 5 *mM* in the same voltage range excluding the depolarizing pulse. *Lower panel:* Steady state *I/V* relationship taken for the same preparation by plotting the current measured at the end of 10 s voltage clamp steps. Closed circles (K_o = I *mM*; *TTX* = 3 *um*), closed triangles (K_o = 5 *mM*; *TTX* = 3 *um*). (See text for details).

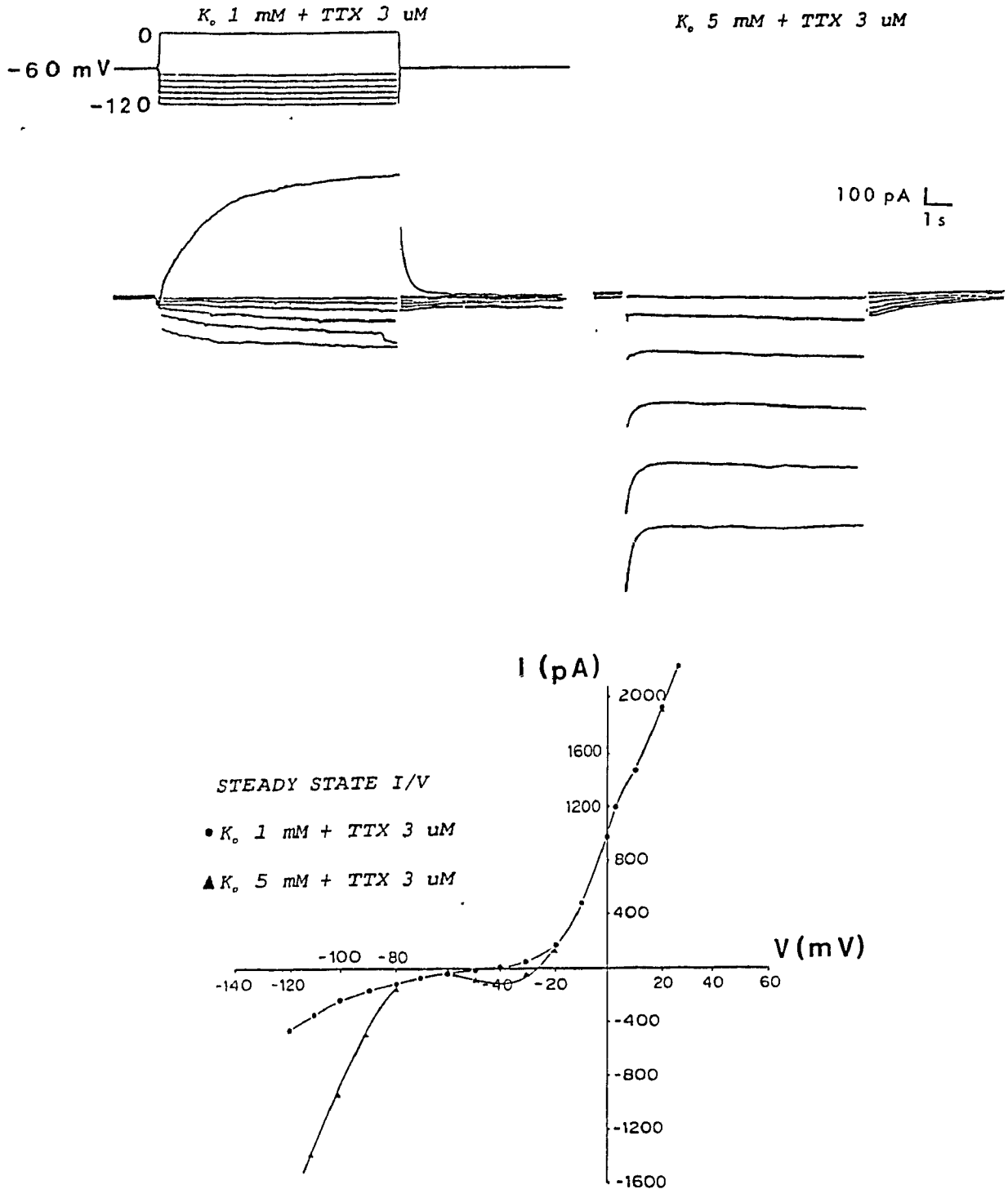
K_o 1 mM + TTX 3 uM
K_o 5 mM + TTX 3 uM
0
-60 mV
-120
100 pA
1 s
I (pA)
2000
1600
1200
800
400
-400
-800
-1200
-1600
V (mV)
-100 -80
-140 -120
-40 -20
20 40 60
STEADY STATE I/V
• K_o 1 mM + TTX 3 uM
▲ K_o 5 mM + TTX 3 uM

Fig. 3.2

Fig. 3.3 *Upper left hand panel:* Taken from the right hand panel of figure 3.2 for comparison. *Upper right hand panel:* ▫ effect of adding *Ba* (5 *mM*). *Ba* blocked the background current (I_{k1}), as is evidenced by a decrease in instantaneous current, and revealed the pacemaker current. *Lower panel:* Steady state I/V relationship: closed triangles (K_o = 1 *mM*; *TTX* = 3 um), and open triangles (K_o = 5 *mM*; *TTX* = 3 *um*; *Ba* = 5 *mM*).

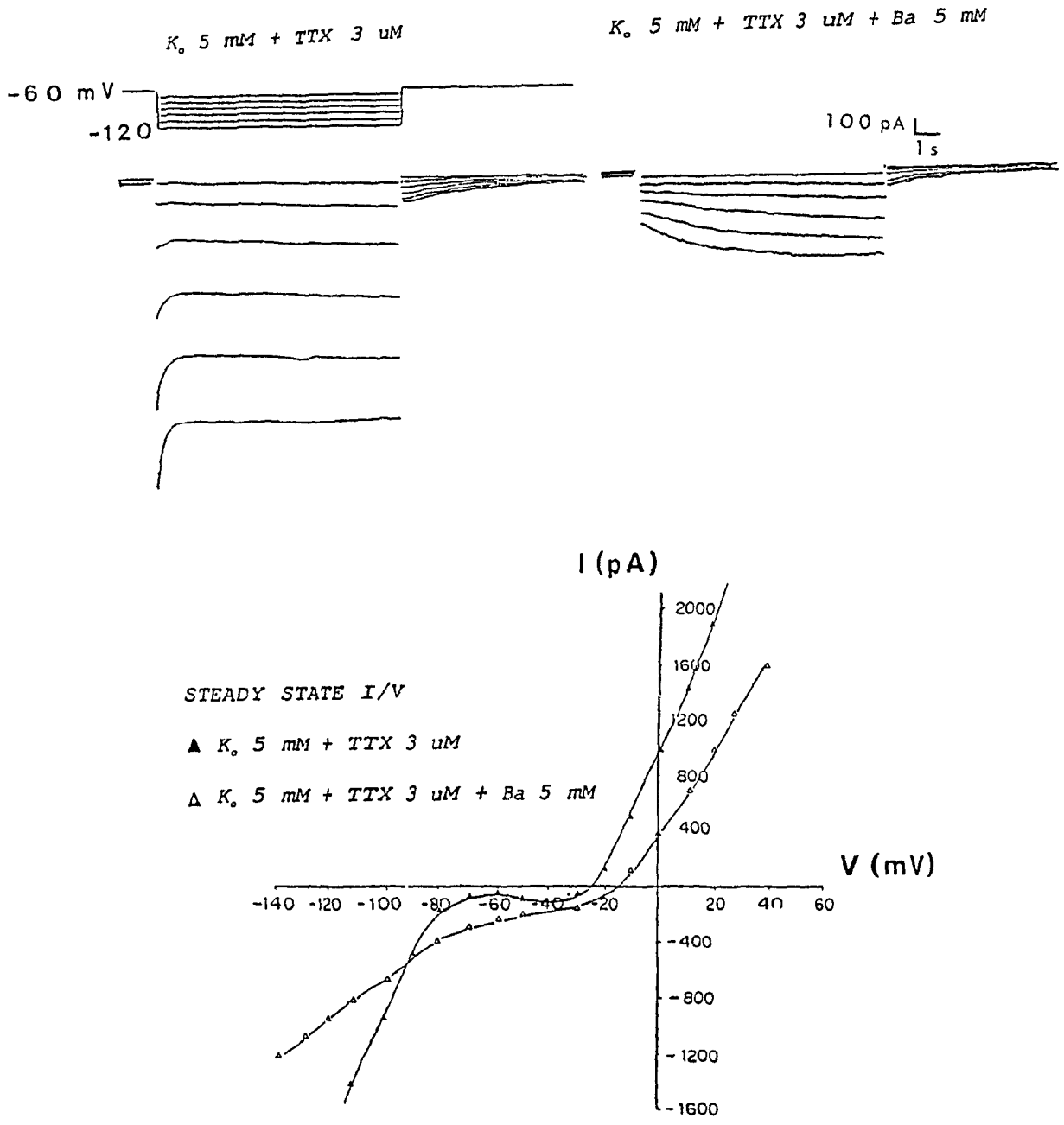
K_o 5 mM + TTX 3 uM
K_o 5 mM + TTX 3 uM + Ba 5 mM
-60 mV
-120
100 pA
1 s
I (pA)
2000
1600
1200
800
400
V (mV)
-140 -120 -100 -80 -60 -40 -20 20 40 60
-400
-800
-1200
-1600
STEADY STATE I/V
▲ K_o 5 mM + TTX 3 uM
Δ K_o 5 mM + TTX 3 uM + Ba 5 mM

Fig. 3.3

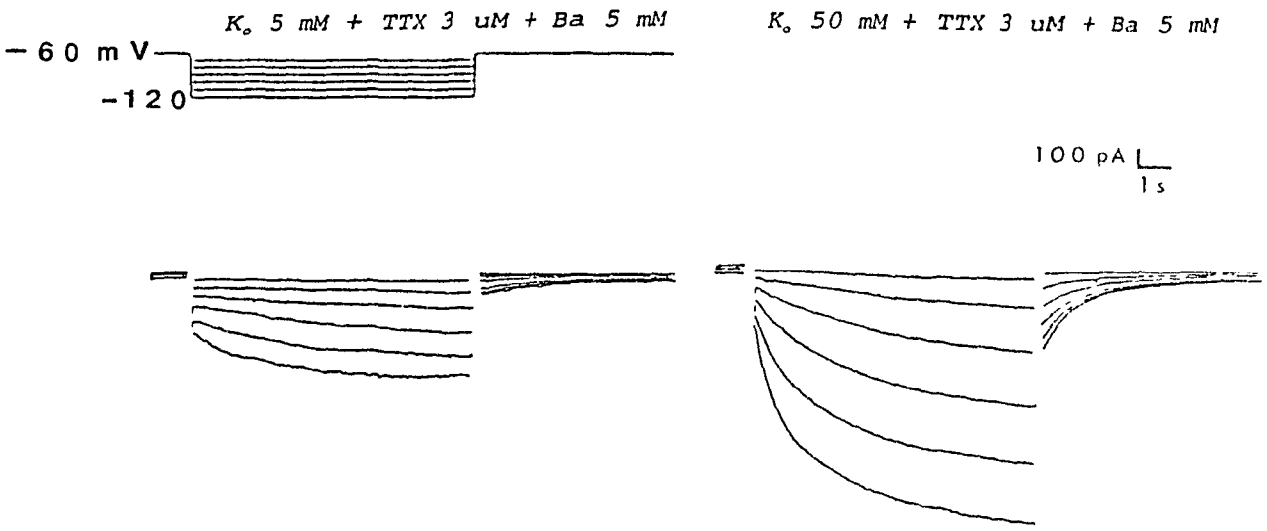

Fig. 3.4 Left hand panel: Taken from the right hand panel of figure 3.3 for comparison. Right hand panel: The effect of increasing K_o (50 mM). An increase in K leads to an increase in the conductance of I_t at all potentials and a 20 mV positive shift of its activation range.

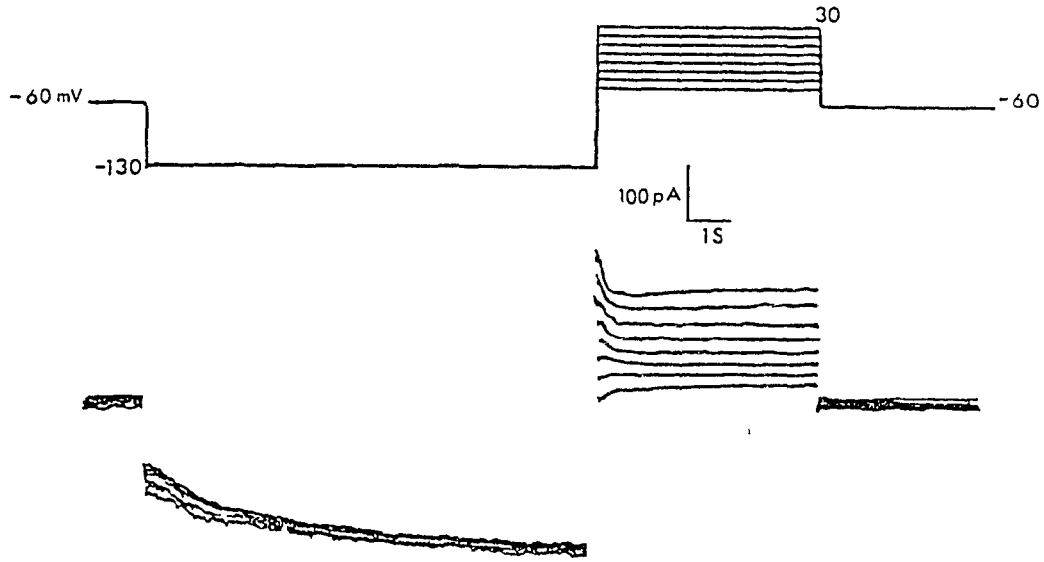

Fig. 3.5 A two step protocol for determining the reversal potential of I_t (K_o = 5 mM; TTX = 3 um; Ba = 5 mM) for a 14 day old ventricular preparation. The holding potential was -60 mV and the initial conditioning hyperpolarizing pulse was down to -130 mV. The second pulse was depolarizing from -40 to +30 mV in 10 mV increments E_t was determined to be around -30 mV.

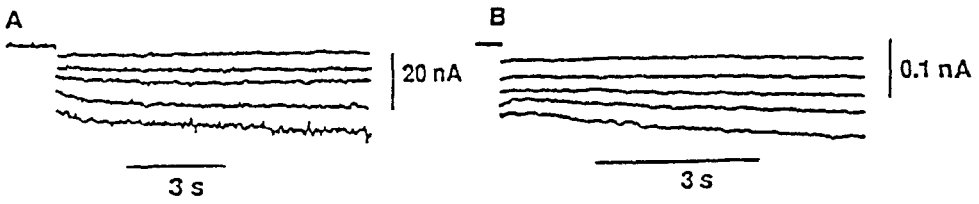

Fig. 3.6a Membrane currents recorded from a 7 day old atrial aggregate (K_o = 1.3 mM; TTX = 3 um; diameter = 200 um; T = 37 °C) Fig. 3.6 b: represents the membrane currents recorded from a small cluster of 7 day old atrial cells (K_o = 3 um; TTX = 3 um, T = 24 °C). The holding potentials for both preparations was -60 mV, and the step potentials were in 10 mV increments from -70 to -110 mV. (See text for details).

CHAPTER IV

CHAPTER IV:
I_{k1} IN SINGLE CELLS
FROM EMBRYONIC CHICK VENTRICULAR HEART

IV.1 SUMMARY:

Hyperpolarizing voltage clamp steps from a holding potential of - 60 mV revealed a current which displayed a time-dependent inactivation. The instantaneous current versus voltage plot of this current showed inward-going rectification for steps positive to -70 mV and a linear voltage dependence for hyperpolarizing steps. The blocking effects of *Cs* and *Ba*, the inward-rectifying nature of the *I/V* relationship, and the K_o sensitivity of the current slope conductance suggests that this current is similar to the inward rectifying *K*-current (I_{k1}). However, the kinetics of the inactivation process was slower than that seen in other cardiac preparations.

IV.2 INTRODUCTION:

The properties of the anomalous or inward rectifier were first described in skeletal muscle by Katz (1949). The term "*anomalous*" rectification was used to describe the conductance of this current which increases upon hyperpolarization and decreases upon depolarization. More extensive studies of the inward rectifier (I_{k1}) were performed on skeletal muscle (Adrian et al., 1970; Leech and Stanfield, 1981), and were extended to other preparations such as the starfish and tunicate egg cell preparations (Hagiwara et al., 1976; Ohmori, 1978, 1980).

Weidmann (1955) was the first to record an increase in membrane resistance during the plateau of the action potential in Purkinje fibers.

Voltage clamp experiments suggested that this was due to the presence of an inwardly-rectifying *K* current (Hutter and Noble, 1960). The presence of this current in Purkinje fibers helped to explain the higher membrane resistance measured during the plateau of the action potential and the decrease in slope conduction during the pacemaker depolarization described by Weidmann (1955). The absence of the inward rectifier at depolarized potentials helps to minimize the amount of inward current necessary to maintain the plateau of the action potential (Noble, 1965). The inward-rectifying *K* current (I_{k1}) was first considered to be an instantaneous current that was not associated with any time dependence (Beeler and Reuter, 1977; McDonald and Trautwein, 1978; Cleeman and Morad, 1979). Clay and Shrier (1981a) also described the background component in embryonic chick ventricular heart cells as being time-independent and showing inward-going rectification. These studies assumed that I_{k1} would be the *K* current left after the subtraction of the slow time-dependent currents from the total membrane currents. Carmeliet (1982) was the first to provide evidence that changes in I_{k1} were not instantaneous in sheep cardiac Purkinje fibers. More recent reports, including single channel studies of I_{k1}, have confirmed that it undergoes time-dependent inactivation upon membrane hyperpolarization (Sakmann and Trube, 1984a,b; Kameyama *et al.*, 1983; Kurachi, 1985, Josephson and Brown, 1986; Harvey and Ten Eick, 1988) The experiments in this chapter were designed to provide further evidence for the presence of a time-dependent I_{k1} current in embryonic cardiac cells. The biphasic nature of the pacemaker current in embryonic ventricular cells appears to be associated with I_{k1} and I_f not an accumulation/depletion process

IV.3 MATERIALS AND METHODS:

Tissue culture techniques

Fertilized White Leghorn *(Gallus gallus)* chicken eggs were incubated at 37 °C and 95 % humidity for a period of 7 days. The hearts of

the chicken embryos were extracted following decapitation under aseptic conditions. The tissue culture technique has already been described in detail in chapter III. Hearts were isolated and the ventricular portions were separated. The tissue was diced into small fragments and then dissociated into its component cells using a multiple-cycle trypsinization process (DeHaan, 1967, 1970). An inoculum of cells was added to the well formed in the bottom of a 35 *mm* tissue culture dish. The plated cells were then incubated for 3-7 days at 37 *°C* in an atmosphere consisting of 5 % CO_2, 10 % O_2, and 85 % N_2. The incubation media *818A* (DeHaan, 1970) consisted by volume of 25 % *M199* (*Gibco*), 2 % heat-inactivated horse serum (*Gibco*), 4 % heat-inactivated fetal calf serum (*Gibco*), and 0.5 % Gentamicin (*Schering*) in a balanced salt solution which contained (in *mM*): *NaCl*, 116.0; $MgSO_4$, 0.8; NaH_2PO_4, 0.9; $CaCl_2$, 1.8; $NaHCO_3$, 26.6; *glucose*, 5.5, and *KCl*, 1.3.

Electrophysiology

The whole cell configuration of the patch clamp technique (Hamill et al., 1981) was used for voltage clamping single dissociated cells, or small clusters at room temperature (T = 20 to 24 *°C*). The patch electrodes were filled with intracellular medium composed of (in *mM*): *KCl*, 119.8; K_2EGTA, 5; $MgCl_2$, 4; Na_2CP, 5; Na_2ATP, 3.1; Na_2GTP, 0.42; $CaCl_2$, 0.062; *HEPES buffer* (*N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid*), 10. The *pH* of this solution was adjusted to 7.1 with KOH, and all solutions were filtered (0.22 *um* pore size) The extracellular medium contained (in *mM*): *NaCl*, 140; $MgSO_4$, 1; NaH_2PO_4, 0.2; $CaCl_2$, 1.8; *HEPES*, 10; *glucose*, 5; *KCl*, 10. Tetrodotoxin (*TTX*, 3 *um*) was used to block the fast inward sodium current. Cesium (*Cs*, 2 *mM*) was used in some experiments to block I_f. Electrode resistances were typically in the range of 1-5 *M ohms*. The liquid junction potential between the intracellular medium of the patch electrode and the extracellular medium was compensated. The average resting potential for these preparations was -37 *mV* (K_o = 10 *mM*; *TTX* = 3 *um*; n = 6). The

method of data recording and analysis, and the suitability of these cells for whole cell recordings was described in chapter III.

IV.4 RESULTS:

The left hand panel of figure 4.1 represents the membrane current recorded from a 7 day old ventricular preparation (K_o = 10 *mM*; *TTX* = 3 *um*). The holding potential was -60 *mV* and 10 *s* steps were imposed with increments of 10 mV. The depolarizing pulse to 0 *mV* elicited the delayed rectifier (I_k), a *K* current which is partially responsible for repolarization of these cardiac cells (Shrier and Clay, 1986, Clapham and Logothetis, 1988). The amplitude of this plateau current can be compared with the relatively large inward currents activated by hyperpolarizing pulses from the same holding potential. The step to -70 *mV* was time-independent except for a very small decaying component at the beginning of the step. For stronger hyperpola :zations (-80 to -120 *mV*) there was a gradual increase in the amplitude of the instantaneous component. Beyond -90 *mV* the current record also took on a biphasic appearance due to the activation of an additional inwardly activated current, I_f. The appearance of this biphasic record at potentials negative to -90 *mV* is consistent with the results presented in chapters II and III, where the membrane current exhibited a biphasic appearance due to a mixture of two current components I_{kf} with I_f. The right hand panel of figure 4.1 represents the effects of cesium (*Cs*, 2 *mM*) on the same preparation. The voltage clamp protocol was identical except that the hyperpolarizing pulses were from -70 to -110 mV and the depolarizing pulse to 0 *mV* was excluded. Cesium decreased the amplitude of the instantaneous current jumps by partially blocking the decaying component, and abolishing the biphasic appearance of the current records by blocking I_f. Similar results to these were found in 5 out of 5 other preparations of 7 day old embryonic chick ventricular heart cells which had been in culture for periods varying from 3 to 7 days The bottom panel of figure 4.1 is a plot of the instantaneous *I/V* taken by measuring the current

amplitude just after the decay of the capacitive transient. The open circles represent the instantaneous *I/V* relationship with (K_o = 10 *mM*; *TTX* = 3 *uM*) which illustrates the inward-going rectification of this component permitting *K* ions to enter more easily upon hyperpolarization than to exit upon depolarization. The closed circles illustrate the blocking effect of *Cs* (2 *mM*). At potentials negat e to -100 *mV Cs* appears to be more effective in blocking the current as is illustrated by a curvature in the *I/V* relationship between -100 and -110 *mV*. Whereas, the *I/V* relationship is barely affected by *Cs* at potentials positive to -70 *mV*.

Figure 4.2 represents a two step protocol which was used to determine the voltage dependence of the inactivation process. Prepulses were applied for a duration of 2 *s* to different potentials in 10 *mV* increments ranging from -50 to -130 *mV*, followed by a brief return to the holding potential of -60 *mV* for 80 *ms*, and then followed by a test pulse to -120 *mV* for 2 *s*. The amplitude of the test pulse to -120 mV, measured just after the decay of the capacitive transient, depended upon the degree of hyperpolarization during the prepulse. The smaller current amplitudes measured for the test pulses were associated with the larger prepulses. Similar results were observed in 4 other ventricular preparations. These results suggest that the voltage range of inactivation extends from -70 mV to -130 mV, in 10 mM K_o.

Hyperpolarizing voltage clamp pulses were limited to the first 2 to 4 *s* in an attempt to study I_{k1} in isolation of I_f, and without the voltage-dependent blocking effects of *Cs*. Most of the current elicited during the first few seconds was attributed to I_{k1} because of the relatively slow activation and small amplitude of I_f in this time frame. Figure 4.3 represents a two step protocol to determine E_{rev} for the current. The holding potential was -60 *mV* and the first conditioning prepulse was down to -120 *mV* for 4 *s*, a potential which elicited a large decaying component. The second test pulse was imposed in 10 mV increments, from -70 to -130 *mV*, for 4 *s*. The current tail

analysis was complicated by the absence of a clear reversal over the entire range of potentials examined.

IV.5 DISCUSSION:

The results presented in this chapter and earlier chapters suggest that the inward rectifying current component found at hyperpolarized membrane potentials in embryonic chick ventricular heart is qualitatively similar to the I_{k1} current in other cardiac preparations in a number of respects: 1) the *I/V* relationship shows marked inward-rectification, 2) the current is blocked by *Ba*, and to some extent by Cs, and 3) the magnitude of the current seems to be sensitive to changes in K_o. The effect of Cs on the inward rectifier in embryonic heart cells is similar to the voltage-dependent block of I_{k1} by Cs described for the starfish egg (Hagiwara *et al.*, 1976), for squid axon (French and Shoukimas, 1985) and for cardiac Purkinje fibers (Isenberg, 1976). Cesium was also shown to be effective in blocking I_f (chapter II; and DiFrancesco, 1982), and barium (5 *mM*) was effective in blocking I_{k1} as was demonstrated in chapters II and III. The stimulatory effect of an increase in *K* on I_{k1} was discussed in chapter III (see fig. 3.2). Sakmann and Trube (1984) reported that the conductance of the inward rectifier channel depended upon the square root of $[K]_o$. From the sort of data presented in figures 3.2 an 3.3 it is impossible to directly measure changes in the conductance of I_{k1} as a function of K_o. However, it is evident that the slope of the inward portion of the I/V relationship, in the potential range negative to -80 mV, becomes markedly steeper when K_o is elevated from 1 to 5 mM. Moreover, the *Ba* sensitive component in figure 3.3, which is presumably I_{k1}, represents a significant fraction of this relationship and the slope of this component is relatively steep by comparison with the slope of the I/V in 1 mM Ko, which is clearly an overestimate of the slope due to I_{k1} alone.

However, some discrepancies exist between the inward rectifier in the chick heart cells and I_{k1} in other preparations, in particular: 1) in the embryonic cells a time dependent increase in inward current did not follow the initial instantaneous inward current jump during hyperpolarizing voltage steps, as had been observed in other cardiac preparations, and 2) the inactivation of this current was much slower (in the range of seconds) compared to I_{k1} in cat myocytes where inactivation occurred in tens of milliseconds (Harvey and Ten Eick, 1988). These differences may reflect the fact that more than one type of inwardly rectifying *K* channel may exist (ie nodal vs atrial or ventricular tissue) (Sakmann *et al.*, 1983, Sakmann and Trube, 1984 a,b). In fact, Josephson and Brown (1986) reported three different inward-rectifying channels which could contribute to the inactivating whole cell current which they described in neonatal and adult rat myocytes. The inactivation of I_{k1} in skeletal muscle (Standen and Stanfield, 1979) and in cardiac muscle (Harvey and Ten Eick, 1988) has been suggested to be partially due to a time and voltage-dependent blockade by extracellular *Na* . In fact, this mechanism can be used to explain the results in figure 4.2, if it is assumed that the inactivation of I_{k1} during the hyperpolarizing step to -120 mV is due to a Na blockade that is more pronounced than the Na blockade occurring at less negative potentials. During the second step, to more positive potentials, this blockade is relieved and I_{k1} increases to its steady state level. In frog skeletal muscle the inactivation of I_{k1} was separated into two processes: 1) a *K* depletion process, and 2) a voltage and time-independent decrease in permeability (Almers, 1972 a,b). Evidence from experiments on isolated cat ventricular myocytes suggest that the depletion of extracellular K plays a relatively minor role in the inactivation process (Harvey and Ten Eick, 1988). Since there is no evidence of any reversal potential during the second step in figure 4.2, over the range between -60 and -120 mV, it is unlikely that K reaccumulation plays a dominant role in this potential range. In myocytes isolated from newborn rat I_{k1} was found to be mainly selective to *K*, but *Na* ions were also shown to pass through the channel with a P_{Na}/P_k = 0.056 (Payet *et al.*, 1985).

The background current in embryonic chick heart was first described by Clay and Shrier (1981a). They separated a time-independent background component from the steady state pacemaker current (I_{k2}). They then modelled this background current result with three time-independent currents: an inwardly rectifying current (I_{k3}), a background sodium current ($I_{Na,b}$), and an outwardly rectifying current (I_{k4}). They assumed that the combination of I_{k3} with I_{k4} was similar to the time-independent I_{k1} current in Purkinje fibers (McAllister and Noble, 1966). They also determined that this current was partly, but not exclusively carried by potassium ions by looking at changes in E_{rev} in response to changes in external K Clay and Shrier (1981b) found little change in the shape of the inward rectifying component over the developmental stages of 7 to 17 days. Since they determined that the background component became less inward with development without a change in the inward rectifying component; they attributed this result to a developmental loss of $I_{Na,bg}$. The inward rectifying component in this study, contrary to the results of Clay and Shrier (1981a), showed time dependence at hyperpolarized potentials which may be associated with a voltage dependent change in conductance. Sakmann and Trube (1984a,b) identified single K channels which had many similarities to the macroscopic inward rectifier and having a single channel conductance of 3.6 *pS* in 5 *mM K*. Mazzanti and DeFelice (1988) recorded single inward rectifier channels on cell attached patches of beating embryonic chick ventricles (K_o = 1.3 *mM*). They reported a single channel conductance of 2-3 *pS* with an E_{rev} of -80 *mV* in 1.3 *mM* K_o. They also reported that the channel was only active during late repolarization and diastolic phases of the action potential, and no outward current was measured through this channel during beating. Since this component has a slow time course of inactivation and because it is activated during the diastolic phase of the action potential it could interact with the pacemaker current I_f. In fact, the *pacemaker depolarization* in embryonic chick ventricular aggregates may involve a mixture of these two components.

FIGURES

Fig. 4.1 The left hand panel of figure 4.1 represents the membrane currents recorded from a 7 day old ventricular preparation (K_o = 10 *mM*; *TTX* = 3 *um*). The holding potential was -60 *mV* and the steps were on for 10 *s*. The depolarizing pulse was to 0 mV and the hyperpolarizing pulses were in 10 *mV* increments from -70 to -120 *mV*. The right hand panel represents the effect of adding cesium (*Cs* = 2 *mM*; K_o = 10 *mM*; *TTX* = 3 *um*). The voltage clamp protocol was identical except that both the hyperpolarizing pulse to -120 *mV* and the depolarizing pulse to 0 *mV* were omitted. The bottom panel is an instantaneous *I/V* plot taken by measuring the current amplitude just after the decay of the capacitive transient. The closed circles represent the control data (K_o = 10 *mM*; *TTX* = 3 *um*) and the open circles represent the data in the presence of cesium (*Cs* = 2 *mM*; K_o = 10 *mM*; *TTX* = 3 *um*) (see text).

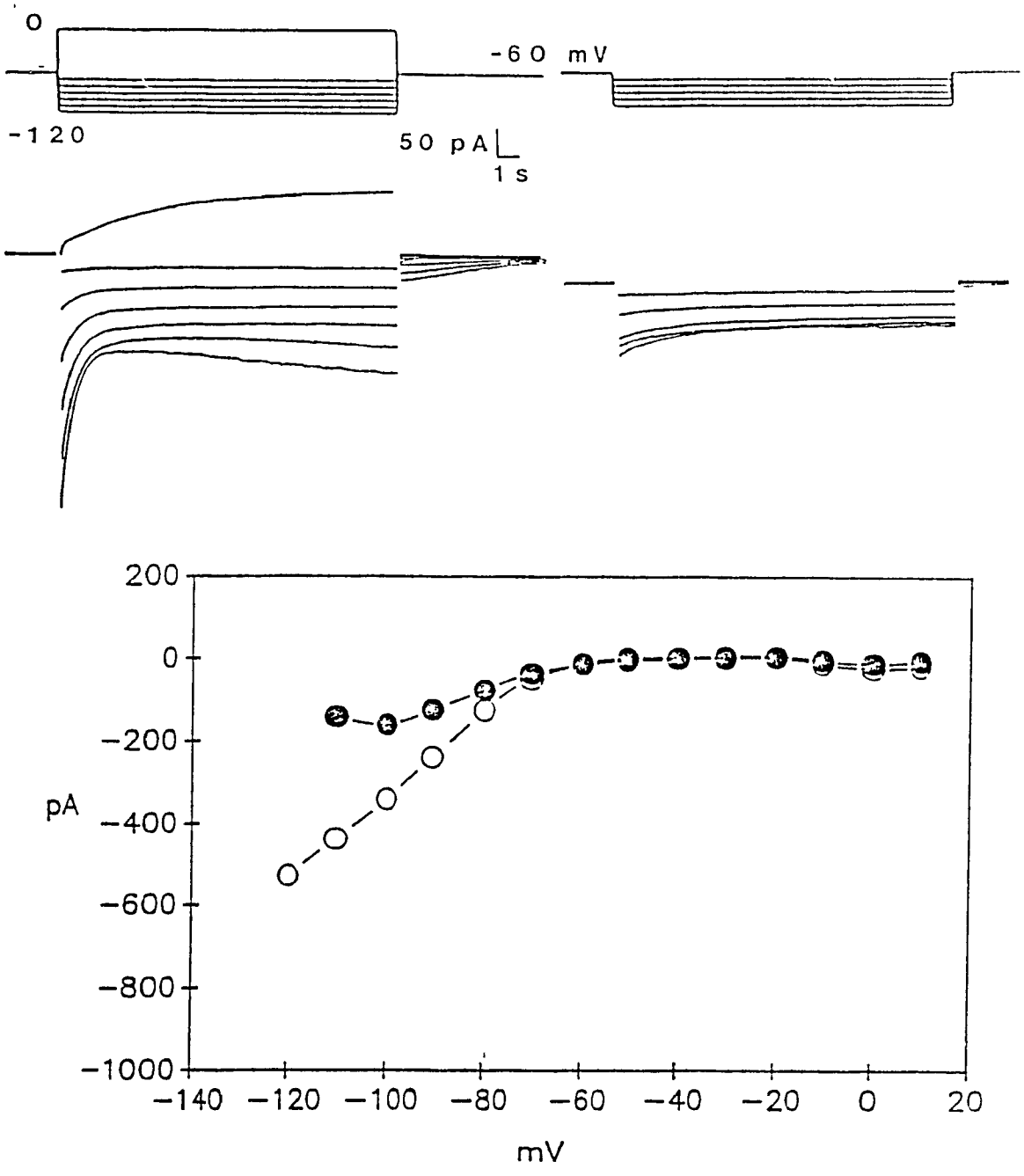
0
-60 mV
-120
50 pA
1 s
200
0
-200
-400
-600
-800
-1000
pA
-140 -120 -100 -80 -60 -40 -20 0 20
mV

Fig. 4.1

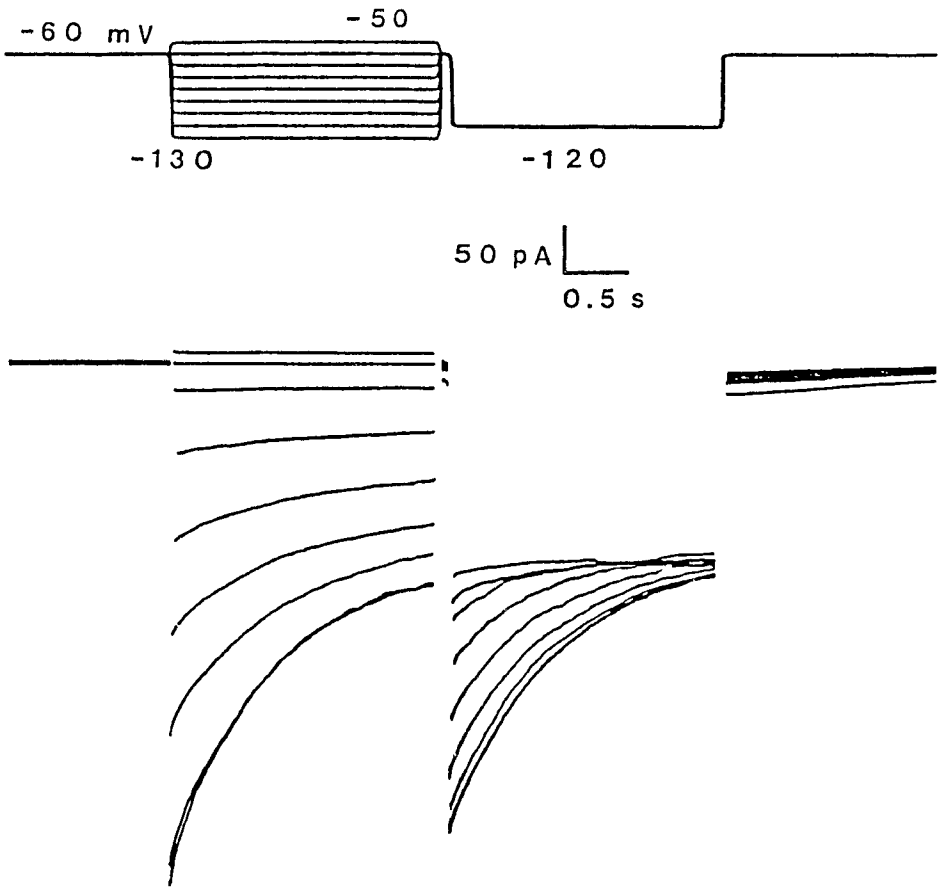

Fig. 4.2 A two step protocol was used to characterize the voltage dependence of inactivation. Prepulses were applied for 2 s to different potentials ranging from -50 to -130 mV in 10 mV increments followed by a brief return to the holding potential of -60 mV for 80 ms and then followed by a test pulse to -120 mV for 2 s (see text).

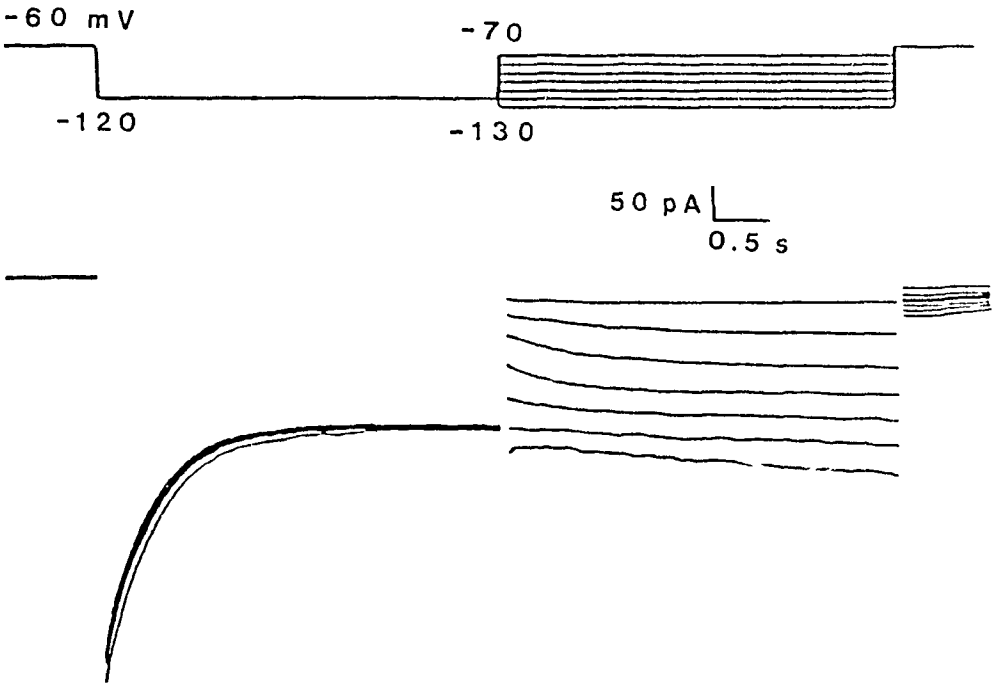

Fig. 4.3 *A two step protocol was used to generate tail currents (K_o = 10 mM; TTX = 3 uM). The holding potential was -60 mV and the conditioning prepulse was down to -120 mV for 4 s, a potential which elicited a large decaying instantaneous component The second test pulse was on for 4 s to different potentials in 10 mV increments from -70 to -130 mV (see text for details)*

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