# RECEPTOR BINDING OF SOMATOSTATIN-14 AND SOMATOSTATIN-28 IN RAT BRAIN: DIFFERENTIAL MODULATION BY NUCLEOTIDES AND IONS

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**Short title:**

MODULATION OF RAT BRAIN SOMATOSTATIN RECEPTORS BY NUCLEOTIDES AND IONS

To my Mother

Portions of this work were presented at the International Conference on Somatostatin, Washington D.C., May 1986 (Abstract 1-7), and at the Canadian Society for Clinical Investigation (CSCI), Toronto, September 1986 (Abstracts 242 & 247) (see Appendix).

# ABSTRACT

Somatostatin-14 (S-14) and somatostatin-28 (S-28) exhibit same spectrum of biological actions but different spectrum of potencies. This is reflected in part in their different relative affinities for receptor binding between tissues. These data coupled with preferential uptake of labelled S-28 demonstrated in some areas of rat brain by in vivo and in vitro autoradiography, suggest the possible existence of distinct receptors for S-14 and S-28. In the present study, an attempt was made to identify these receptors by investigating the effects of nucleotides and ions on the binding of [$^{125}$I-Tyr$^{11}$] S-14 (T* S-14) and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 (LTT* S-28) to rat brain membranes.

Results: (1) The binding of both radioligands was inhibited by adenine and guanine nucleotides. (2) This inhibition was due to a decrease in $B_{max}$. (3) T* S-14 binding sites were inhibited by ATP to a greater extent than by GTP (46.5% vs 33.2%), while GTP was more potent than ATP in reducing LTT* S-28 binding sites (54.7% vs 28.9%). (4) The affinities of these binding sites were not altered by nucleotides.

(1) Monovalent cations ($K^+$, $Na^+$, $Li^+$) inhibited the specific binding of T* S-14 and LTT* S-28. (2) In contrast divalent cations (2mM) stimulated the binding of these radioligands: T* S-14 binding sites were more sensitive to $Ba^{2+}$, $Mg^{2+}$, and $Co^{2+}$ compared to LTT* S-28 binding sites. (3) $Mg^{2+}$ up to 20 mM caused a sustained increase in binding of both ligands whereas $Ca^{2+}$ ($> 10^{-7}$ mM) inhibited the binding of LTT* S-28 without affecting that of T* S-14. (4) The increased specific binding in presence of 2 mM $Ca^{2+}$ was due to a decrease in non-specific binding. (5) The affinity of S-14 for LTT* S-28 but not for T* S-14 binding sites was directly proportional to $Ca^{2+}$. (6) This increase in affinity was accompanied by a decrease in LTT* S-28 binding sites.

These results demonstrate that nucleotides and $Ca^{2+}$ differentially modulate the receptor binding of S-14 and S-28 in rat brain providing evidence for distinct S-14/S-28 receptors.

## RESUME

La somatostatine-14 (S-14) et la somatostatine-28 (S-28) partagent un même éventail d'actions biologiques mais diffèrent de part leur potentiel respectif. Ces faits sont appuyés en partie à travers leurs différentes affinités relatives envers leurs récepteurs tissulaires. Ces découvertes auxquelles viennent s'ajouter l'incorporation préférentielle de S-28 radioactive démontrée à l'intérieur de certaines régions spécifiques du cerveau du rat par l'autoradiographie in vivo et in vitro, suggèrent l'existence de différents récepteurs pour S-28 distinct de ceux appartenant à S-14. La présente étude se voulait une tentative d'identification de tels récepteurs en analysant les effets des nucleotides et des ions sur la liaison de [$^{125}$I-Tyr$^{11}$] S-14 (T* S-14) et de [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 (LTT* S-28) au niveau des membranes cervicales chez le rat.

Résultats: (1) La liaison des 2 radioligands fut inhibée par les nucleotides à adenine et à guanine. (2) Cette inhibition était due à une diminution du Bmax. (3) Le nombre de sites récepteurs appartenant à T* S-14 fut diminué à un plus bas degré par ATP que par GTP (46.5% vs 33.2%), tandis que GTP était plus puissant que ATP pour réduire le nombre total de sites récepteurs pour LTT* S-28 (54.7% vs 28.9%). (4) Les affinités de ces sites récepteurs ne furent pas influencées par les nucleotides.

(1) Les cations monovalents ($K^+$, $Na^+$, $Li^+$) inhibèrent la liaison spécifique de T* S-14 et de LTT* S-28. (2) Les cations divalents (2 mM) cependant eurent un effet stimulateur sur la liaison de ces 2 radioligands: Les sites récepteurs de T* S-14 étant plus sensibles que ceux de LTT* S-28 au $Ba^{2+}$, $Mg^{2+}$, et $Co^{2+}$. (3) $Mg^{2+}$ (jusqu'à 20 mM) créa une stimulation maintenue de la liaison des 2 radioligands tandis que les ions $Ca^{2+}$ (> 10 mM) inhibèrent la liaison LTT* S-28 sans affecter celle de T* S-14. (4) L'augmentation de la liaison spécifique en présence de $Ca^{2+}$ 2 mM était due à une diminution de la liaison non-spécifique. (5) L'affinité de S-14 pour les sites récepteurs de LTT* S-28 mais non pour ceux de T* S-14 fut directement proportionnelle au $Ca^{2+}$. (6) Cette augmentation de l'affinité fut accompagnée par une diminution du nombre de sites récepteurs appartenant à LTT* S-28.

Ces résultats démontrent que les nucleotides et les ions $Ca^{2+}$ exercent un effet contrastant sur la liaison S-14 et S-28 au niveau du cerveau du rat, appuyant ainsi l'hypothèse de différents récepteurs pour S-14 et S-28.

## ACKNOWLEDGEMENTS

I wish first to thank Drs. C. B. Srikant and Y. C. Patel for their invaluable aid and encouragement in all aspects of this investigation. I would also like to express my appreciation to Ms. C. Craig for excellent technical assistance. Finally, I would like to thank Dr. R. Dykes for his constant support throughout this project.

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## ABBREVIATIONS

| | |
|---|---|
| S-14 | somatostatin 14 |
| S-28 | somatostatin 28 |
| $B_{max}$ | maximum binding capacity |
| $K_D$ | affinity dissociation constant |
| ACTH | adrenocorticotropic hormone |
| CRF | corticotropin-releasing factor |
| BSA | bovine serum albumin |
| RRA | radio-receptor assay |
| GH | growth hormone |
| $N_i$ | inhibitory guanyl nucleotide - binding regulatory subunit of adenylate cyclase |
| $N_s$ | stimulatory guanyl nucleotide - binding regulatory subunit of adenylate cyclase |

| | |
|---|---|
| GTP | guanosine triphosphate |
| GDP | guanosine diphosphate |
| GMP | guanosine monophosphate |
| cGMP | guanosine 5'-3' monophosphate |
| GMP-PMP | guanyl-5'-yl imidodiphosphate |
| ATP | adenosine triphosphate |
| ADP | adenosine diphosphate |
| cAMP | adenosine 5'-3' monophosphate |

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# INTRODUCTION

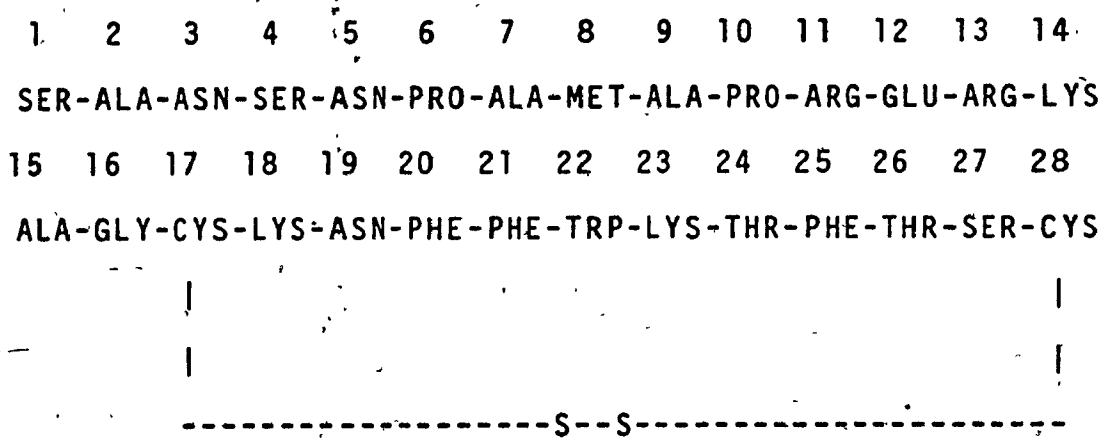
The tetradecapeptide somatostatin (S-14) has been shown to act via specific receptors (1, 2, 12, 14, 43-44, 55, 59-62, 64-65). The S-14 receptors initially demonstrated in pituitary $GH_4C_1$ tumor cells (55) have now been characterized in several tissues such as the brain (14, 43, 60), the exocrine pancreas (54, 70), and the pituitary (1, 12, 44, 62) in the normal rat. Subcellularly these receptors were localised to plasma membranes (1, 12, 60, 62). In the CNS, synaptosomal membranes were found to contain the highest concentration of S-14 binding sites (60). In the CNS, S-14 receptor concentration was found to be maximal in the cerebral cortex, followed by thalamus, hypothalamus, striatum, amygdala and hippocampus, while medulla and pons, cerebellum and spinal cord exhibited negligible binding. Outside the CNS, S-14 receptors were found to be in greater number in the adrenal cortex followed by pituitary and pancreatic acini (4).

Structure activity relationship studies using several synthetic S-14 analogs have shown that the cyclic structure of the molecule is essential for its biological activity and that amino acid residues 7-11 constitute the recognition site for receptor binding.

It is now established that in addition to S-14, two related molecules exist in tissues. These are somatostatin-28 (S-28), a 14 amino acid N-terminal extension of S-14 (Figure A) and a 92 amino acid higher molecular weight form (prosomatostatin) which contains the S-28 sequence at the C-terminal end of the molecule (41). Both S-14 and S-28 act through the same active site located at the C-terminal end of the molecule. The N-terminal fragment of S-28 appears to be biologically inactive (4).

Both S-14 and S-28 share a number of common actions. A comparison of some of these actions show that each peptide possesses its own selective specificity. For example, S-28 is more potent than S-14 in inhibiting GH and insulin but not glucagon secretion (35). It also appears that centrally administered S-28 but not S-14 inhibits pituitary ACTH and adrenomedullary epinephrine secretions (7). These data suggest that S-14 and S-28 may act as independent hormones subserving specific functions.

**Figure A: Amino acid structure of somatostatin-28.** The 15-28 sequence corresponds to somatostatin-14


1 2 3 4 5 6 7 8 9 10 11 12 13 14
SER-ALA-ASN-SER-ASN-PRO-ALA-MET-ALA-PRO-ARG-GLU-ARG-LYS
15 16 17 18 19 20 21 22 23 24 25 26 27 28
ALA-GLY-CYS-LYS-ASN-PHE-PHE-TRP-LYS-THR-PHE-THR-SER-CYS
S--S

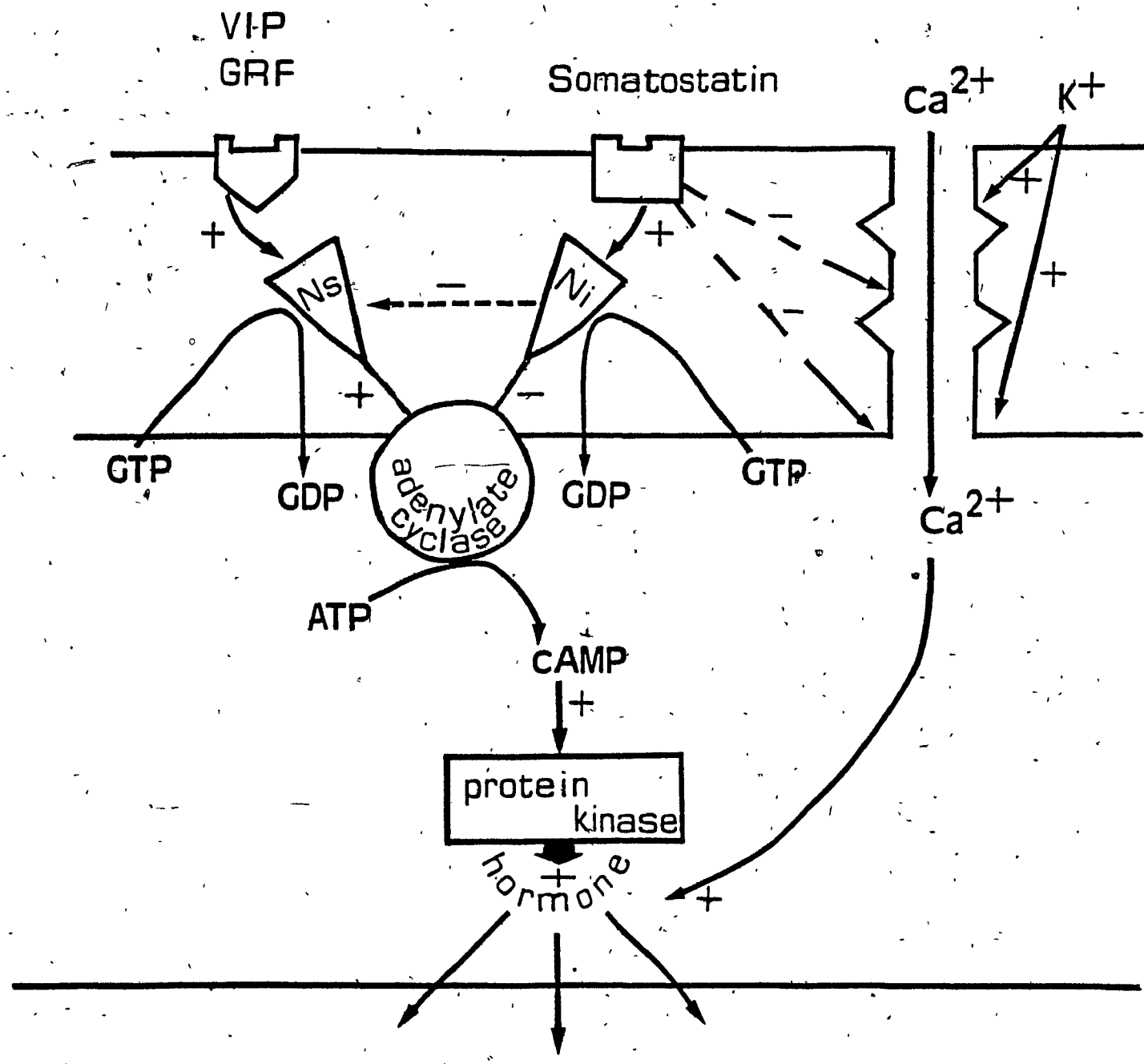
In spite of the fact that S-14 and S-28 act through the same recognition site, S-14 receptors interact with these two peptides with different relative affinities in different tissues. For example, S-28 is more potent than S-14 for binding to S-14 receptors in the pituitary, but not in the brain and the exocrine pancreas, while these two peptides bind to adreno-cortical S-14 receptors with almost equal avidity (59, 65, 66).

The fact that S-14 receptors in different tissues interact with S-14 and S-28 with different affinities coupled with the observed differences in their biological potencies strongly suggest the possible existence of S-28 receptors distinct from those of S-14 (59). Initial studies by Reubi et al. (43) on the binding of a radioligand prepared from an S-28 analog ([Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28) suggested binding parameters for rat brain receptors different from those of [$^{125}$I-Tyr$^{11}$] S-14. However, detailed binding studies using these two radioligands have failed to identify distinct S-28 receptors (63-64). In vivo autoradiographic studies however suggest that discrete areas such as the median eminence may have receptors that bind preferentially [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 but not [$^{125}$I-Tyr$^{11}$] S-14 (39).

At a post-receptor level, the actions of somatostatin are known to be mediated via a cAMP dependent-and-independent (presumably involving $Ca^{2+}$ ions and inositol triphosphate: $IP_3$) mechanisms (Figure B). S-14 is believed to act at the level of cAMP formation by inhibiting the adenylate cyclase enzyme. S-14 receptors are coupled to the adenylate cyclase via a guanine regulatory inhibitory protein ($N_i$) (27). In addition it has been shown that S-14 receptor binding could inhibit adenylate cyclase stimulation by a variety of agents (18, 27, 29). Moreover the coupling of S-14 receptors with the adenylate cyclase was demonstrated to be GTP-dependent, and blocked by non-reducible analogs of GTP such as GMP-PNP (27, 29). Therefore, nucleotides appear to be obligatory mediators of S-14 actions. Such an involvement of nucleotides in mediating hormone-receptor interactions has also been demonstrated with binding sites for substance P (3, 58), glucagon (31, 46-47), TRH (21), CRF (40), and beta-adrenergic receptors (34, 50, 67).

Studies from Enjalbert et al. (13) indicate that guanine nucleotides inhibit S-14 receptor binding in rat brain and pituitary leading to a decrease in receptor number without any alteration of the affinity while

**Figure B:** **Molecular mechanisms following somatostatin receptor binding.** (See text for details).


VIP
GRF
Somatostatin
Ca2+
K+
+
+
Ns
Ni
−
+
−
GTP
GDP
adenylate cyclase
GDP
GTP
Ca2+
ATP
cAMP
+
protein kinase
hormone
+
+

adenine nucleotides were found to have no effect on S-14 receptor binding.

By contrast it was reported that in $GH_4C_1$ cells, guanine nucleotides inhibited S-14 receptor binding by increasing the dissociation rate thereby decreasing the binding affinity (56). However, the modulation of the binding of S-28 by these mediators in any of these systems has not been investigated. Studies from Srikant and Heisler (63) in AtT20 pituitary tumor cells have demonstrated that the cAMP-dependent mechanism is more sensitive to S-14 than to S-28 whereas the cAMP-independent pathway is more sensitive to S-28 than to S-14. It is still not clear whether the differential potencies of S-14 and S-28 arise due to distinct binding sites or if these peptides, acting via the same binding sites are differentially coupled to these processes. In order to determine if distinct receptors for S-14 and S-28 could be identified by possible differences in their sensitivity to nucleotides in direct binding studies, we investigated the modulation by guanine and adenine nucleotides of the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28.

In addition, many receptor systems coupled to Ac (adenylate cyclase) via $\overline{N_i}$ or $N_s$ (stimulatory regulatory component of the guanine protein) are sensitive to ions (5, 9-10, 23, 30, 33, 40, 73). Divalent ions ($Mg^{2+}$ in particular) have been shown to modify receptors to high affinity conformation, guanine nucleotides reverse this effect by converting the receptors to low affinity forms (3, 9, 23). To determine if such an effect occurs with somatostatin receptors, we investigated the modulation by $Mg^{2+}$ and other divalent cations such as $Ba^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Ca^{2+}$ as well as by monovalent cations: $Na^+$, $K^+$, $Li^+$ on the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^8$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28.

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# METHODS

Preparation of synaptosomal membranes:

Brains were removed from adult male Sprague-Dawley rats (150-160 g) immediately following decapitation. The cerebral cortex was dissected, homogenised with a Dounce homogenizer under isoosmolar conditions in 20 mM Tris HCl (pH = 7.5) containing 0.32 M sucrose. Synaptosomal membranes were prepared according to Srikant and Patel (60). Briefly, the homogenate was centrifuged at 800 x g for 5 min. in order to remove the nuclear debris. The supernatant obtained was resuspended in 0.32 M sucrose buffer and centrifuged at 10,000 x g for 10 min. The resulting pellet containing the crude mitochondrial fraction was resuspended in 0.32 M sucrose, applied to the top of a discontinuous Ficoll gradient (8-20 %) in 0.32 M sucrose, and centrifuged at 63,580 x g for 30 min. in a Beckman L5-65 ultracentrifuge. Synaptosomal fraction sedimenting at the Ficoll (16-12 %) interface, which had been shown to contain the highest concentration of S-14 receptors, was collected and hypoosmotically lysed by swelling in 20 mM Tris-HCl (pH = 7.5) overnight. This procedure removed > 95 % of endogenous somatostatin which was a prerequisite for demonstrating optimal binding of

S-14 receptors. Membranes were centrifuged at 10,000 x g the following day and the pellets obtained were resuspended in 20 mM Tris-HCl and stored in liquid nitrogen for no more than 3 months until the day of use.

Preparation of radioiodinated peptides:

Radioiodinated derivatives of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 were prepared by modification of the chloramine-T method using Na$^{125}$I and purified by chromatography on Sephadex G-25 ([$^{125}$I-Try$^{11}$] S-14) or Sephadex G-50 ([Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28) (65). The specific activities of these two radioligands were 1850 and 1050 Ci/mmol respectively for [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28.

Equilibrium binding studies:

Somatostatin receptor binding was carried out as described by Srikant and Patel (60). Briefly, membranes

(30 µg) were incubated at 30°C for 40 min. with 0.1 nM [$^{125}$I-Tyr$^{11}$] S-14 until equilibrium binding was achieved in a 50 mM Hepes KOH buffer (pH = 7.5) containing BSA (10 mg/ml), $MgCl_2$ (5 mM), Trasylol (200 KIU/ml), bacitracin (0.02 µg/ml) and phenylmethylsulfonyl fluoride (0.02 µg/ml) (RRA buffer). Radioligand bound to the membranes was separated by centrifugation (3000 x g for 5 min.). Subsequently these membranes were washed twice with 1 ml of 50 mM Hepes KOH (pH = 7.5) containing 1 % albumin. The radioactivity in the resulting pellet was quantitated in a gamma spectrometer. Specific binding was defined as the difference in the amount of radioligand bound in the absence (total binding) and presence (non-specific binding) of 100 nM S-14. Competitive inhibition of the radioligand binding was investigated using unlabelled S-14 over a concentration range varying from $10^{-10}$ M to 5 x $10^{-8}$ M. Calculation of the binding data and determination of binding parameters ($K_d$ and $B_{max}$) were performed by Scatchard analysis with the aid of the computer program of Rodbard and Faden (45).

Binding studies using [Leu$^8$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 as the radioligand were carried out using the exact procedure described for [$^{125}$I-Tyr$^{11}$] S-14 binding.

Effect of nucleotides on the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28:

To determine the effect of nucleotides on somatostatin receptors in rat brain, the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 was assessed in presence and absence of guanine or adenine nucleotides. The nucleotides tested for their effects on somatostatin receptors were: GTP, GDP, GMP-PNP, cGMP, GMP, and ATP, cAMP, ADP at concentrations varying from $10^{-7}$ M to $10^{-3}$ M. To determine the nature of the effects of these nucleotides, competitive studies using S-14 and S-28 vs [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 were carried out in the absence or presence of $10^{-4}$ M nucleotides.

Kinetic studies:

A- Association experiments

Synaptosomal membranes were incubated at 30°C with [$^{125}$I-Tyr$^{11}$] S-14 or [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28

in the presence and absence of GTP or ATP ($10^{-4}$ M). At different time intervals during the approach to equilibrium binding, the amount of radioligand specifically bound was determined. The association and dissociation rate constants of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$-I-Tyr$^{25}$] S-28 binding were analysed as pseudo-first order kinetics (24) defined by the equation:

$$\ln \frac{[B_{eq}]}{[B_{eq}]-[B]} = ([L]\, k_{on} + k_{off})\, t$$

where $[B_{eq}]$ = concentration of bound somatostatin at equilibrium; [B] = concentration of bound peptide at a given time t; [L] = concentration of the ligand; $k_{on}$ = association rate constant; $k_{off}$ = dissociation rate constant.

The rate constant for the approach to equilibrium (kobs.) was determined by plotting $(1-[B]/[B_{eq}])$ as

a function of time. In the straight line obtained, the half-time for approach to equilibrium corresponded to the association rate constant - Kobs. Moreover, because of the linearity of the plot (pseudo-first order kinetics) the rate to approach equilibrium was described by the differential equation:

$$\frac{d[HR]}{dt} = k_{on}[H][R] - k_{off}[HR]$$

where [H] = total hormone concentration; [R] = receptor concentration.

Under these conditions, integration of this equation results in:

$$k_{obs} = \frac{\ln 2}{t_{1/2}} = k_{on} + k_{off}$$

in which $t_{1/2}$ = time at which [HR] reaches one half of its equilibrium value

B- Dissociation experiments

Membranes were incubated at 30°C with [$^{125}$I-Tyr$^{11}$] S-14 or [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 until equilibrium binding was achieved. After incubation, membrane-bound radioligand was pelleted by centrifugation, washed twice with 1 ml 50 mM Hepes KOH buffer containing 10 mg/ml BSA, resuspended in a large volume of the RRA buffer with the addition of $10^{-7}$ M unlabelled peptide, GTP or ATP ($10^{-4}$ M); and reincubated at 30°C. At specified times, during 120 min. incubation, duplicate 100 µl aliquots were withdrawn and centrifuged and the radioactivity still remaining bound to the membrane receptors was measured. The dissociation rate constant ($k_{off}$) was derived from the equation:

$$\ln \frac{[B]}{[B_{eq}]} = k_{off} \cdot t$$

plot of $[B]/[B_{eq}]$ as a function of time gives a straight line, the slope of which corresponds to a dissociation constant $k_{off}$.

Therefore the association constant ($k_{on}$) was calculated from:

$$k_{on} = \frac{K_{obs} - k_{off}}{[H]}$$

From association and dissociation rate constants, we determined the affinity constant ($K_D$) by the equation:

$$K_D = \frac{k_{off}}{k_{on}}$$

Effect of ions on the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28:

To assess the effect of ions on the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28, membranes were incubated with these radioligands in the RRA buffer from which $Mg^{2+}$ ions were omitted and replaced with the various ions to be tested.

Monovalent ions ($Na^{+}$, $K^{+}$, and $Li^{+}$) were tested over a concentration varying from 0 to 160 mM. Divalent ions ($Ba^{2+}$, $Co^{2+}$, $Mn^{2+}$, and $Ca^{2+}$) were tested over a concentration range of 0 to 20 mM.

Effect of $Ca^{2+}$ on binding affinities of S-14 and S-28:

Because high $Ca^{2+}$ concentrations inhibited only the binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 without affecting that of [$^{125}$I-Tyr$^{11}$] S-14 (vide results), we investigated the effect of this divalent cation at 2 and 10 mM concentrations on the competitive inhibition by S-14 and S-28 of the binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 in

order to evaluate the nature of this inhibition and to determine if this would affect the potencies of S-14 and S-28 to compete against this radioligand in relation to their affinities for [$^{125}$I-Tyr$^{11}$] S-14 binding sites.

The binding affinity of the peptides and the relative affinity of S-28, compared to that of S-14 were calculated as follows. The data obtained from the dose-dependent inhibition of the radioligand by S-14 and S-28 were analysed by the computer program of Rodbard and Faden (45). The parallelism between the inhibition curves of S-14 and S-28 was analysed by an F-test for homogeneity and lines were checked for parallelism by a t-test using this computer program. The relative affinities were calculated from this program as well as the concentration of each peptide required to effect 50 % inhibition of the radioligand binding.

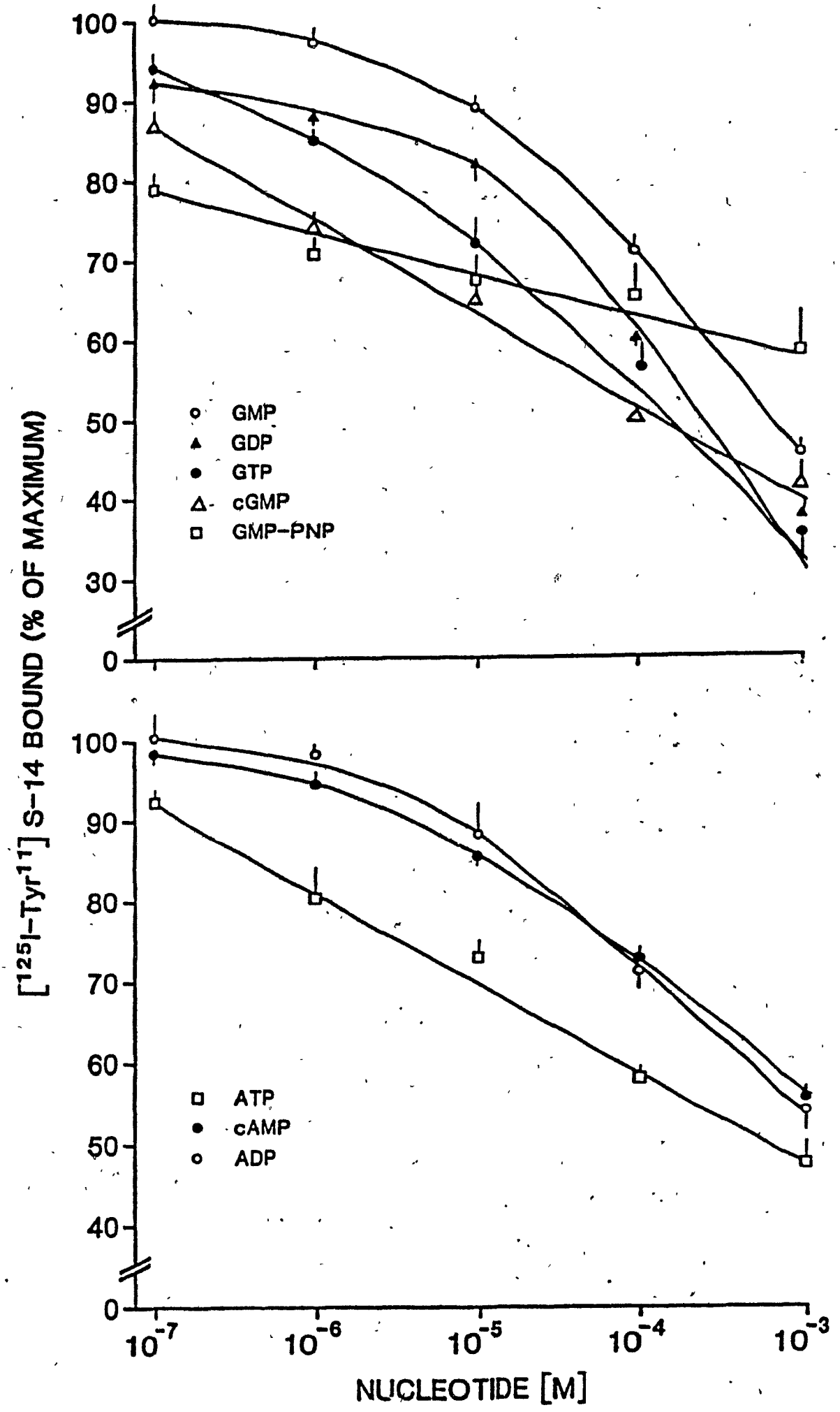
# RESULTS

The binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28 to rat brain synaptosomal membranes was specific and could be inhibited by S-14 in a dose-dependent manner. Furthermore, using these two ligands, similar binding parameters were observed: $B_{max}$ = 340 ± 15 fmol/mg prot.; $K_D$ = 1.2 ± 0.3 nM for [$^{125}$I-Tyr$^{11}$] S-14 and $B_{max}$ = 320 ± 20 fmol/mg prot.; $K_D$ = 1.6 ± 0.3 nM for [Leu$^{8}$,D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28. It has also been reported that both S-14 and S-28 compete against these radioligands for their binding to rat brain membranes with similar relative potencies (64).

Effect of nucleotides on [$^{125}$I-Tyr$^{11}$] S-14 binding:

The binding of [$^{125}$I-Tyr$^{11}$] S-14 to rat brain membranes was inhibited by guanine nucleotides. As shown in Fig. 1 (top panel) GMP, GDP, GTP, cGMP, and GMP-PNP inhibited the specific binding of this radioligand in a dose-dependent manner over a concentration range varying from $10^{-7}$ M to $10^{-3}$ M. Maximal inhibition of the binding ranging from 40.2 ± 2.6 % to 66.0 ± 2.0 % was observed with guanine nucleotides at $10^{-3}$ M. The dose-dependent inhibition of the radioligand binding by GMP, GDP, GTP,

**Fig. 1:** **Inhibition of the specific binding of [$^{125}$I-Tyr$^{11}$] S-14 by guanine nucleotides** (top panel) **and adenine nucleotides** (bottom panel) to rat brain synaptosomal membranes. Amount of [$^{125}$I-Tyr$^{11}$] S-14 specifically bound expressed as a % of maximum specific binding observed in the absence of nucleotides is plotted as a function of nucleotide concentration. Values represent mean $\pm$ S.E. (n = 4).


100
90
80
70
60
50
40
30
0
GMP
GDP
GTP
cGMP
GMP–PNP
100
90
80
70
60
50
40
0
ATP
cAMP
ADP
[125I–Tyr11] S–14 BOUND (% OF MAXIMUM)
10-7
10-6
10-5
10-4
10-3
NUCLEOTIDE [M]

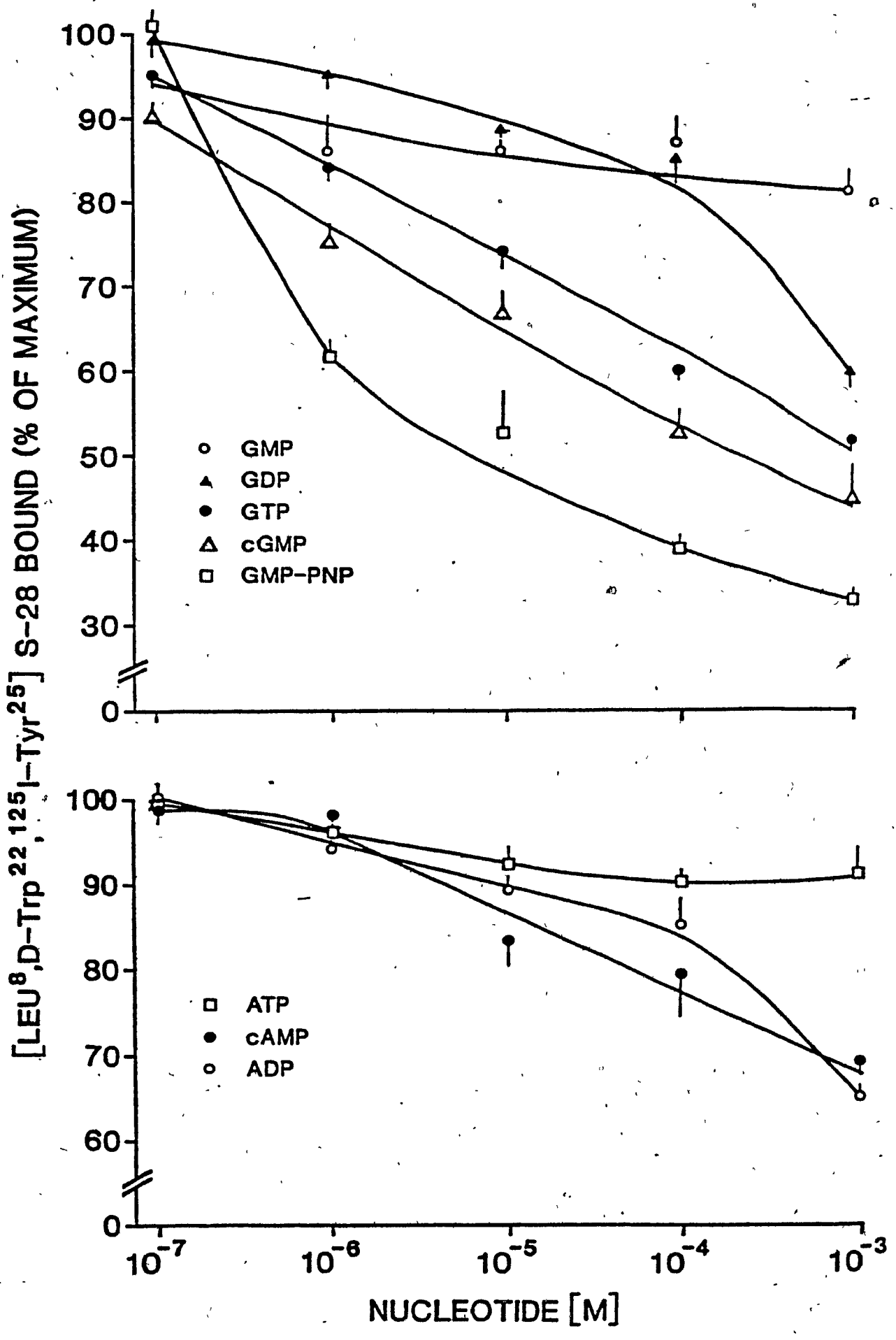
and CGMP were parallel and the order of potency was cGMP > GTP > GDP > GMP. At low concentrations ($\simeq 10^{-7}$ M), the non-reducible analog of GTP: GMP-PNP was more potent than the other guanine nucleotides in decreasing [$^{125}$I-Tyr$^{11}$] S-14 binding. However its inhibitory effect at the highest concentration tested ($10^{-3}$ M) was lower than that observed with the other guanine nucleotides (40.0 $\pm$ 2.6 % inhibition with GMP-PNP compared to $\simeq$60 % inhibition with the other nucleotides).

Amongst adenine nucleotides ATP, cAMP, and ADP reduced [$^{125}$I-Tyr$^{11}$] S-14 binding in a dose-dependent fashion (Fig. 1; bottom panel). ATP was the most potent adenine nucleotide followed by cAMP and ADP (47.1 $\pm$ 0.9 % and 56.0 $\pm$ 0.8 %, 57.3 $\pm$ 1.2 % inhibition for ATP, cAMP and ADP respectively compared to control).

Effect of nucleotides on [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding:

The binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 was also inhibited by guanine nucleotides (Fig. 2; top panel). GTP, GDP, cGMP, GMP, and GMP-PNP maximally reduced the binding of this radioligand at $10^{-3}$ M (40.5 $\pm$ 1.9 % to

**Fig. 2: Inhibition of the specific binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 by guanine nucleotides** (top panel) **and adenine nucleotides** (bottom panel) to rat brain synaptosomal membranes. Amount of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 specifically bound expressed as % of maximum specific binding observed in the absence of nucleotides is plotted as a function of nucleotide concentration. Values represent mean + S.E. (n = 4).

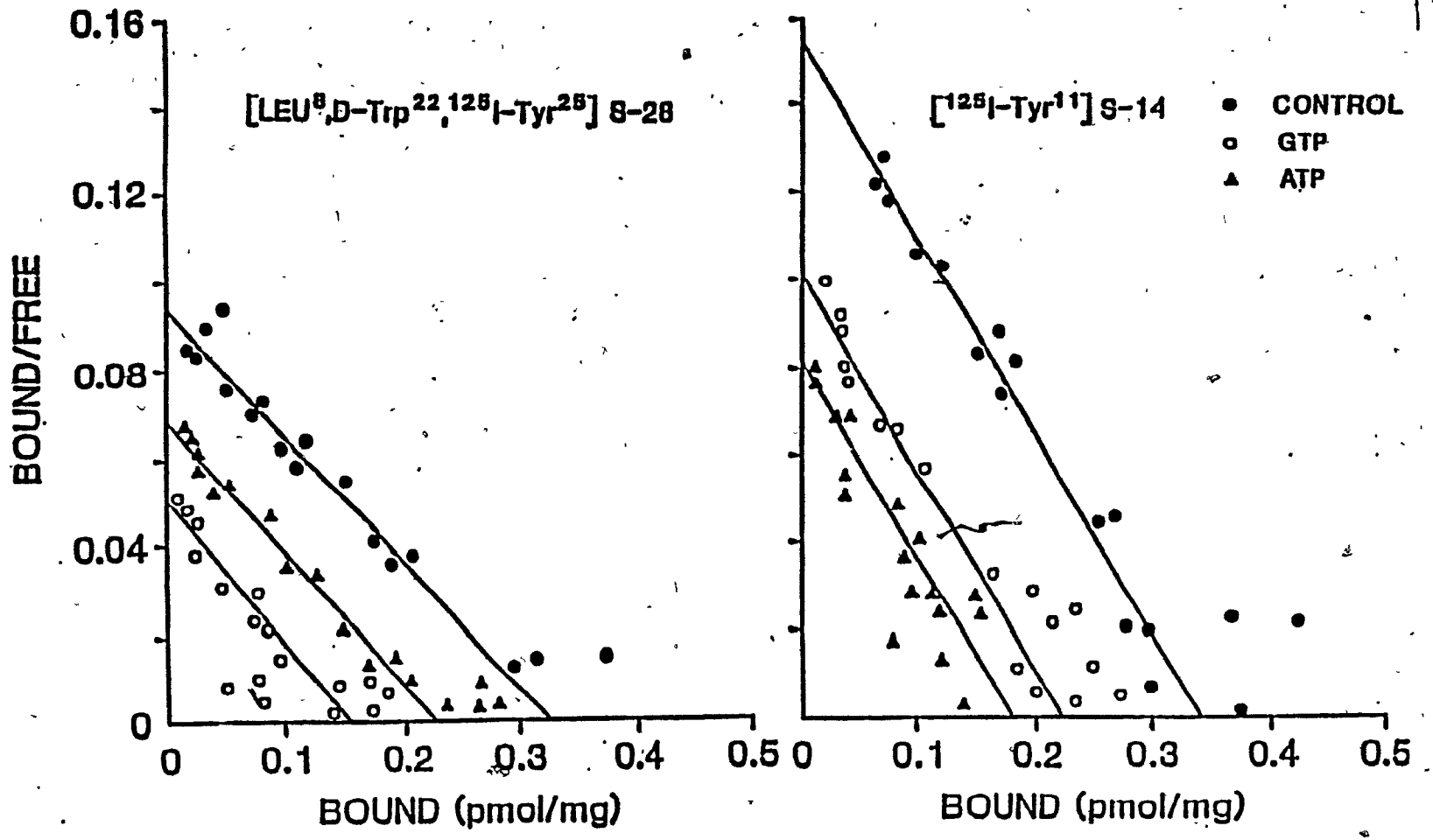

100
90
80
70
60
50
40
30
0
GMP
GDP
GTP
cGMP
GMP-PNP
100
90
80
70
60
0
ATP
cAMP
ADP
$10^{-7}$
$10^{-6}$
$10^{-5}$
$10^{-4}$
$10^{-3}$
NUCLEOTIDE [M]
[LEU$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 BOUND (% OF MAXIMUM)

65.2 ± inhibition). GMP-PNP was more potent than cGMP > GTP while GMP even at maximum concentration tested had very little effect on the binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28.

Amongst adenine nucleotides ATP, cAMP, and ATP reduced [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding in a dose-dependent manner from $10^{-7}$ M to $10^{-3}$ M (fig. 2; bottom panel). Maximal inhibition observed in the presence of these nucleotides was only 36.8 ± 2.0 %. cAMP and ADP were almost equipotent in inhibiting [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding whereas ATP had almost no effect on the specific binding of this radioligand, even at the highest concentration tested (8.2 ± 0.9 % inhibition at $10^{-3}$ M).

To determine the nature of the inhibition of the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 by adenine and guanine nucleotides, we investigated the competitive inhibition of the binding of these radioligands by S-14 in the absence and presence of nucleotides. Scatchard analyses of the binding data, in presence and absence of GTP and ATP ($10^{-4}$ M) (Fig. 3) revealed that this inhibition was due to a decrease in the number of binding sites without any concomitant change in affinity. ATP effected greater reduction in $B_{max}$ of

**Fig. 3: Effects of GTP and ATP ($10^{-4}$M) on the competitive inhibition of the binding of [$^{125}$I-Tyr$^{11}$] S-14 (right panel) and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 (left panel) to rat brain synaptosomal membrane receptors by unlabelled peptide (S-14). Data represent means of 4 experiments in duplicate.**


[LEU8,D-Trp22,125I-Tyr25] S-28
[125I-Tyr11] S-14
CONTROL
GTP
ATP
BOUND/FREE
0.16
0.12
0.08
0.04
0
0
0.1
0.2
0.3
0.4
0.5
BOUND (pmol/mg)
BOUND (pmol/mg)

[$^{125}$I-Tyr$^{11}$] S-14 binding sites than GTP: 183 ± 15 and 227 ± 18 ($p < .001$ and $p < .01$) respectively compared to 340 ± 15 fmol/mg for control. The reduction in $B_{max}$ of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites was greater in presence of GTP than with ATP (145 ± 10 and 228 ± 16 ($p < .001$) respectively compared to 320 ± 20 fmol/mg for control ($p < .001$)). As shown in Table 1, all guanine nucleotides reduced the number of binding sites interacting with [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 the order of potency being: GTP > GMP-PNP > GMP = GDP = cGMP for [$^{125}$I-Tyr$^{11}$] S-14 binding sites. GMP, GDP, and GMP-PNP produced comparable inhibition of [Leu$^{8}$,D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28 binding sites but were less potent than GTP and cGMP. Amongst adenine nucleotides, ATP was more potent than ADP & cAMP for reducing [$^{125}$I-Tyr$^{11}$] S-14 binding sites and cAMP > ADP > ATP for reducing [Leu$^{8}$, D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites respectively (Table 2).

**Table 1:**

EFFECT OF GUANINE NUCLEOTIDES ON [$^{125}$I-Tyr$^{11}$] S-14 AND [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 BINDING SITES IN RAT BRAIN

| | Bmax (fmol/mg) | |
|---|---|---|
| NUCLEOTIDE (M) | [$^{125}$I-Tyr$^{11}$] S-14 BINDING SITES | [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 BINDING SITES |
| NONE | 340 ± 15 | 320 ± 20 |
| GTP | 227 ± 18* | 145 ± 10* |
| GMP-PNP | 250 ± 10* | 161 ± 10* |
| GDP | 283 ± 10* | 156 ± 18* |
| cGMP | 285 ± 17* | 140 ± 13* |
| GMP | 276 ± 20* | |

MEAN ± SE, n=4

* p <.01

**Table 2:**

EFFECT OF ADENINE NUCLEOTIDES ON [$^{125}$I-Tyr$^{11}$] S-14 AND [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 BINDING SITES IN RAT BRAIN

| | Bmax (fmol/mg) | |
|---|---|---|
| NUCLEOTIDE (M) | [$^{125}$I-Tyr$^{11}$] S-14 BINDING SITES | [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 BINDING SITES |
| NONE | 340 ± 15 | 320 ± 20 |
| ATP | 183 ± 15** | 228 ± 16* |
| cAMP | 258 ± 14* | 196 ± 2** |
| ADP | 240 ± 15* | |

MEAN ± SE, n = 4

* $p < .01$

** $p < .001$

Kinetic studies:

To determine if adenine and guanine nucleotides alter the association and dissociation rates of these receptors, kinetic studies were carried out in the absence and presence of nucleotides. Addition of nucleotides increased both association and dissociation rates of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding (Figs 4 & 5). However this increase was found to be non-significant. Dissociation constants ($K_D$) calculated from these kinetic studies were comparable to those derived from Scatchard analyses: 1.0 $\pm$ 0.3 vs 1.2 $\pm$ 0.3 nM in presence of GTP for [$^{125}$I-Tyr$^{11}$] S-14 binding and 1.1 $\pm$ 0.2 vs 1.6 $\pm$ 0.3 nM in presence of GTP for [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding (Table 3).

**Fig. 4:** **Rate of binding of somatostatin receptors in rat brain membranes** in the absence or presence of $10^{-4}$ M GTP. Specific binding of [$^{125}$I-Tyr$^{11}$] S-14 (top panel) and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 (bottom panel) was plotted as a function of time. $B_t$ = specific binding at time t; $B_{max}$ = specific binding at equilibrium. Data represent mean $\pm$ S.E. (n=4).

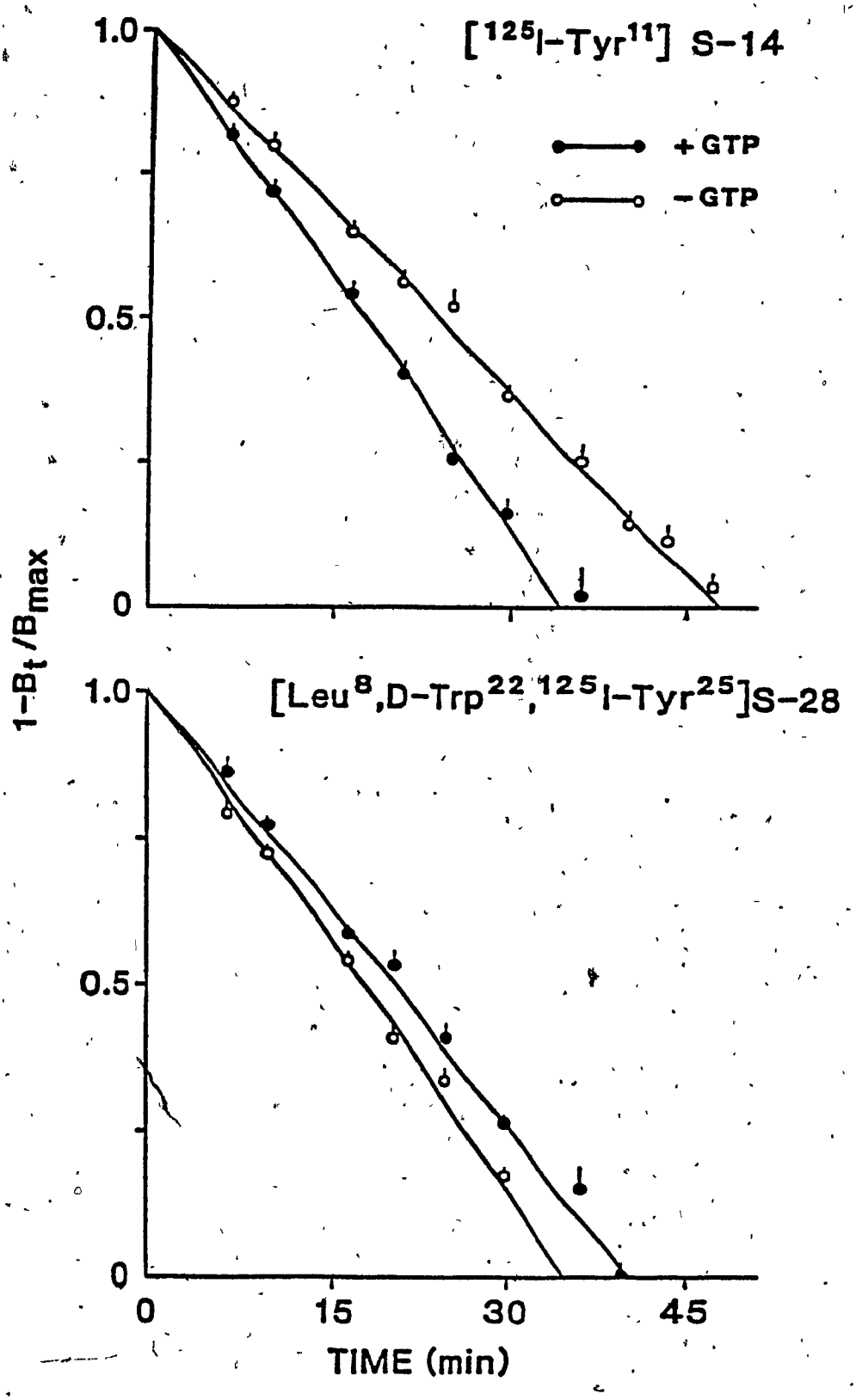

[125I-Tyr11] S-14
+GTP
-GTP
1.0
0.5
0
1-Bt/Bmax
[Leu8,D-Trp22,125I-Tyr25]S-28
1.0
0.5
0
0
15
30
45
TIME (min)

**Fig. 5: Rate of dissociation of receptor bound radioligand from rat brain synaptosomal membranes** in the absence or presence of $10^{-4}$ M GTP. Following equilibrium binding, the binding medium was removed ($t = 0$) and 2 ml of fresh buffer with or without addition of $10^{-4}$ M GTP. The amount of [$Leu^{8}$,D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28 (bottom panel) remaining bound at different times (B) was determined. The ln of the ratio of $B/B_{eq}$ was plotted as a function of time. Values represent mean $\pm$ S.E. (n = 4).

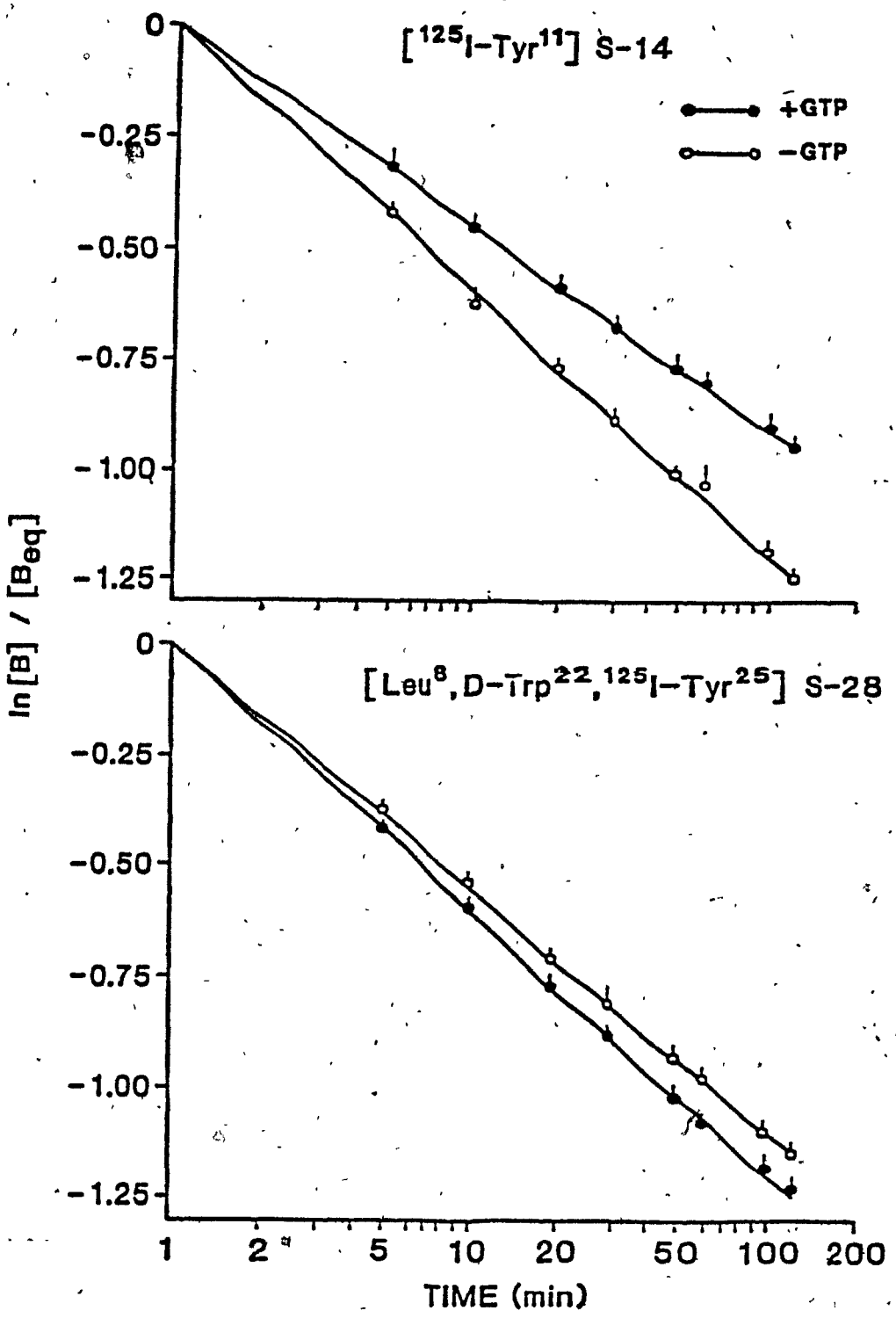

[125I-Tyr11] S-14
+GTP
−GTP
0
-0.25
-0.50
-0.75
-1.00
-1.25
[Leu8, D-Trp22, 125I-Tyr25] S-28
ln [B] / [Beq]
1
2
5
10
20
50
100
200
TIME (min)

**Table 3:**

EFFECT OF GTP ON RATE AND EQUILIBRIUM CONSTANTS OF
[$^{125}$I-Tyr$^{11}$] S-14 AND [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28
BINDING TO RAT BRAIN MEMBRANES

| | $k_{on}$ ($\times 10^4 M^{-1}S^{-1}$) | $k_{off}$ ($\times 10^4 S^{-1}$) | $k_{obs}$ ($\times 10^4 S^{-1}$) | $k_d$ (nM) |
|---|---|---|---|---|
| [$^{125}$I-Tyr$^{11}$] S-14 | | | | |
| CONTROL | 5.1 ± .7 | 5.0 ± .6 | 5.0 ± .6 | 1.0 ± .2 (1.2 ± .3*) |
| GTP | 6.7 ± .5** | 6.6 ± .3** | 6.6 ± .7** | 1.0 ± .3 (1.2 ± .3) |
| [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 | | | | |
| CONTROL | 5.8 ± .2 | 6.5 ± .2 | 6.5 ± .5 | 1.1 ± .2 (1.6 ± .3) |
| GTP | 5.6 ± .2** | 6.0 ± .2** | 6.0 ± .5** | 1.1 ± .2 (1.6 ± .3) |

MEAN ± SE, n = 4

* EQUILIBRIUM DISSOCIATION CONSTANTS SHOWN IN PARENTHESES CALCULATED FROM SCATCHARD ANALYSES.

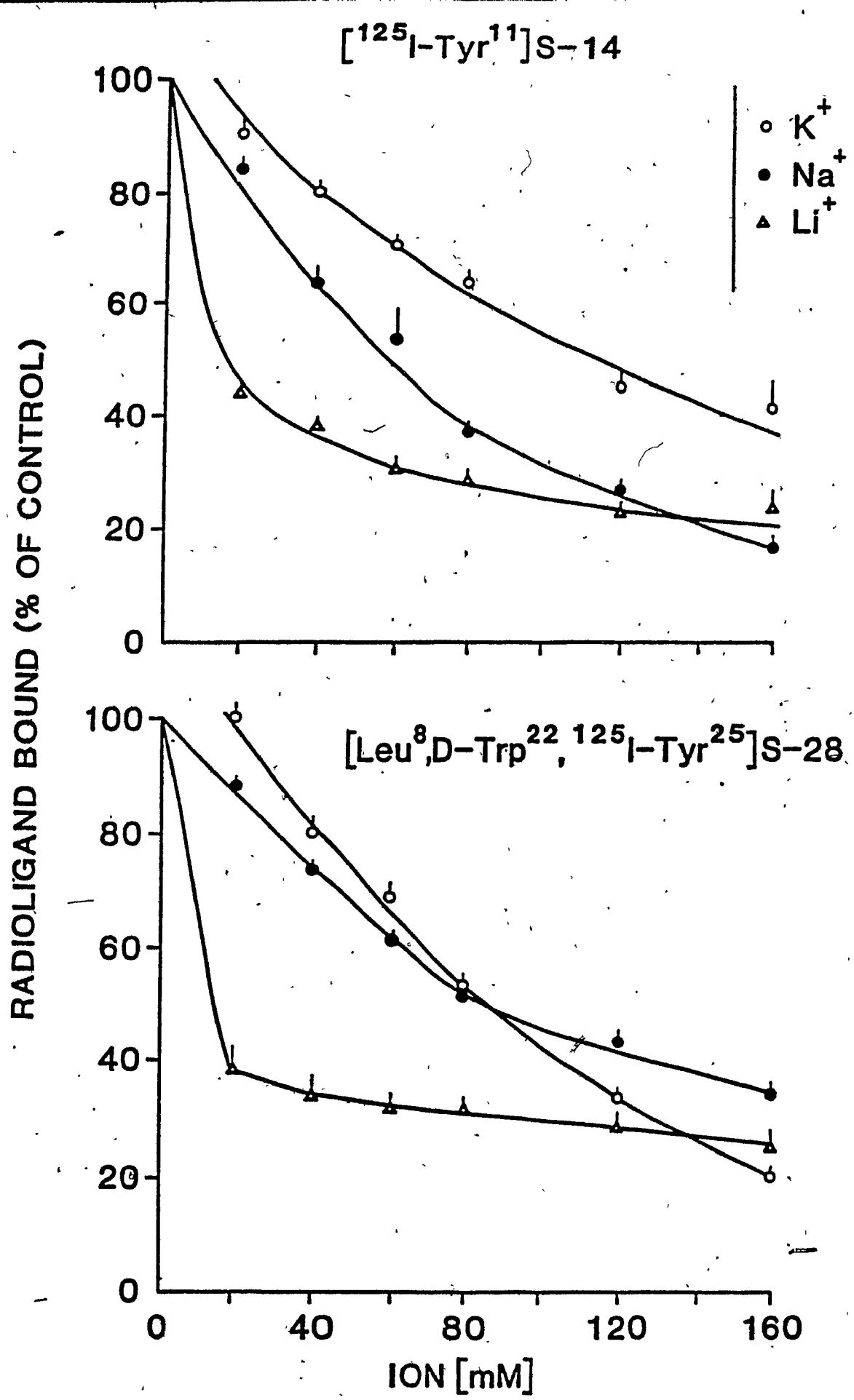
** $p \leq .1$

Effect of monovalent cations on [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding:

Amongst monovalent cations, $K^+$, $Na^+$, and $Li^+$ inhibited [$^{125}$I-Tyr$^{11}$] S-14 binding in a dose-dependent fashion over a concentration range varying from 0 to 160 mM (Fig. 6; top panel). Maximal inhibition of [$^{125}$I-Tyr$^{11}$] S-14 specific binding (from 60. $\pm$ 1.9 % to 84.5 $\pm$ 2.0 %) was observed with all monovalent ions at 160 mM. $Li^+$ and $Na^+$ were more potent than $K^+$ in reducing [$^{125}$I-Tyr$^{11}$] S-14 binding.

The binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 was also decreased by monovalent ions (Fig. 6; bottom panel). At low concentrations, $Li^+$ markedly reduced [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding. However, no further reduction of the binding of this radioligand was seen at high concentrations of $Li^+$. In addition, $K^+$ had a greater inhibitory effect than $Na^+$ (75.0 $\pm$ 0.9 % vs 60.1 $\pm$ 1.2 %) on the binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28.

**Fig. 6:** **Effect of monovalent cations on the specific binding of [$^{125}$I-Tyr$^{11}$] S-14** (top panel) **and of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28** (bottom panel) to rat brain synaptosomal membranes. Amount of the radioligands specifically bound in presence of ions expressed as % of specific binding observed in the absence of ions is plotted as a function of ionic concentration. Values represent mean ± S.E. (n = 4).


[125I-Tyr11]S-14
100
80
60
40
20
0
K+
Na+
Li+
RADIOLIGAND BOUND (% OF CONTROL)
[Leu8,D-Trp22,125I-Tyr25]S-28
100
80
60
40
20
0
0
40
80
120
160
ION [mM]

Effect of divalent ions on [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding:

Amongst divalent ions $Mg^{2+}$, $Co^{2+}$, $Mn^{2+}$, $Ba^{2+}$, and $Ca^{2+}$ increased [$^{125}$I-Tyr$^{11}$] S-14 binding (Fig. 7, top panel) in a dose dependent manner. At low concentrations ($\simeq$ 2mM) all of these ions increased the specific binding of this radioligand by 0.6 to 1.8 fold ($p < .001$); at these concentrations, the order of potency was $Ba^{2+} > Ca^{2+} > Mg^{2+} \simeq Co^{2+} > Mn^{2+}$ (Fig. 7, top panel). However at higher concentrations (up to 20 nM) $Ba^{2+}$ decreased [$^{125}$I-Tyr$^{11}$] S-14 binding and $Co^{2+}$ inhibited the binding of this radioligand below the basal binding (Fig. 8; top panel). In contrast, stimulation of the binding of [$^{125}$I-Tyr$^{11}$] S-14 by $Mg^{2+}$, $Ca^{2+}$, and $Mn^{2+}$ remained sustained (1.7, 1.8, and 0.7 fold for $Mg^{2+}$, $Ca^{2+}$, and $Mn^{2+}$ respectively) at high concentrations (up to 20 mM).

The binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 was also increased by low concentrations of $Mg^{2+}$, $Ca^{2+}$, and $Ba^{2+}$, $Mn^{2+}$, $Co^{2+}$. The order of potency was: $Ca^{2+} > Mn^{2+} > Mg^{2+} > Ba^{2+} > Co^{2+}$ ($p < .001$) (Fig. 7, bottom panel). Although

**Fig. 7:** **Comparison of the stimulatory effect of 2 mM concentration of divalent cations on the binding of [$^{125}$I-Tyr$^{11}$] S-14** (top panel) **and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28** (bottom panel) to rat brain synaptosomal membranes. Values represent mean $\pm$ S.E. (n = 4). (*: $p < .001$).

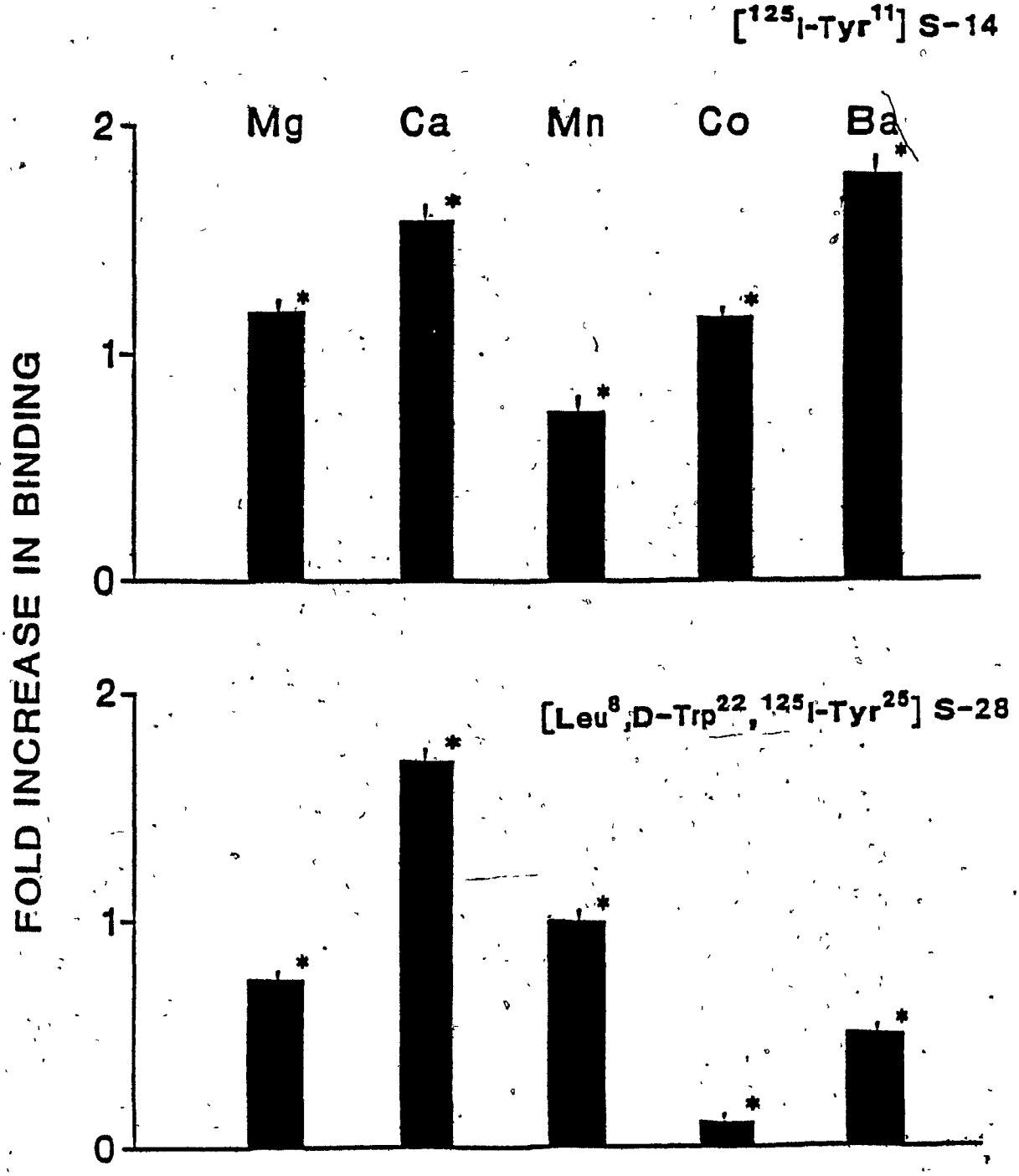
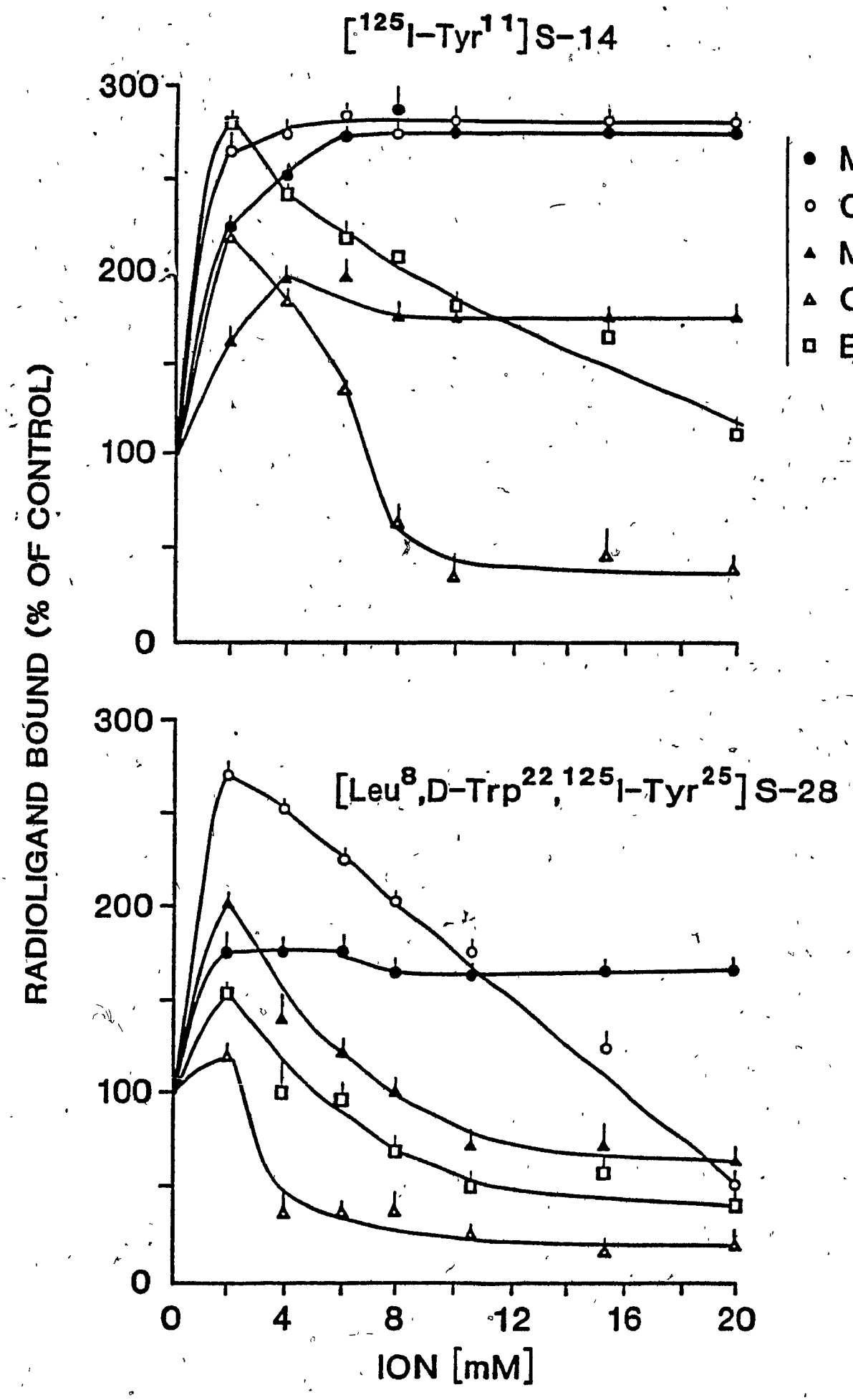

[125I-Tyr11] S-14
Mg
Ca
Mn
Co
Ba
2
1
0
FOLD INCREASE IN BINDING
[Leu8,D-Trp22,125I-Tyr25] S-28
2
1
0

**Fig. 8:** **Effect of divalent cations on the specific binding of [$^{125}$I-Tyr$^{11}$] S-14** (top panel) **and (Leu$^{8}$,D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28** (bottom panel) to rat brain synaptosomal membranes. Amount of the radioligands specifically bound expressed as % of maximum specific binding observed in the absence of these ions is plotted as a function of ionic concentration. Values represent mean ± S.E. (n = 4).

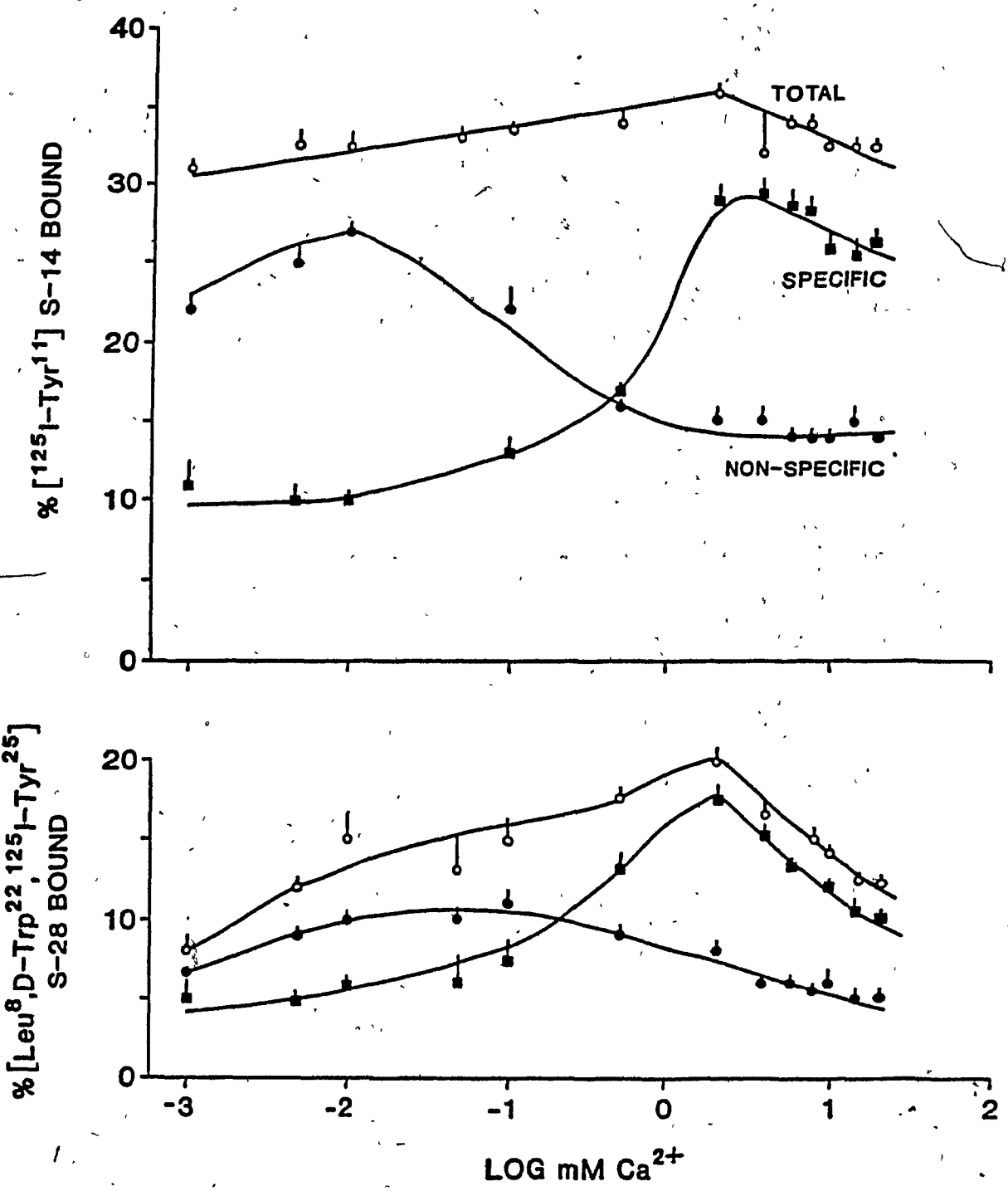

[125I-Tyr11]S-14
300
200
100
0
Mg2+
Ca2+
Mn2+
Co2+
Ba2+
RADIOLIGAND BOUND (% OF CONTROL)
[Leu8,D-Trp22,125I-Tyr25]S-28
300
200
100
0
0
4
8
12
16
20
ION [mM]

low concentrations of $Mn^{2+}$, $Co^{2+}$, and $Ba^{2+}$ increased [$Leu^8$,D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28 binding (1.0; 0.1; and 0.5 fold respectively) concentrations of these ions exceeding 4 mM reduced the binding of this radioligand. Stimulation of [$Leu^8$,D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28 binding by $Mg^{2+}$ (0.7 fold) was sustained even at maximal concentrations of this ion ( ≈ 20mM), however $Ca^{2+}$ at high concentrations inhibited the binding of [$Leu^8$.D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28.

Effect of $Ca^{2+}$ on [$^{125}$I-$Tyr^{11}$] S-14 and [$Leu^8$,D-$Trp^{22}$, $^{125}$I-$Tyr^{25}$] S-28 binding:

Since differences in the binding of these two radioligands were observed only with $Ca^{2+}$ at high concentrations, we decided to investigate the effect of this divalent ion on total, specific and non-specific binding over a wider range of concentration (from 1 uM to 20 mM). Fig. 9 illustrates that although the total binding of [$^{125}$-$Tyr^{11}$] S-14 (top panel) remained constant, the non-specific binding of this radioligand decreased at high $Ca^{2+}$ concentrations. In presence of 20 mM $Ca^{2+}$ the non-specific binding corresponded only to 42.5 $\pm$ 2.9 % of the

**Fig. 9:** **Effect of $Ca^{2+}$ ions on the binding of $^{125}I$-Tyr$^{11}$] S-14** (top panel) **(Leu$^{8}$,D-Trp$^{22}$,$^{125}I$-Tyr$^{25}$] S-28** (bottom panel) to rat brain synaptosomal membrane receptors. Specific binding represents the difference between the amounts of radioligand bound in the absence (total) and presence (non-specific binding) of $10^{-7}$ M unlabelled S-14. Values represent mean $\pm$ S.E. (n = 4).


40
30
20
10
0
TOTAL
SPECIFIC
NON-SPECIFIC
% [125I-Tyr11] S-14 BOUND
20
10
0
% [Leu8,D-Trp22,125I-Tyr25] S-28 BOUND
-3
-2
-1
0
1
2
LOG mM Ca2+

total binding of this radioligand. In a similar way, the non-specific binding of [Leu$^8$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 (bottom panel) was altered by high concentrations of $Ca^{2+}$ ions (39.2 $\pm$ 2.3 % compared to the total binding). In addition $Ca^{2+}$ concentrations greater than 4 mM had a tendency to reduce the total binding of this radioligand.

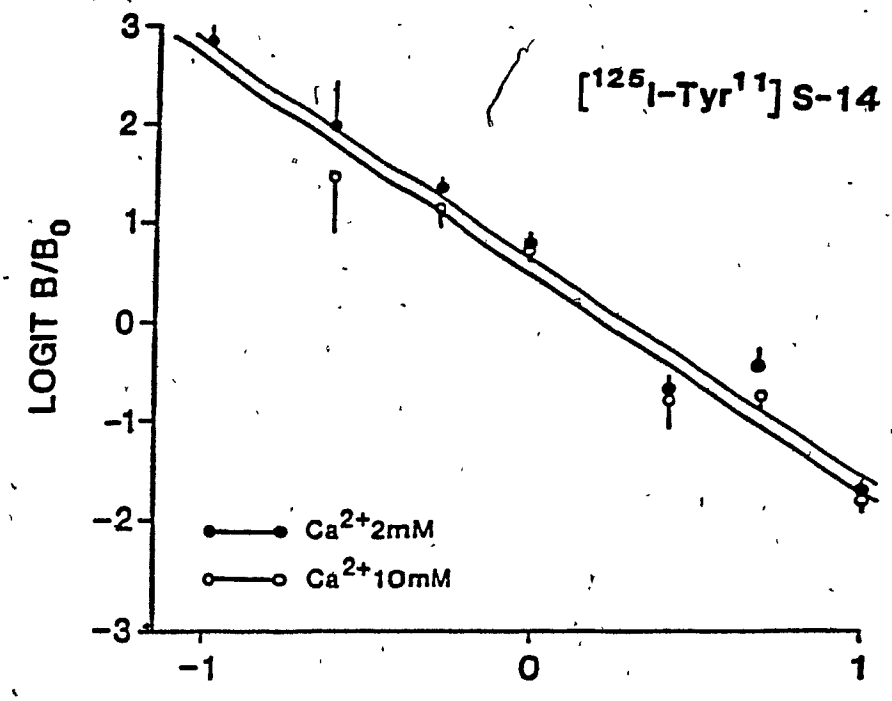
Effect of $Ca^{2+}$ on binding affinities of S-14 and S-28:

Competitive inhibition studies in presence of $Ca^{2+}$ at 2 mM and 10 mM demonstrated that the relative potency of S-14 to inhibit [Leu$^8$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding was 4 times greater at 10 mM $Ca^{2+}$ (Fig. 10, top panel). By contrast, the potencies of S-14 to inhibit [$^{125}$I-Tyr$^{11}$] S-14 binding was not influenced by these $Ca^{2+}$ concentrations (Fig. 10, bottom panel). Additionally, the relative potency of S-28 to inhibit [$^{125}$I-Tyr$^{11}$] S-14 or [Leu$^8$, D-Trp$^{22}$,I-Tyr$^{25}$] S-28 was not altered by change in $Ca^{2+}$ concentration (Fig. 11). The number of [Leu$^8$,D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28 binding sites calculated from competitive binding studies using S-14 in presence of $Ca^{2+}$ was: 158 $\pm$ 61 and 399 $\pm$ 35 fmol/mg at 10 mM vs 2 mM respectively.

There was no change in the concentration of these receptors calculated from binding studies using S-28 (300 ± 52 vs 299 ± 47 fmol/mg at 2 mM vs 10 mM $Ca^{2+}$ respectively (Fig. 12). Finally there was no change in the number or the affinity of [$^{125}$I-Tyr$^{11}$] S-14 binding sites using either S-14 or S-28 as unlabelled peptide (Fig. 13).

**Fig. 10: Effect of $Ca^{2+}$ on the inhibition by S-14 of the specific binding of [$^{125}$I-Tyr$^{11}$] S-14 (top panel) and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 (bottom panel).**

Competitive inhibition data obtained from equilibrium binding studies are expressed as Logit $B/B_0$ (where B and $B_0$ represent the amounts of radioligand specifically bound in the presence and absence of unlabelled peptide respectively) as a function of the peptide concentration. Values represent mean $\pm$ S.E. (n = 4).


[125I-Tyr11] S-14
LOGIT B/B0
Ca2+2mM
Ca2+10mM
3
2
1
0
−1
−2
−3
−1
0
1

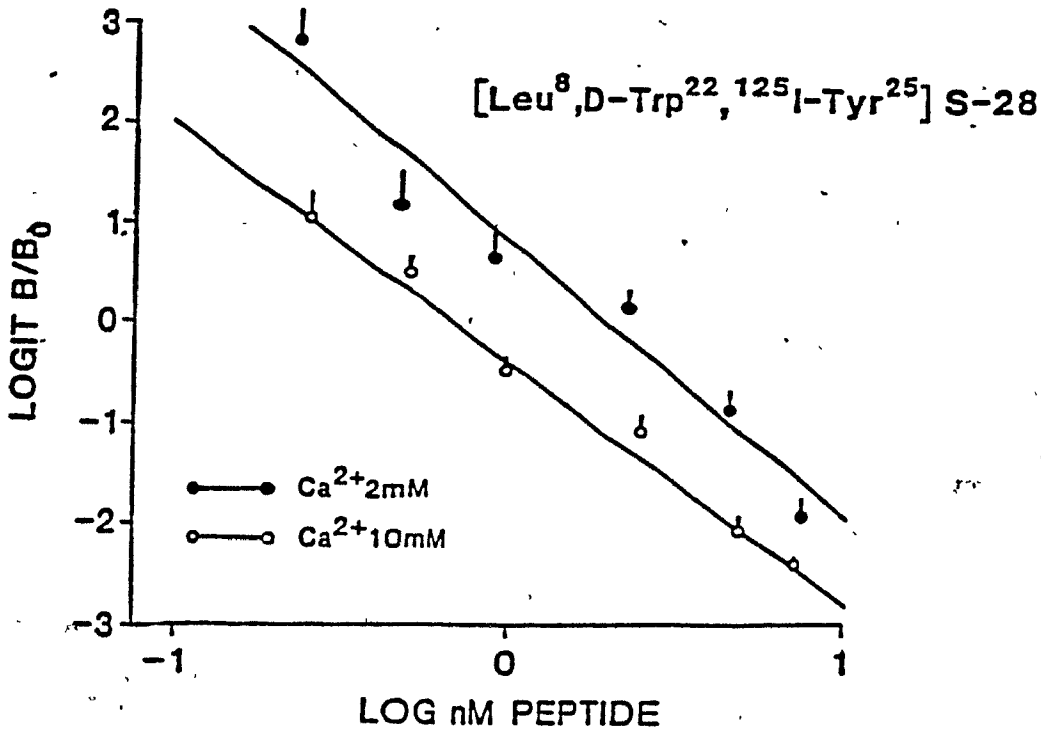
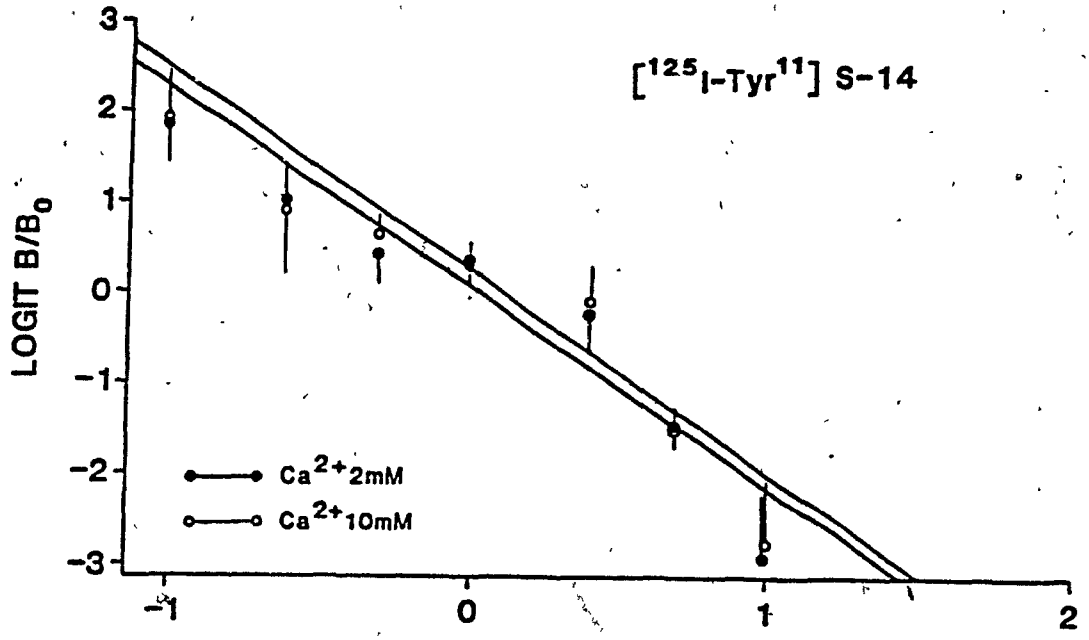

[Leu8,D-Trp22,125I-Tyr25] S-28
LOGIT B/B0
Ca2+2mM
Ca2+10mM
3
2
1
0
−1
−2
−3
−1
0
1
LOG nM PEPTIDE

**Fig. 11:** **Effect of $Ca^{2+}$ on the inhibition by S-28 of the specific binding of [$^{125}$I-Tyr$^{11}$] S-14** (top panel) **and (Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28** (bottom panel). Competitive inhibition data obtained from equilibrium binding studies are expressed as $B/B_0$ (where B and $B_0$ represent the amounts of radioligand specifically bound in the presence and absence of unlabelled peptide respectively) as a function of the peptide concentration. Values represent mean $\pm$ S.E. ($n = 4$).


[125I-Tyr11] S-14
LOGIT B/B0
Ca2+2mM
Ca2+10mM
3
2
1
0
-1
-2
-3
-1
0
1
2

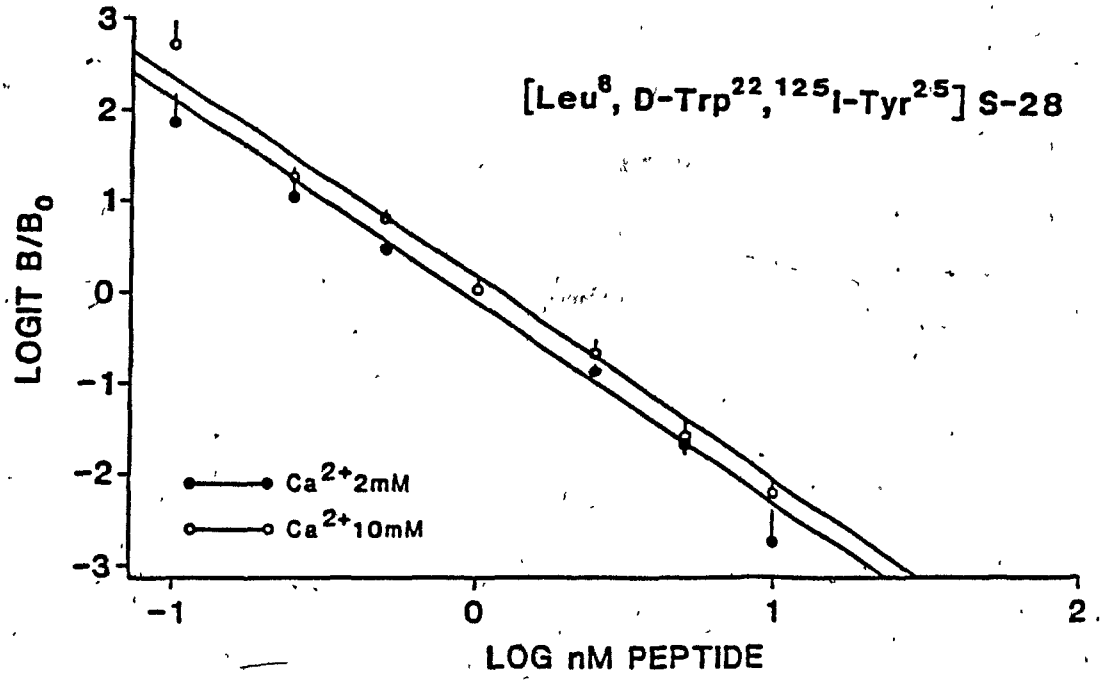

[Leu8, D-Trp22,125I-Tyr25] S-28
LOGIT B/B0
Ca2+2mM
Ca2+10mM
3
2
1
0
-1
-2
-3
-1
0
1
2
LOG nM PEPTIDE

**Fig. 12:** **Comparaison of Scatchard plots showing the effects of 2 and 10 mM $Ca^{2+}$ on the competitive inhibition by S-14** (left panel) **and S-28** (right panel) **of the binding of (Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28** to rat brain synaptosomal membranes.. Values represent means of 4 experiments in duplicate.

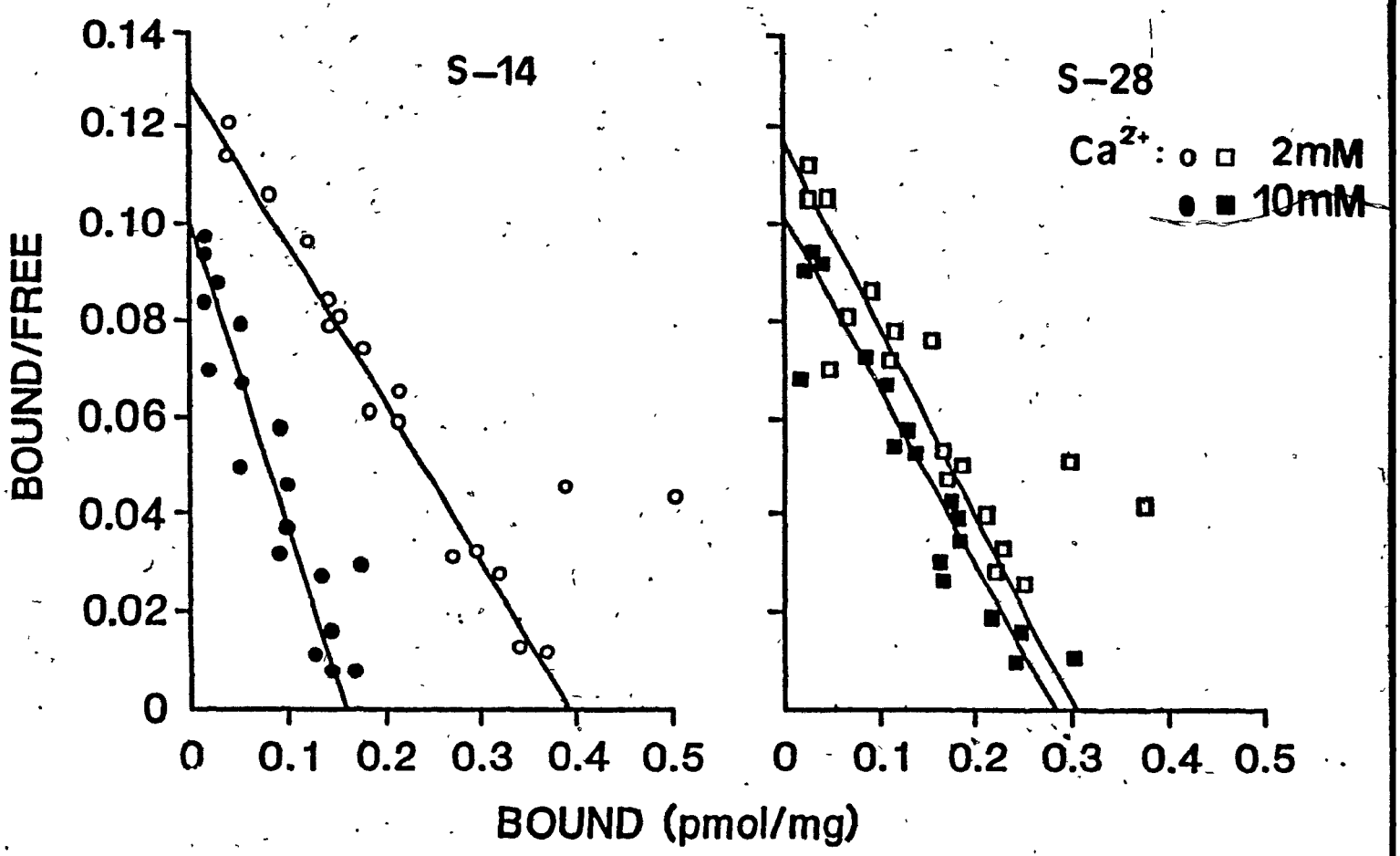
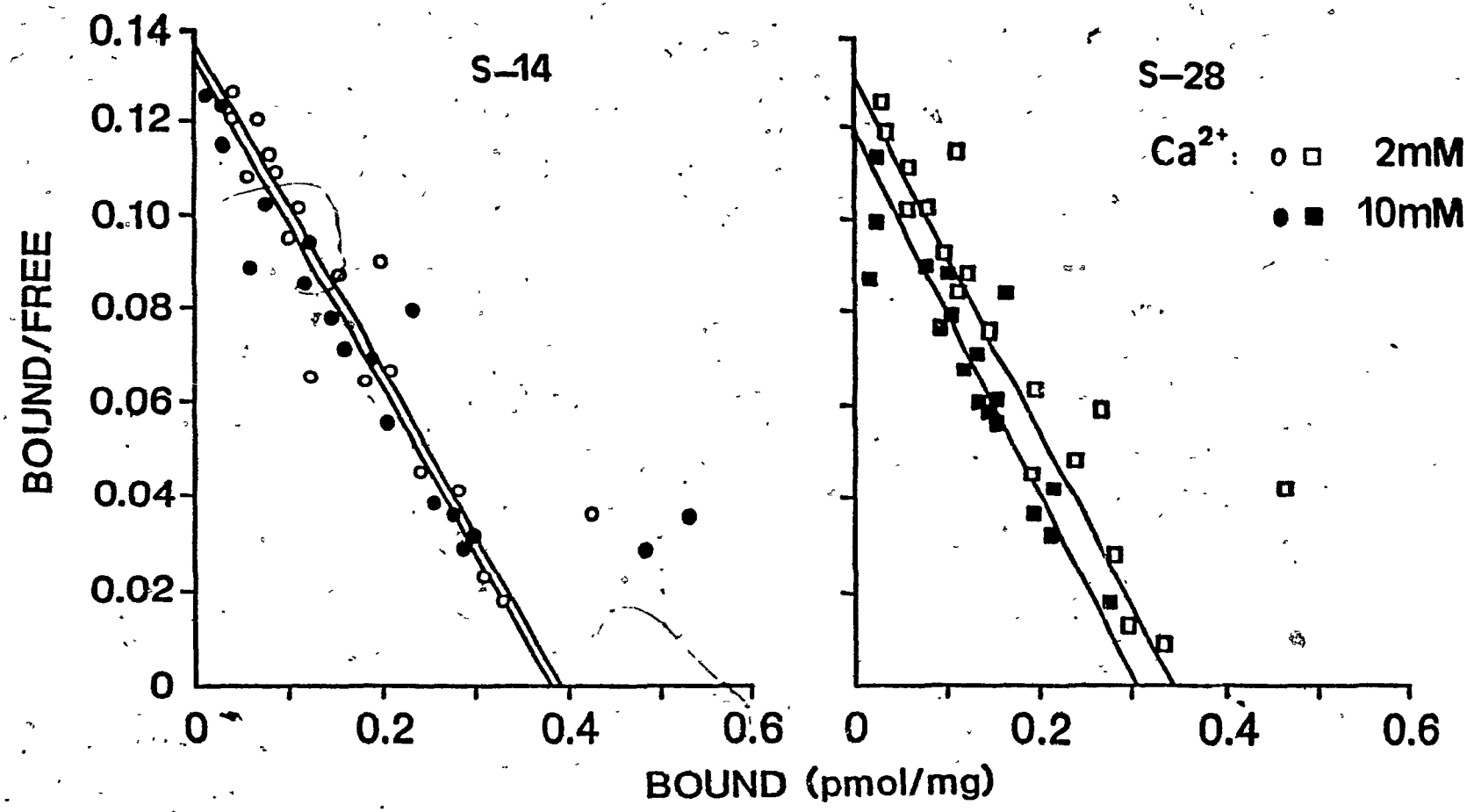

S–14
S–28
$Ca^{2+}$: o □ 2mM
● ■ 10mM
BOUND/FREE
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0 0.1 0.2 0.3 0.4 0.5
0 0.1 0.2 0.3 0.4 0.5
BOUND (pmol/mg)

**Fig. 13: Comparison of Scatchard plots showing the effects of 2 and 10 mM $Ca^{2+}$ on the competitive inhibition of S-14** (left panel) **and S-28** (right panel) **of the binding of [$^{125}$I-Tyr$^{11}$] S-14** to rat brain synaptosomal membranes. Data represent means of 4 experiments in duplicate.


S–14
S–28
$Ca^{2+}$: ○ □ 2mM
● ■ 10mM
BOUND/FREE
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
0
0.2
0.4
0.6
BOUND (pmol/mg)

# DISCUSSION

Effects of nucleotides on [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$, D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28 binding:

The specific binding of [$^{125}$I-Tyr$^{11}$] S-14 to rat brain synaptosomal membranes was inhibited by both guanine and adenine nucleotides. The inhibition of the binding of this radioligand was dependent on the nucleotide concentration and resulted in a reduction in the binding capacity of the receptors. The affinity of [$^{125}$I-Tyr$^{11}$] S-14 binding sites was not altered by the nucleotides. From kinetic studies it was seen that neither the association nor the dissociation rates were significantly altered by the presence of adenine and guanine nucleotides. These data provide further direct experimental evidence for the lack of nucleotide-dependent change in affinity of these receptors. This study also provides the first direct evidence that nucleotides inhibit the binding of labelled S-28 analog to rat brain membrane receptors for somatostatin. Both groups of nucleotides inhibited the binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 resulting in a reduction of the number of binding sites for this ligand without any alteration of the affinity of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites. With this radioligand also no nucleotide-induced change in association or dissociation kinetics of binding was observed.

While both adenine and guanine nucleotides reduced the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28, the extent of inhibition of binding of these radioligands by these nucleotides revealed striking differences. Adenine nucleotides appeared to be less effective than guanine nucleotides in reducing [Leu$^{8}$, D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding. By contrast, [$^{125}$I-Tyr$^{11}$] S-14 binding sites were almost equally affected by both groups of nucleotides. Furthermore, the number of receptors sites for the two ligands was differentially altered by guanine nucleotides. GTP was more potent than GMP-PNP > GMP ≈ GDP ≈ cGMP in decreasing [$^{125}$I-Tyr$^{11}$] S-14 binding sites while cGMP was more effective than GTP > GMP ≈GDP ≈ GMP-PNP in reducing [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites. Differences in the effect of adenine nucleotides on the binding of these radioligands were also evident in that ATP was more potent than ADP and cAMP in reducing [$^{125}$I-Tyr$^{11}$] S-14 and ADP was about equipotent to cAMP and ATP in reducing [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites. These studies demonstrate that the binding of these two radioligands to rat brain membranes is differentially sensitive to adenine and guanine nucleotides and suggest that the binding of S-14 and S-28 to somatostatin receptors could be differentially modulated by nucleotides.

There are only three reports in the literature dealing with the effects of nucleotides on somatostatin receptors. In two of these studies, the effect of nucleotides was assessed on the binding of N-terminally labelled S-14 ligands ($^{125}$I-Tyr S-14 and $^{125}$I-Tyr1 S-14) and contradictory conclusions were reached (13, 28). Enjalbert et al. (13), reported that the modulation of guanine nucleotides on the binding of [$^{125}$I-Tyr] S-14 to adenohypophyseal and cerebrocortical membranes resulted in a reduction in the binding capacity without significantly affecting the affinity, which is in agreement with the present findings. It was also found by these workers that [$^{125}$I-Tyr$^{1}$] S-14 binding sites in rat brain and pituitary were insensitive to adenine nucleotides. This is in striking contrast to our finding that adenine nucleotides cause a marked reduction in [$^{125}$I-Tyr$^{11}$] S-14 binding in rat brain. The S-14 receptors in $GH_4C_1$ cells (28-29) unlike those in normal tissues were found to respond to guanine nucleotides in a different manner. In presence of GTP, the specific binding of [$^{125}$I-Tyr$^{1}$] S-14 in $GH_4C_1$ cells decreased primarily due to a decrease in affinity. This change in affinity was a consequence of an increased dissociation rate. These authors postulated that guanine

nucleotides may regulate the interconversion of low and high affinity states of the somatostatin receptor (28). Thus the tumor cell S-14 receptors appear to differ significantly from those in normal rat tissues. The reasons for the observed differences in normal tissues compared to tumor cells are not clear and may be due to the use of different radioligands. It is well known that N-terminally labelled S-14 ligands are more suceptible to proteolytic degradation (37) and it is likely that the extent of such degradation and its sensitivity to nucleotides may differ between tissues.

The inhibitory effect of guanine nucleotides on the binding of [$^{125}$I-Tyr$^{11}$] S-14 to somatostatin receptors in rat pancreatic acinar cells has also been reported (66). In this tissue also a reduction in the number of receptor sites with no change in affinity was observed. Additionally, adenine nucleotides were found to have no influence on S-14 receptor binding in this tissue (66). Thus, S-14 receptors in the rat brain and exocrine pancreas are distinctly different in their regulation by nucleotides.

Nucleotide inhibition of receptor binding has been demonstrated for a number of other peptide hormones and neurotransmitters. In the majority of these cases, guanine nucleotides appeared to facilitate dissociation of bound ligand resulting in reduced equilibrium binding as it has been shown for CRF (40) and TRH (21). In addition a direct effect of these nucleotides in reducing the concentration of high affinity receptors has also been documented with beta-adrenergic receptors (25). The somatostatin receptor in rat brain appear to differ from these receptors in that guanine nucleotides do not alter their affinity/or dissociation rate but only the number of receptor sites. This is not unique since in the case of substance P receptors, nucleotide-induced reduction in receptor concentration in rat small intestine has been reported (58), while it should be pointed out that in submaxillary glands nucleotides altered the affinity but not the number of substance P binding sites (3).

It has been postulated that guanine nucleotides, more precisely GTP, inhibit agonist binding to receptors coupled to the catalytic subunit of the adenylate cyclase.

via either $N_s$ or $N_i$ by converting these receptors from high affinity to low affinity forms. Such interconversion has been demonstrated for a number of receptors including those for glucagon (31, 46-47), beta-adrenergic agents (34, 50, 67) as well as muscarinic agonists (5,53). In all of these systems it appears that the nature of the effect of guanine nucleotides in changing receptor affinities requires the presence of $Mg^{2+}$ ions. Williams et al. (73) postulated that addition of $Mg^{2+}$ triggered an increase in agonist binding while this increase was abolished by guanine nucleotides. Although somatostatin receptors have been shown to be coupled to $N_i$ and a GTP-dependent interconversion of high and low affinity forms has been postulated for somatostatin receptors in tumor cells (56) this was not observed in normal rat brain. One cannot however rule out the interdependent effects of $Mg^{2+}$ and nucleotides on somatostatin receptors; nevertheless such reciprocal heterotropic effects of divalent ions and guanine nucleotides on somatostatin receptors could not be evaluated because of low binding of somatostatin receptors in rat brain in the absence of divalent ions.

The effect of nucleotides on labelled S-28 ligand has been investigated only in rat brain membranes. A number of studies have raised the possibility that S-28 binding sites distinct from those of S-14 may exist. The strongest evidence for this comes from autoradiographic studies (39) but has not been confirmed in direct binding studies using the labelled S-14 and S-28 ligands employed in this study. We have demonstrated for the first time that the binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 are differentially sensitive to nucleotides providing support to the concept of distinct S-14 and S-28 receptors. The possibility that the changes in local concentrations of nucleotides may determine the extent of interaction of these receptors with S-14 and S-28 in discrete anatomical regions should also be considered. Similar differences in other tissues may exist and further studies using these ligands are clearly required to establish the role of nucleotides in regulating the receptor binding of S-14 and S-28 in these tissues.

Effect of monovalent and divalent cations on [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding:

The binding of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28 to rat brain membranes was also influenced by the ionic milieu. Both radioligands exhibited lower binding in the presence of monovalent cations. $Li^+$ and $Na^+$ were more potent than $K^+$ in reducing [$^{125}$I-Tyr$^{11}$] S-14 binding but in the case of [Leu$^{8}$,D-Trp$^{22}$.$^{125}$I-Tyr$^{25}$] S-28, $Li^+$ at low concentrations ( < 40 mM) produced greater inhibition than other monovalent ions tested. High concentrations of $K^+$ ions inhibited the binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 to a greater extent than $Na^{++}$ whereas $Na^+$ was more effective than $K^+$ in inhibiting [$^{125}$I-Tyr$^{11}$] S-14 binding.

Addition of divalent ions (2 mM) increased the binding of these radioligands to rat brain membrane receptors; [$^{125}$I-Tyr$^{11}$] S-14 binding being more sensitive to $Ba^{2+}$, $Mg^{2+}$, and $Co^{2+}$ compared to [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding whereas low concentrations of $Ca^{2+}$ were about equipotent in increasing the binding of these two ligands. At higher concentrations (of up to 20 mM), $Mg^{2+}$ caused a sustained stimulation of [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$,D-

$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28 binding. However, in striking contrast to its effect on [$^{125}$I-$Tyr^{11}$] S-14 binding, $Ca^{2+}$ when present at concentrations exceeding 10 mM markedly reduced the binding of [$Leu^{8}$,D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28.

In other systems, such as beta-adrenergic receptors, divalent ions have been reported to increase the affinity (73). Ion-dependent increase in receptor number without any change in affinity as been shown with substance P (30). There is also evidence that divalent ions increased the number of high affinity binding sites for CRF in the anterior pituitary (40). Other studies have shown that ions such as $Mg^{2+}$ may also alter the equilibrium between low and high affinity states as has been demonstrated in the case of muscarinic receptors (33). Evidence for an ion-dependent conformational change has also been observed with TRH receptors in $GH_4C_1$ cells (21).

The differential sensitivity of the binding of [$^{125}$I-$Tyr^{11}$] S-14 and [$Leu^{8}$,D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28 to high $Ca^{2+}$ concentrations led us to carry out more detailled studies on the role of $Ca^{2+}$ in modulating the binding of these ligands. It was seen that the increase

in binding of [$^{125}$I-Tyr$^{11}$] S-14 in presence of $Ca^{2+}$ was found to result from a decrease in non-specific binding without the total binding being affected. By contrast, in the case of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28, in addition to the observed decrease in non-specific binding, high concentrations of $Ca^{2+}$ ions also tended to inhibit the total binding. Thus $Ca^{2+}$ at > 10 mM concentrations selectively reduces the specific binding of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 but not that of [$^{125}$I-Tyr$^{11}$] S-14. It is well known that both endo-and exopeptidases are associated with these synaptosomal membranes (8). In previous studies reported from this laboratory it was shown that such degradation can be minimized by proteolytic inhibitors included in the receptor assay buffer containing $Mg^{2+}$ (60). Recently Esteve et al. (15) reported that the binding of [$^{125}$I-Tyr$^{11}$] S-14 but not [$^{125}$I-Tyr$^{1}$] S-14 to guinea pig brain and pancreatic acinar cell membranes was increased in presence of low $Ca^{2+}$ concentrations. Similar increase in binding of [$^{125}$I-Tyr$^{11}$] S-14 to rat pancreatic acinar cell membrane receptors following addition of 0.1 mM $Ca^{2+}$ has also been observed (66). These findings coupled with the present data strongly suggest that $Ca^{2+}$ augments the efficacy of proteolytic inhibitors, particularly that of endopeptidase, and help minimize the degradation of

[$^{125}$I-Tyr$^{11}$] S-14 under experimental conditions. Although the S-28 analog used in this study, was a conformationally restricted and stable analog, it was also seen to undergo some degradation in the absence of $Ca^{2+}$, as evidenced by the high non-specific binding which decreased when $Ca^{2+}$ was included in the assay buffer. Thus divalent ions appear to promote ligand stability and specific binding in the case of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$]S-28 also.

The present study has also shown that alteration in ionic milieu, particularly changes in $Ca^{2+}$ concentration, selectively alters the ability of S-14 to interact with [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites in rat brain. It was found that the affinity of S-14 for [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites was directly proportional to $Ca^{2+}$ concentrations. Scatchard analysis of the dose-dependent inhibition by S-14 of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding revealed that the increase in affinity of S-14 was accompanied by a decrease in the capacity of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-Tyr$^{25}$] S-28 binding sites. Surprisingly, competitive binding studies with S-28 revealed no $Ca^{2+}$ dependent change in the number of [Leu$^{8}$,D-Trp$^{22}$,$^{125}$I-

$Tyr^{25}$] S-28 binding sites as well as in the affinity of this peptide for these sites. Such $Ca^{2+}$-dependent changes in the interaction of S-14 or S-28 were not observed with [$^{125}$I-$Tyr^{11}$] S-14 binding sites.

In conclusion, the present study has provided evidence for the differential sensitivity to adenine and guanine nucleotides as well as to $Ca^{2+}$ ions of the binding of [$^{125}$I-$Tyr^{11}$] S-14 and [$Leu^{8}$,D-$Trp^{22}$,$^{125}$I-$Tyr^{25}$] S-28 to rat brain membranes. These data do not however provide direct evidence for distinct S-14/S-28 receptors. If indeed two populations of receptors exist, extension of this study should lead to delineation of the conditions for identifying such receptors in direct binding studies. It is possible that both S-14 and S-28 act via the same receptor sites but may be differentially sensitive to nucleotides and ions. Further studies should lead to a better understanding of the receptor binding and biological potencies of S-14 and S-28.

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# APPENDIX

International Conference on Somatostatin

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# DIFFERENTIAL EFFECTS OF GUANINE AND ADENINE NUCLEOTIDES ON [$^{125}$I-Tyr$^{11}$] S-14 and [Leu$^{8}$, D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28 BINDING SITES IN RAT BRAIN.

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Somatostatin receptors in rat brain synaprosomal membranes have been quantitated using radioiodinated derivatives of somatostatin-14 ([$^{125}$I-Tyr$^{11}$] S-14, T* S-14) and somatostatin-28 ([Leu$^{8}$, D-Trp$^{22}$, $^{125}$I-Tyr$^{25}$] S-28, LTT* S-28). To determine whether these radioligands are differentially sensitive to nucleotides, we investigated the effects of guanine and adenine nucleotides on their binding. Both groups of nucleotides produced concentration-dependent inhibition of the radioligands. The maximum inhibition (∿ 50%) of T$^{11}$ S-14 binding was observed with all nucleotides at $10^{-3}$ M concentration whereas adenine nucleotides were less effective than guanine nucleotides in inhibiting LTT* S-28 binding (36 and 50% respectively). The non-reducible GTP analog GMP-PNP was more potent than GTP. The ability of adenine nucleotides to inhibit the binding of these radioligands however showed marked differences: ATP > ADP ≈ cAMP for T* S-14 and cAMP > ADP > ATP for LTT* S-28. The inhibition of the binding resulted in a decrease in Bmax without any change in affinity of the receptors. The decrease in Bmax of LTT* S-28 binding sites was greater in the presence of GTP than ATP ($10^{-4}$ M) (145 ± 10 and 228 ± 16 ($p < 0.001$) respectively compared to 320 ± 20 fmol/mg for control). By contrast ATP effected greater reduction in Bmax of T* S-14 binding sites than GTP (182 ± 15 and 227 ± 8 ($p < 0.01$) respectively compared to 340 ± 15 fmol/mg for control). We conclude that (1) LTT* S-28 binding sites in rat brain are more sensitive to guanine nucleotides than adenine nucleotides. (2) The decrease in Bmax of LTT* S-28 in presence of GTP is greater than that induced by ATP. (3) Both GTP and ATP decrease T* S-14 binding sites to the same extent. (4) The affinities of T* S-14 and LTT* S-28 binding sites are not altered by nucleotides. These data indicate that T*S-14 and LTT* S-28 are differentially sensitive to guanine and adenine nucleotides and suggest that the potencies of S-14 and S-28 may be nucleotide dependent.