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^{3}H-Substance P Binding to Salivary Gland Membranes

Regulation by Guanyl Nucleotides and Divalent Cations

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SUMMARY

With appropriate measures to protect ^{3}H-substance P (^{3}H-SP) from proteolytic degradation and from nonspecific adsorption to glass-fiber filters, we have been able to demonstrate reliably a high-affinity specific binding of ^{3}H-SP to rat submaxillary/sublingual gland membranes with a K_D of 1 nM and B_{max} of 6 pmoles/g of tissue. The relative potencies of various SP fragments and related analogues in reducing ^{3}H-SP binding parallel their potencies in stimulating phosphatidylinositol turnover, amylase release, and salivation, thus supporting an association of the observed ^{3}H-SP binding site with the physiological SP receptors in this tissue. This binding is selectively stimulated by some divalent cations ($Mn^{2+} > Mg^{2+} > Ca^{2+}$) but inhibited by several guanyl nucleotides, suggesting a possible linkage to adenylate cyclase. However, no effect of SP on either the basal or the norepinephrine-stimulated adenylate cyclase activity could be demonstrated in salivary gland homogenates.

INTRODUCTION

SP[1] is an undecapeptide (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) with diverse pharmacological effects in numerous peripheral tissues as well as the central nervous system.

Several groups have attempted to label SP receptors by ligand binding techniques. Early reports of ^{3}H-SP (1) or ^{125}I-Tyr^8-SP (2, 3) to brain membranes and synaptic vesicles appear to reflect the high-affinity binding of SP to phospholipids whose peptide specificity differs substantially from that of physiological SP receptors. More recently SP receptor binding to intact salivary gland, pancreatic cells, and cultured brain cells has been reported with derivatives such as ^{125}I-physalaemin (4, 5), ^{125}I-Tyr^8-SP (6), and ^{125}I-Bolton-Hunter reagent-labeled SP (7-9). The only report using native ^{3}H-SP to label receptors with binding properties characteristic of physiological SP receptors has been obtained in brain membranes (10).

For hormones and neurotransmitters which act via adenylate cyclase, ligand binding is often regulated by guanyl nucleotides and divalent cations with a specific pattern involving selective decreases and increases in agonist but not antagonist affinity in the presence of guanyl nucleotides and divalent cations, respectively (11-16). In the present paper we have developed conditions permitting reliable estimation of ^{3}H-SP binding to membranes of rat submaxillary-subligual gland membranes. We report the specific regulation of this binding by guanyl nucleotides and divalent cations.

MATERIALS AND METHODS

SP receptor binding assay. Submaxillary and sublingual glands from male, adult Sprague-Dawley rats were homogenized in 10 volumes (w/v) of ice-cold Buffer A (50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl and 5 mM KCl) with a Brinkmann Polytron PT-10 at a setting of 7 for 10-15 sec. The homogenate was incubated on ice in the presence of 300 mM KCl and 10 mM EDTA for 30 min with intermittent mixing and then centrifuged at 50,000 × g for 10 min. The pellet was resuspended in 20 volumes of ice-cold Buffer B (50 mM Tris-HCl, pH 7.4) by a Polytron at setting 7 for 5-10 sec and centrifuged again. This washing procedure was repeated twice, and the final pellet was resuspended in 60 volumes of Buffer B.

For binding experiments, the reaction was initiated by adding 400 μl of the freshly prepared membrane preparation in triplicate to 0.2-10 nM ^{3}H-SP at 20° for 10 min in a final volume of 500 μl containing 50 mM Tris-HCl (pH 7.4), 0.02% BSA, 1 μg of chymostatin, 2 μg of leupeptin, 20 μg of bacitracin, and various tested drugs. At the end of the incubation period, 5 ml of ice-cold Buffer A were added to each tube and its content was filtered immediately under reduced pressure through Whatman GF/F glass-fiber filters (pretreated with 0.1% polyethyleneimine in water for >3 hr prior to use). Each of the filters was then washed two times with 5 ml of ice-cold Buffer A and radioactivity was determined by liquid scintillation spectrometry. Nonspecific bind-

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[1] The abbreviations used are: SP, substance P; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; GMP-PNP, guanylyl-5'-imidodiphosphate.

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ing was defined as binding in the presence of 1 μM SP. Specific binding was calculated by subtracting the nonspecific binding from total binding.

Adenylate cyclase assay. Rat submaxillary and sublingual glands were homogenized in 20 volumes (w/v) of ice-cold Buffer A with a glass homogenizer and a Teflon pestle. The homogenate was preincubated at 37° for 10 min and assayed for adenylate cyclase activity immediately. The assay medium (final volume 500 μl) contained 50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 5 mM KCl, 10 mM theophylline, 10 mM $MgCl_2$, 50 μM GTP, 3 mM ATP, 0.05% BSA, 10 μg of bacitracin, 1 μg of chymostatin, 2 μg of leupeptin, 50 μl of salivary gland homogenate, and various tested substances as indicated.

The enzyme was preincubated in duplicate with all components of the assay mixture, except ATP, for 3 min on ice. The reaction was initiated by the addition of freshly prepared ATP and carried out for 5 min at 37°. The reaction was terminated by boiling for 3 min. Boiled enzyme preparations were used for the determination of tissue blanks. The denatured proteins in the reacted mixtures were sedimented by centrifugation at 2000 × *g* for 10 min. The cyclic AMP content in 30-μl aliquots of the supernatants was measured in duplicate with the cyclic AMP ^{125}I-radioimmunoassay kit purchased from New England Nuclear Corporation (Boston, Mass.).

HPLC of SP and its related fragments. The purity and stability of SP and its related peptides were checked by reverse-phase HPLC on a μBondapak C_{18} column (0.4 × 30 cm) with two different solvent systems. In System A, the peptides were eluted isocratically at 2 ml/min with acetonitrile/0.1% phosphoric acid, pH 2.0 (26:74, v/v). The retention times for SP, SP sulfoxide, SP_{4-11}, SP_{6-11}, SP_{7-11}, SP_{8-11}, and SP_{9-11} were 9.4, 3.8, 7.6, 10.8, 12.2, 4.4, and 2.4 min, respectively. In System B, the peptides were eluted with a linear gradient of acetonitrile/0.1% phosphoric acid, pH 2.0, from 5:95 (v/v) to 65:35 (v/v). The retention times for SP, SP_{1-6}, SP_{1-7}, SP_{1-8}, SP_{7-11}, SP_{8-11}, and SP_{9-11} were 12.8, 2.7, 7.4, 10.4, 14.6, 11.8, and 7.6 min, respectively. The SP-related peptides used in the present study were found to be >90% pure with the exception of SP_{6-11}, which is contaminated about 10–15% with the pyroglutamyl form. Since the pyroglutamyl form did not differ greatly from the hexapeptide in biological activity (17), all peptides were used as supplied without further purification.

^{3}H-SP was found to be of >95% radiochemical purity by HPLC analyses. However, it was noted that about 20% accumulation of ^{3}H-SP sulfoxide occurred over a period of 3 months storage at −20°C. The procedure of Floor and Leeman (18) was used to purify ^{3}H-SP in "aged" preparations.

Materials. Chymostatin, leupeptin, bacitracin, BSA (radioimmunoassay grade), polyethyleneimine, *N*-ethylmaleimide, *p*-chloromercuriphenylsulfonate, and (−)-norepinephrine were purchased from Sigma Chemical Company (St. Louis, Mo.). Guanyl and adenyl nucleotides were obtained from Sigma Chemical Company or P-L Biochemicals (Milwaukee, Wisc.). Angiotensin II, Leu-enkephalin, and Met-enkephalin were from Bachem Fine Chemicals (Torrance, Calif.). SP tripeptide and SP tetrapeptide were from Beckman (Mountainside, N. J.). All other peptides used were from Peninsula Laboratories (San Carlos, Calif.).

[^{3}H]Dihydroalprenolol (41.8 Ci/mmole) and L-[2-*prolyl*-3,4-^{3}H-(N)] ^{3}H-SP (28 Ci/mmole, in ethanol containing 0.2% β-mercaptoethanol) were obtained from New England Nuclear Corporation.

RESULTS

General properties of ^{3}H-SP binding. Several technical problems make it difficult to detect ^{3}H-SP binding reliably. SP is a very basic peptide and so is positively charged at physiological pH values and adsorbs extensively to most negatively charged filters. However, pretreating glass-fiber filters with polyethyleneimine greatly reduces this nonspecific association of ^{3}H-SP with the filters.

SP is very susceptible to proteolytic degradation even with extensively washed membrane preparations. In preliminary experiments we found that the thiol-peptidase inhibitors *N*-ethylmaleimide and *p*-chloromercuriphenylsulfonate, which have been used to protect ^{3}H-SP from proteolytic degradation for brain membrane binding (10), markedly inhibit SP binding in salivary gland membranes. Pretreatment of membranes with 2 mM *N*-ethylmaleimide or 1 mM *p*-chloromercuriphenylsulfonate at 37° for 30 min reduces specific ^{3}H-SP binding to 39% and 18% of control, respectively. The peptide thiol-peptidase inhibitor leupeptin does not, however, appear to inhibit receptor binding. In the presence of a mixture of bacitracin (a wide-spectrum protease inhibitor), chymostatin (a serine peptidase inhibitor), and leupeptin we can inhibit almost fully SP degradation by salivary gland membranes. The "cocktail" of protease inhibitors is prepared fresh before each experiment, as preliminary experiments indicate that maintaining this mixture for as little as 12 hr is associated with much higher levels of nonspecific ^{3}H-SP binding. In incubations with a wide range of ^{3}H-SP under these conditions, HPLC analysis of acid extracts of membranes at the end of incubations shows that no degradation of ^{3}H-SP has occurred in the course of standard incubations.

^{3}H-SP binding is temperature- and time-dependent. At 0° ^{3}H-SP binding increases slowly and does not fully plateau at 30 min. At 37° preliminary experiments suggest some ^{3}H-SP degradation, so no detailed studies have been conducted at this temperature. At 20°, binding reaches steady-state levels within 5 min with no change for at least 20 min. An incubation time of 10 min is used for routine assays. The pH optimum for binding is fairly broad with similar levels between pH 7.0 and 7.8.

^{3}H-SP binding to salivary gland membranes is saturable. In typical experiments with 2 nM ^{3}H-SP the total binding is about 1800 cpm, whereas nonspecific binding assayed in the presence of 1 μM SP is 350 cpm. ^{3}H-SP binding approaches plateau level at about 10 nM with half-maximal binding at about 1.0 nM (Fig. 1). Scatchard analysis indicates a single population of binding sites with a dissociation constant (K_D) of 1.0 nM and a maximal number of binding sites (B_{max}) of about 6 pmoles/g of tissue.

Peptide specificity of ^{3}H-SP binding to salivary gland membranes. Differences in relative potencies of SP fragments in eliciting various pharmacological effects have suggested the existence of multiple receptors. In some instances peptide specificity for binding sites has not been the same as relative pharmacological potencies. For instance, in competing for ^{3}H-SP binding sites in rat brain membranes, SP_{4-11} is only 5% as potent as SP (10), whereas it has the same potency as SP neurophysiologically in rat central nervous system neurons (19). The relative potencies of most SP fragments and related peptides in competing for ^{3}H-SP binding to salivary gland membranes in our study correlate well with relative potencies of these peptides in stimulating salivary gland secretion, amylase release, and phosphatidylinositol turnover (Table 1). However, there are discrepancies between the potencies of the fragments at ^{3}H-SP sites in salivary gland and rat brain membranes. Thus, SP_{6-11} is only 5% as potent as SP at ^{3}H-SP binding sites in salivary

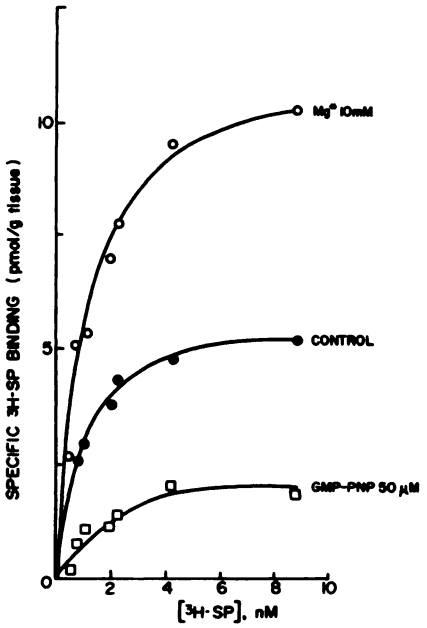

FIG. 1. *Saturation of specific ^{3}H-SP binding to rat submaxillary-sublingual membranes with increasing concentrations of ^{3}H-SP under standard assay conditions and in the presence of 10 mM $MgCl_2$ or 50 μM GMP-PNP*

Data shown are from a typical experiment.

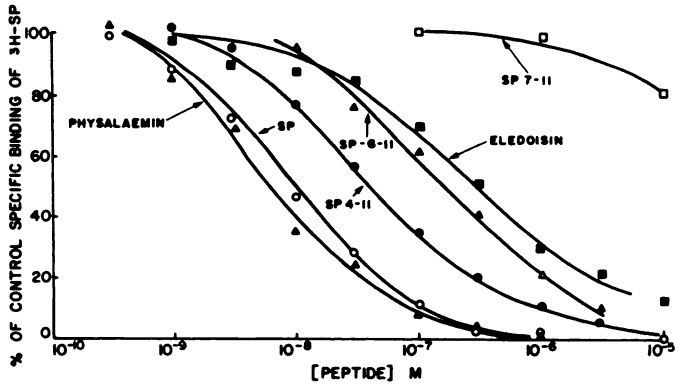

FIG. 2. *Displacement of ^{3}H-SP (2 nM) binding to rat submaxillary-sublingual membranes by SP, physalaemin, SP_{4-11}, SP_{6-11}, eledoisin, and SP_{7-11}*

Results represent the mean of two or three experiments performed in triplicate, which varied less than 15%.

gland membranes but is actually more potent than SP in competing for ^{3}H-SP binding in rat brain membranes. Moreover, SP is 38 times more potent than eledoisin at salivary gland membranes but only 3–4 times more potent than eledoisin in rat brain membranes.

Competition curves for the SP fragments and related tachykinins are parallel and appear to be monophasic with calculated Hill coefficients of about 1.0 (Fig. 2). Thus, we do not detect evidence for positive or negative cooperativity in the binding sites or binding of ^{3}H-SP to more than one class of binding sites in salivary gland membranes.

The specificity of the binding sites is further supported by the failure of a substantial number of other neurotransmitters, drugs, and peptides to influence ^{3}H-SP binding (Table 1).

Influences of guanyl nucleotides and divalent cations upon ^{3}H-SP binding to salivary gland membranes. Guanyl nucleotides influence ^{3}H-SP binding in a specific fashion (Fig. 3A). GTP and GDP are most potent whereas GMP-PNP, the nonmetabolized analogue of GTP, is somewhat less potent. GMP and the adenine

TABLE 1

Inhibition of specific ^{3}H-SP binding to rat submaxillary-sublingual gland membranes

Binding of ^{3}H-SP was assayed at 2 nM radioligand concentration. IC_{50} is defined as the concentration of drug required to inhibit 50% of specific binding. The following compounds had no significant effect on ^{3}H-SP binding when tested at 10 μM: γ-aminobutyric acid, picrotoxin, serotonin, cinanserin, histamine, doxepin, dopamine, haloperidol, norepinephrine, propanolol, yohimbine, prazosin, phenylephrine, phentolamine, benztropine, desipramine, mianserin, mazindol, amphetamine, cyproheptidine, carbachol, atropine, Leu-enkaphalin, Met-enkephalin, naloxone, thyrotropin-releasing hormone, His-Pro-diketopiperazine, somatostatin, angiotensin II, and neurotensin.

Peptides	Inhibition of ^{3}H-SP binding in salivary gland[a]		Relative potencies			
	IC_{50}	Relative potency	Rat brain binding[b]	Amylase release[c]	Salivation[d]	[^{3}H]inositol incorporation[d]
	nM					
SP	9.0	1.0	1.0	1.0	1.0	1.0
Tyr^8-SP	5.6	1.6	ND[e]	ND	ND	ND
SP_{4-11}	42.0	0.21	0.05	0.27	0.37	0.28
Tyr^8-SP_{5-11}	160.0	0.06	ND	ND	ND	ND
SP_{6-11}	180.0	0.05	1.7	0.13	0.17	0.38
SP_{7-11}	>10,000	<0.001	0.01	0.003	<0.003	0.00017
SP_{8-11}	>10,000	<0.001	ND	ND	<0.002	ND
SP_{9-11}	>10,000	<0.001	ND	ND	<0.002	ND
Eledoisin	340.0	0.03	0.3	1.1	ND	ND
Physalaemin	6.2	1.5	1.9	2.6	2.7	3.4

[a] Values are the means of two or three independent triplicate determinations which varied by less than 20%.

[b] Data from Hanley *et al.* (20).

[c] Data from Brown and Hanley (21).

[d] Data from Hanley *et al.* (10).

[e] ND, Not determined.

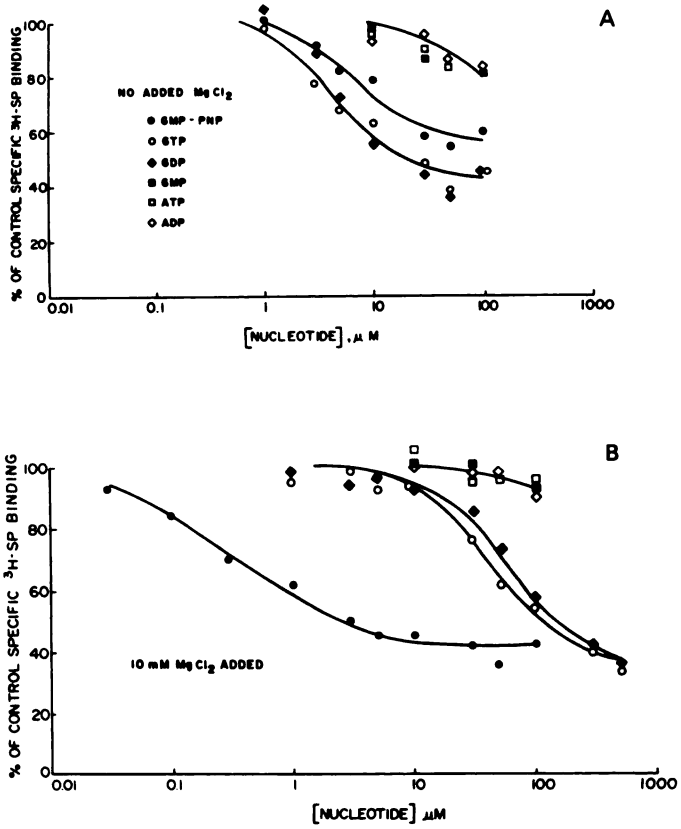

FIG. 3. *Effects of guanyl and adenyl nucleotides on ^{3}H-SP (2 nM) binding to rat submaxillary-sublingual membranes in the absence (A) or the presence (B) of 10 mM $MgCl_2$*

Specific binding levels of ^{3}H-SP in the absence and presence of 10 mM $MgCl_2$ were 4.4 ± 0.2 and 6.6 ± 0.8 pmoles/g of tissue, respectively. Data represent the means of three to six separate experiments which varied less than 15%. Nucleotides used are listed in A; *symbols* in A and B are identical.

nucleotides ATP and ADP are much less active, producing only 20% inhibition of ^{3}H-SP binding at 100 μM. GTP, GDP, and GMP-PNP do not abolish all ^{3}H-SP binding even when added at 1 mM concentration (data not shown). There is a plateau of maximal inhibition of about 60% of the binding observed between 0.1 and 1 mM for GTP and GDP. With GTP and GDP this 60% reduction of ^{3}H-SP binding takes place over about 2 orders of magnitude, between 1 and 100 μM, providing a pseudo-Hill coefficient of 1.0.

Saturation analysis of ^{3}H-SP binding in the presence of guanyl nucleotides indicates that the decrease in binding is largely associated with a reduced B_{max} with relatively little change in K_D (Fig. 1; Table 2). GTP does elicit almost a 2-fold increase in K_D, but GMP-PNP does not significantly increase the K_D. On the other hand, both GTP and GMP-PNP reduce the B_{max} of ^{3}H-SP binding about 65%.

Some divalent cations stimulate ^{3}H-SP binding (Fig. 4; Table 2). Manganese is most potent, producing a maximal 60% augmentation of binding at 1 mM and half-maximal increased binding at about 50 μM. Magnesium is second most potent, with half-maximal binding apparent at 0.3 mM. Barium is almost as potent as magnesium

TABLE 2

Effects of guanyl nucleotides and cations on ^{3}H-SP binding constants in rat submaxillary-sublingual membranes

Saturation experiments were performed using seven concentrations of ^{3}H-SP (0.4–10 nM). K_d and B_{max} values were determined by linear regression analysis of a Scatchard plot. Values given are the means ± standard deviation of n separate experiments.

Chemical	Concentration	No. of experiments	Binding constants	
			K_D	B_{max}
			nM	*pmoles/g tissue*
Control		4	1.02 ± 0.05	6.13 ± 0.39
$MnCl_2$,	0.5 mM	2	0.98 ± 0.14	9.87 ± 0.54[a]
$MgCl_2$,	10 mM	4	0.89 ± 0.17	9.36 ± 0.97[a]
$MgCl_2$ + GTP	10 mM 50 μM	2	1.99 ± 0.54[a]	8.25 ± 1.01[a]
GTP	50 μM	3	1.85 ± 0.05[a]	2.31 ± 0.07[a]
GMP-PNP	50 μM	2	1.29 ± 0.17	2.39 ± 0.00[a]
NaCl	150 mM	3	1.38 ± 0.23	5.37 ± 0.85

[a] $p < 0.05$ compared with control.

whereas calcium is less active, providing half-maximal enhancement at 1 mM. The enhanced binding elicited by manganese and magnesium is associated with an increased B_{max} with no change in K_D (Table 2). By contrast, the monovalent cation sodium at 150 mM fails to change ^{3}H-SP binding at all (Table 2).

Zinc and cobalt, on the other hand, reduce ^{3}H-SP binding. At 1 mM, zinc abolishes all ^{3}H-SP binding, and half-maximal reduction is apparent at 0.5 mM. Cobalt is less active, reducing binding 50% at 5–10 mM. The percentage inhibition of ^{3}H-SP binding by cobalt and zinc is similar in the presence as in the absence of 10 mM $MgCl_2$.

Adding magnesium (10 mM) alters the pattern of inhibition of ^{3}H-SP binding by guanyl nucleotides (Fig. 3). GTP and GDP produce half-maximal decreased binding (IC_{50}) at 7.4 and 7.0 μM, respectively, in the absence of Mg^{2+}. In the presence of 10 mM magnesium, the IC_{50}

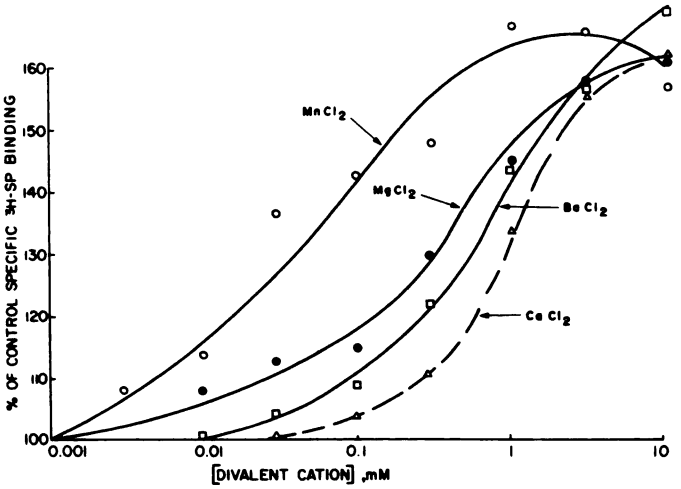

FIG. 4. *Effects of divalent cations on ^{3}H-SP (2 nM) binding to rat submaxillary-sublingual gland membranes*

Stimulation by $MnCl_2$, $MgCl_2$, $BaCl_2$, and $CaCl_2$. Data represent the means of three or four separate experiments which varied less than 12%.

values for GTP and GDP are 48 and 56 μM, respectively. This may be explained in part by our preliminary observation that magnesium accelerates the breakdown of GTP and GDP into inactive products (GMP and guanosine) upon incubation with salivary gland membranes. However, in spite of this marked decrease in their relative potencies in inhibiting ^{3}H-SP binding in the presence of magnesium, the slope of displacement curves for GTP and GDP are very similar in the presence as in the absence of magnesium. In contrast, when magnesium is added, GMP-PNP (the nonmetabolized analogue of GTP) becomes substantially more potent and the slope of its displacement curve becomes more shallow. Even in the presence of magnesium the plateau for maximal inhibition by guanyl nucleotides remains, so that a maximal 60% inhibition by GMP-PNP of control binding is apparent between 10 and 1000 μM. This 60% inhibition by GMP-PNP takes place over 3 orders of magnitude, between 0.1 and 10 μM.

Magnesium (10 mM) alone increases B_{max} 50% without changing K_D, whereas GTP (50 μM) alone reduces B_{max} two-thirds while almost doubling the K_D (Table 2). When these treatments are combined, the doubling of K_D re mains, while B_{max} is increased by only 35%.

Since the divalent cation enhancement of ^{3}H-SP binding involves an increased number of binding sites, we wondered whether it might be secondary to physical changes such as altered permeability of membrane vesicles, thus exposing binding sites on the inside surface of vesicles. Accordingly, we evaluated the effect of saponin treatment on manganese and magnesium enhancement of ^{3}H-SP binding. Saponin increases membrane vesicle permeability in concentrations that do not destroy membrane-bound receptors and so has been employed to localize α-bungarotoxin binding sites in such vesicles (22, 23). Thirty-minute exposure at 4° to 0.01–0.5% saponin, conditions known to enhance markedly vesicle membrane permeability, does not significantly alter ^{3}H-SP binding. Moreover, at 0.01%, 0.03%, 0.1%, and 0.5% saponin, $MgCl_2$ (10 mM) and $MnCl_2$ (0.5 mM) stimulation of ^{3}H-SP is the same as in membranes not treated with saponin. Thus it seems unlikely that divalent cation changes in membrane permeability account for the increases of ^{3}H-SP binding. Moreover, the relative potencies of the ions $Mn^{2+} > Mg^{2+} > Ca^{2+}$ fit closely with observations at numerous other neurotransmitter receptor binding sites (13, 17, 24–29).

Effect of SP on adenylate cyclase activity in homogenates of submaxillary-sublingual glands. Because regulation of hormone and neurotransmitter receptor binding by guanyl nucleotides and divalent cations often indicates a linkage to adenylate cyclase, we evaluated the influence of SP on adenylate cyclase activity of rat salivary gland homogenates. The basal adenylate cyclase activity in submaxillary-sublingual homogenates is 2.14 ± 0.14 (mean ± standard error of the mean; $N = 7$) pmoles of cyclic AMP formed per milligram of tissue per minute. The addition of 100 μM (−)-norepinephrine or 10 mM sodium fluoride stimulates this activity to 7.92 ± 0.55 ($N = 4$) and 14.4 ± 1.16 ($N = 3$) pmoles/milligram of tissue per minute, respectively. Adding 0.01 μM–10 μM SP does not significantly alter the basal or the norepinephrine-stimulated adenylate cyclase activity. For instance, at 10 μM SP the basal and norepinephrine-stimulated adenylate cyclase activities are 2.29 ± 0.05 ($N = 4$) and 7.24 ± 0.66 ($N = 4$), respectively.

DISCUSSION

In the present study we have developed incubation conditions under which ^{3}H-SP binding to salivary gland membranes can be readily detected with low levels of nonspecific binding and binding properties characteristic of physiological SP receptors. In some studies ^{3}H-SP (1) and ^{125}I-Tyr^8-SP (2, 3) binding to brain membranes and synaptic vesicles have appeared to reflect high-affinity interactions with phospholipids. The relative peptide potencies at these phospholipid binding sites differ from the relative potencies observed in the present study. Moreover, we have found that trypsin and chymotrypsin pretreatment greatly reduce ^{3}H-SP binding to salivary gland membranes (data not shown), indicating the protein nature of the binding sites.

The ^{3}H-SP binding sites observed here have a substrate specificity similar to those observed with ^{125}I-Bolton-Hunter-labeled SP in intact cells of rat parotid gland (7). In preliminary experiments we have found the peptide specificity of ^{3}H-SP binding to membranes of rat parotid to be essentially the same as for rat submaxillary-sublingual glands. The latter preparation has been used routinely for binding studies, since larger amounts of tissue are more readily obtained and since the submaxillary-sublingual preparation has lower levels of nonspecific binding. The peptide specificity of salivary ^{3}H-SP binding differs somewhat from that of rat brain membranes. Since binding to salivary gland and rat brain membranes have been conducted under conditions in which SP metabolism does not occur, these differences in specificity presumably reflect differences in receptor properties rather than merely variations in the extent of peptide degradation. Accordingly, our binding data might support the existence of discrete subtypes of SP receptors. Interestingly, the peptide specificity for SP receptor binding sites labeled with ^{125}I-Bolton-Hunter-labeled SP in intact mouse brain cells in culture (8, 9) more closely resembles the present results in rat salivary gland than those in rat brain membranes (10).

A major finding of the present study is that ^{3}H-SP binding is regulated by guanyl nucleotides and divalent cations. The pattern of these effects with GTP, GMP-PNP, and GDP being active but GMP and adenine nucleotides being inactive fits with what has been observed at other receptors for hormones and neurotransmitters. Similarly, the relative potencies of divalent cations at SP binding sites is similar to what has been observed with other receptors. This unique pattern of effects has been noted at receptors that are linked to adenylate cyclase (11–17, 24–30). This raises the question about a possible association of SP receptors to adenylate cyclase. In limited experiments we have not found any influence of SP on adenylate cyclase activity of submaxillary-sublingual gland homogenates. SP increases phosphatidylinositol turnover in salivary glands (6–9). It has been suggested that hormones and neurotransmitters can be divided into two classes: those that influence adenyl-

ate cyclase and those that influence phosphatidylinositol turnover and related molecular events (31). For some neurotransmitter receptor interactions, such as muscarinic cholinergic and angiotensin II effects, adenylate cyclase changes are obtained in some tissues and phosphatidylinositol metabolism is altered in others. It remains to be established whether, as with $alpha_2$ (negatively coupled to adenylate cyclase) and $alpha_1$ (stimulates phosphatidylinositol turnover) adrenergic receptors, there are subtypes of muscarinic and angiotensin II receptors that are pharmacologically and biochemically distinct from each other. We do not know whether our findings of guanyl nucleotide and divalent cation regulation for SP binding indicate that phosphatidylinositol mechanisms can be linked to GTP binding proteins or whether there exists an adenylate cyclase linked to SP which has escaped detection. Muscarinic cholinergic (30, 32) and $alpha_1$-adrenergic (33) receptors, that may act in some instance through phosphatidylinositol, can be regulated by GTP.

Although saturation analysis suggests that there is only one population of ^{3}H-SP binding sites in salivary glands, the effects of guanyl nucleotides suggest the possibility of heterogeneity. Thus, guanyl nucleotides maximally reduce only 60% of ^{3}H-SP binding, with about 40% being resistant to as much as 1 mM nucleotide. The lowering of ^{3}H-SP binding elicited by guanyl nucleotides involves a reduced number of binding sites with lesser changes in affinity. For several other receptors, guanyl nucleotides reduce the affinity of agonists. It is possible that for SP receptors, as has been shown for $alpha_2$-adrenergic receptors (24), guanyl nucleotides lower the affinity of some SP binding sites so markedly that they can no longer be detected by a filtration binding assay using the agonist ligand ^{3}H-SP. Such an effect will result in an apparent B_{max} change. Perhaps sites of such markedly reduced affinity would still be detected with a labeled SP antagonist, in which case one would be able to quantify reduced agonist affinity by measuring competition for the binding of radiolabeled antagonist.

Another item suggesting the existence of multiple SP binding sites is the selective change in the pattern of GMP-PNP inhibition of binding in the presence of magnesium. With magnesium, GMP-PNP becomes more potent and its displacement curve becomes much more shallow. This suggests that magnesium introduces a new population of binding sites, evidenced in the increase in B_{max}, where GMP-PNP has selectively high affinity whereas its lower affinity at other sites does not change. Further kinetic analysis on the association and dissociation rates of ^{3}H-SP binding in the presence of guanyl nucleotides and/or divalent cations may provide more insight into this apparent multiplicity of ^{3}H-SP binding sites.

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