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Specific Binding of [^{3}H-Tyr^8]Physalaemin to Rat Submaxillary Gland Substance P Receptor

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SUMMARY

[^{3}H]Physalaemin ([^{3}H]PHY) binds to a single class of noninteracting sites on rat submaxillary gland membranes suspended in high ionic strength media with a K_D of 2.7 nM, a B_{max} of 240 fmol/mg of protein, and low nonspecific binding. The relative potencies of substance P (SP) and its fragments in competing with [^{3}H]PHY correlate with their relative salivation potencies. This indicates that [^{3}H]PHY interacts with a physiologically relevant SP receptor. In low ionic strength media, the K_D of [^{3}H]PHY does not change, but SP and some of its fragments are more potent than PHY in competing with[^{3}H] PHY. Computer-assisted analysis of [^{3}H]PHY and [^{3}H]SP binding in high and low ionic strength media demonstrated that both peptides are equipotent in high ionic strength but that the affinity of SP increases by 70-fold in low ionic strength. The SP fragments that contain a basic residue in positions 1 and/or 3 also display an increased affinity in low ionic strength. These findings document that [^{3}H]PHY binding in high ionic strength (μ = 0.6) accurately reflects the pharmacological potencies of agonists on the SP-P receptor. The binding of [^{3}H]PHY, like that of [^{3}H]SP, increases by the addition of divalent cations ($Mg^{2+} > Ca^{2+} > Mn^{2+}$). Guanine nucleotides decrease [^{3}H]PHY binding by decreasing the B_{max} to the same level (160 fmol/mg of protein), in the presence or absence of Mg^{2+}.

INTRODUCTION

SP[1] (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is a mammalian putative neurotransmitter/modulator that has several central and peripheral effects. PHY (pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2) is an amphibian undecapeptide, that like SP, belongs to the tachykinin group of peptides (2). Both peptides have a similar spectrum of biological effects and interact with the same receptor subtype (3, 4).

The SP receptor on rat salivary glands has been previously labeled with [^{125}I]BH-SP (5), with [^{3}H]SP after preincubating the membranes in high ionic strength buffer (6) and with [^{125}I-Tyr^1,Nle^{11}]SP in the presence of 200 mM NaCl in the binding medium (7). In low ionic strength media, SP is 70-fold more potent than PHY in competing for the [^{3}H]SP binding to rat submaxillary gland membranes, even though both peptides are equipotent sialogogues (8, 9). In contrast, we have found that, in the presence of monovalent cations, SP and PHY are equipotent in competing with [^{3}H]SP (8). To resolve these apparent discrepancies, we synthesized tritium-labeled PHY and used it as an additional ligand for the salivary gland SP-P receptor (4).

In this paper, we have characterized the binding properties of [^{3}H]PHY in high and low ionic strength media, determined that PHY and SP bind to the same receptor site despite the differential effect of ionic strength on both peptides and report the optimal conditions to label the SP-P submaxillary gland receptor with this novel ligand.

MATERIALS AND METHODS

Materials. Polyethylenimine, HEPES, TLCK, TPCK, PCMB, NEM, iodoacetamide, bacitracin, chymostatin, and the various guanine and adenine nucleotides were from Sigma Chemical Co. (St. Louis, MO). All peptides were purchased from Peninsula (Belmont, CA). GF/B filters were from Whatman. [^{3}H]SP (21.6 Ci/mmol) was a generous gift from Dr. R. Wade, Ciba-Geigy Pharmaceuticals, Horsham, England. This label was stored in liquid nitrogen and purified biweekly by HPLC.

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[1] The abbreviations used are: SP, substance P; PHY, physalaemin; BH-SP, Bolton Hunter SP; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, tosylphenylalanyl chloromethyl ketone; HPLC, high performance liquid chromatography; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide.

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Synthesis, storage, and assay of [^{3}H]PHY. [^{3}H-Tyr^8]Physalaemin was prepared from physalaemin (Cambridge Research Biochemicals, Cambridge, England) by catalytic tritiation of iodinated PHY in which the methionine residue was temporarily protected as the sulfoxide. The product was isolated by semipreparative high performance liquid chromatography and had satisfactory amino acid analysis after acid hydrolysis. The specific activity was 30.4 ± 2.3 Ci/mmol and the radiochemical purity was 99%, inclusive of not more than 1% of the sulfoxide. The labeled peptide, 10 μM, was kept in small aliquots in 10 mM acetic acid in liquid nitrogen and diluted when needed to 0.1 μM with 5% ethanol, 1 mM mercaptoethanol, and kept under nitrogen at −20°. More than 96% of the [^{3}H]PHY co-migrated with PHY after 3 weeks of storage at −20°, as judged by the HPLC. We tested the biological activity of the tritiated PHY on the guinea pig ileum myenteric plexus-longitudinal muscle preparation (10) and found that [^{3}H]PHY and PHY had the same biological activity and cross-desensitized each other.

Preparation of the tissue. Rat submaxillary glands were homogenized in 20 volumes of 20 mM HEPES, pH 7.4 at 4° (Polytron setting 8 for 30 sec) and then centrifuged at 37,000 × *g* (20 min, 4°). The pellet was resuspended in the same buffer containing 0.1 mM TLCK and TPCK and incubated in a shaking water bath for 15 min at 20° to protect the peptides from degradation during the binding assay. The homogenate was centrifuged at 37,000 × *g* for 20 min and the pellet was resuspended in 40 volumes of the appropriate binding medium containing bacitracin (0.3 mg/ml) and chymostatin (0.05 mg/ml). For studies in low ionic strength binding media, the pellet was suspended in 0.36 M sucrose, 20 mM HEPES, pH 7.4 at 20°. For high ionic strength binding studies, the pellet was suspended in 0.25 M Na_2SO_4, 20 mM HEPES, pH 7.4 at 20°. Unless otherwise stated, the binding media contained 20 mM HEPES and the pH was adjusted to 7.4 after the addition of the sucrose or salts.

Binding assay. The binding assay was initiated by adding 0.4 ml of the final homogenate suspended in the appropriate binding medium to aliquots of radiolabel, unlabeled peptides, or water to a final volume of 0.5 ml. The samples were incubated in a shaking water bath at 20° for 30 min, and the binding was terminated by cooling for 2 min on ice. The bound [^{3}H]PHY was quantitated by filtering the homogenate onto 0.05% polyethylenimine-treated GF/B filters (8). Prior to filtering the homogenate, the filter was rinsed with 5 ml of wash buffer composed of 50 mM sodium phosphate, 100 mM choline chloride in 0.1% albumin, pH 7.4 at 0°. After adding the membranes, the filter was immediately rinsed three times with 5 ml of wash buffer, placed in counting vials and shaken for 30 min with 1 ml of NCS tissue solubilizer (Amersham, Arlington Heights, IL). Then, 10 ml of toluene-based scintillation fluor (0.4% 2,5-diphenyloxazole and 0.02% *p*-bis[2-(5-phenyloxazolyl)benzene] were added to each vial, and radioactivity was determined by liquid scintillation spectrometry. For competition experiments between [^{3}H]PHY and the unlabeled ligands, the peptides were diluted from the stock in albumin-coated polypropylene tubes to prevent their adsorption. The tubes were coated by filling them with 0.1% ultrapurified albumin, kept for 6 hr at 4°, and then emptied by suction. Specific [^{3}H]PHY binding was defined as the difference in binding of [^{3}H]PHY in the presence or absence of 1 μM PHY. The guanine nucleotides were quantitated at the end of the incubation by HPLC as described (11). Protein content was measured by the method of Peterson (12). The results were obtained from triplicate determinations that varied by less than 15%.

Effect of protein-modifying reagents. After homogenization and subsequent centrifugation, the pellet was suspended in 40 volumes of 20 mM HEPES, pH 7.4 at 25°. The membranes were incubated with the appropriate protein-modifying reagent in a shaking water bath for 30 min at 25°. TLCK and TPCK were added 15 min after the start of the incubation. Thereafter, the pellet was prepared by centrifugation, suspended in the appropriate binding medium, and incubated with [^{3}H]PHY as described above.

Calculation and data preparation. The data from each saturation with [^{3}H]PHY and competition experiment with either PHY or the other unlabeled peptides were entered in the appropriate subroutine of the equilibrium binding data analysis program (EBDA, Ref. 13). This program was designed to process raw data from radioligand-binding experiments into a form suitable for use by the nonlinear least squares fitting program SCAFIT (14) where the main analysis of saturation and competition data was conducted. The EBDA program is especially suitable for competition studies because it calculates the IC_{50} through an iterative curve-fitting program (15), and from this the K_I is calculated (16). In addition, the program prints the Hofstee plot (17) and, if it appears that the competing drug is binding to two or more sites, each segment is analyzed separately prior to SCAFIT analysis to reduce the residual variance (18). The EBDA and SCAFIT programs were adapted to the IBM-PC computer and supplied by G. A. McPherson, Victorian College of Pharmacy, Australia.

RESULTS

[^{3}H]PHY binding in low ionic strength media. Since previous results showed that PHY was less potent than SP and some of its fragments in competing for [^{3}H]SP binding (7, 8), despite that PHY is a more potent sialogogue (5, 19), we decided to reinvestigate these apparent discrepancies under several different conditions. We found that in low ionic strength media [^{3}H]PHY binding was time dependent and linear with tissue concentration. Saturation of the binding site with increasing concentrations of [^{3}H]PHY resulted in a half-maximal saturation at about 3 nM (Fig. 1A) and a very low nonspecific binding. Scatchard analysis (seven independent experiments) indicated a single population of binding sites with a dissociation constant (K_D) of 3.3 ± 0.6 nM and a maximal receptor concentration (B_{max}) of 22 ± 3 pmol/g wet weight (345 ± 46 fmol/mg of protein) (Fig. 1C).

Inhibition of [^{3}H]PHY binding by SP fragments and analogues in low ionic strength binding media. SP was more potent than PHY in competing for [^{3}H]PHY bound to membranes suspended in low ionic strength binding medium (Fig. 2A). The competition was analyzed by the computer programs EBDA, and the results are represented in Table 1. The apparent IC_{50} of SP was 0.57 nM while that of PHY was 7 nM. Since the binding of [^{3}H]SP was extensive in low ionic strength (20), the free concentration of SP was determined from a parallel experiment using [^{3}H]SP. By this analysis, the true IC_{50} of SP was 0.14 nM (Table 1), resulting in an affinity about 60-fold higher than PHY. The rank order of the various peptides in inhibiting [^{3}H]PHY binding was: SP > SP(3-11) > SP(2-11) > PHY > SP(4-11) > pGlu-SP(5-11) (Fig. 2A). Therefore, in addition to SP, SP(2-11) and SP(3-11) had a higher affinity than PHY in competing with [^{3}H]PHY.

The apparent Hill slope of the inhibition of [^{3}H]PHY binding by SP determined by the usual method (21) of $\log[B/100 - B]$ versus log[peptide concentration added] was −1.67 (21). The Hill coefficient changed to −0.94 ± 0.14 when the corrected value for the free SP concentration was used (Table 1 and Fig. 2A). Since the other radioactive SP fragments were not available, their free concentration could not be determined, and the data for calculating their IC_{50} and Hill slope were based on the amount of peptide added (Table 1).

The low affinity of PHY compared to that of SP in competing for [^{3}H]PHY in 0.3 M sucrose, was similar to the results obtained with [^{3}H]SP. Analysis of the com-

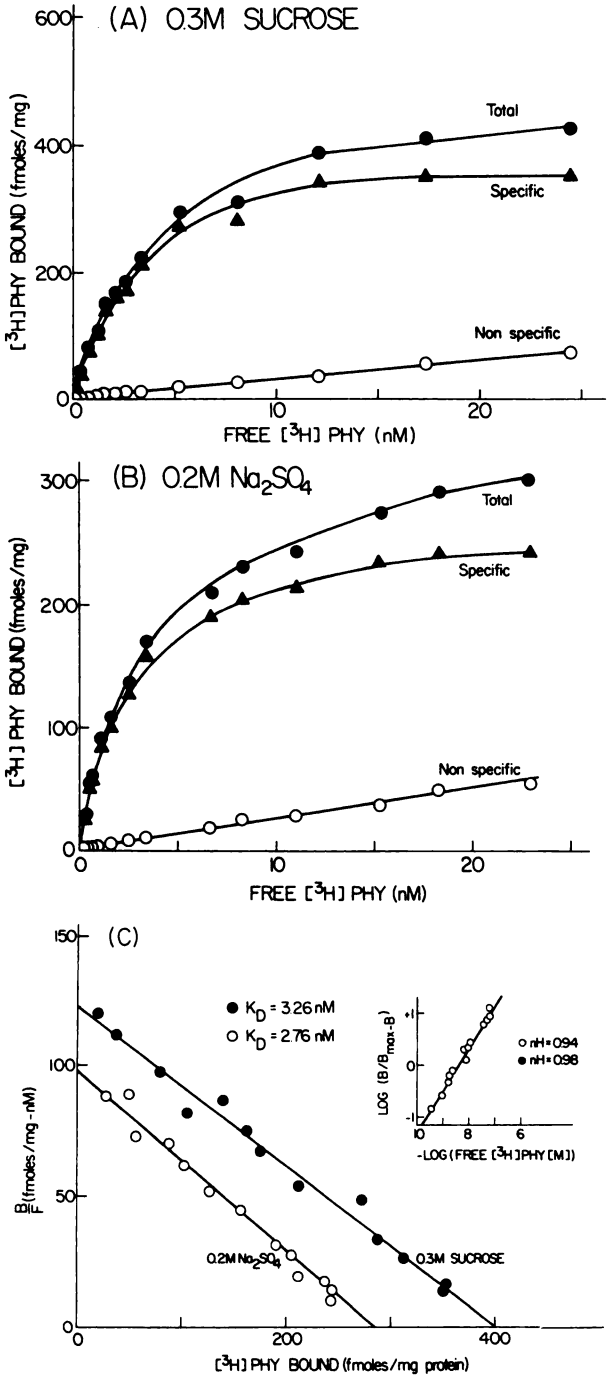

FIG. 1. *Saturation of the rat submaxillary gland membranes suspended in 0.3 M sucrose or 0.2 M Na_2SO_4, 20 mM HEPES (pH 7.4) with [^{3}H]PHY*

The rat submaxillary gland homogenate was prepared as described. The binding reaction was initiated by adding 0.4 ml of the homogenate to increasing concentrations of [^{3}H]PHY in the presence or absence of 1 μM PHY in a total volume of 0.5 ml. A: a direct plot of the data in low ionic strength (0.3 M sucrose, 20 mM HEPES, pH 7.4). Specific [^{3}H]PHY binding (▲) represents the difference between total (●) and nonspecific binding (○). B: same as A except high ionic strength binding medium. C: Scatchard analysis of the specific [^{3}H]PHY binding in 0.3 M sucrose (●) or 0.2 M Na_2SO_4 (○). The K_D of [^{3}H]PHY in low and high ionic strength was 3.26 and 2.76 nM, respectively, and the corresponding B_{max} was 400 and 285 fmol/mg of protein. Inset: Hill slope of the saturation data in 0.2 M Na_2SO_4 (n_H = 1.0). A similar slope was obtained for the 0.3 M sucrose data.

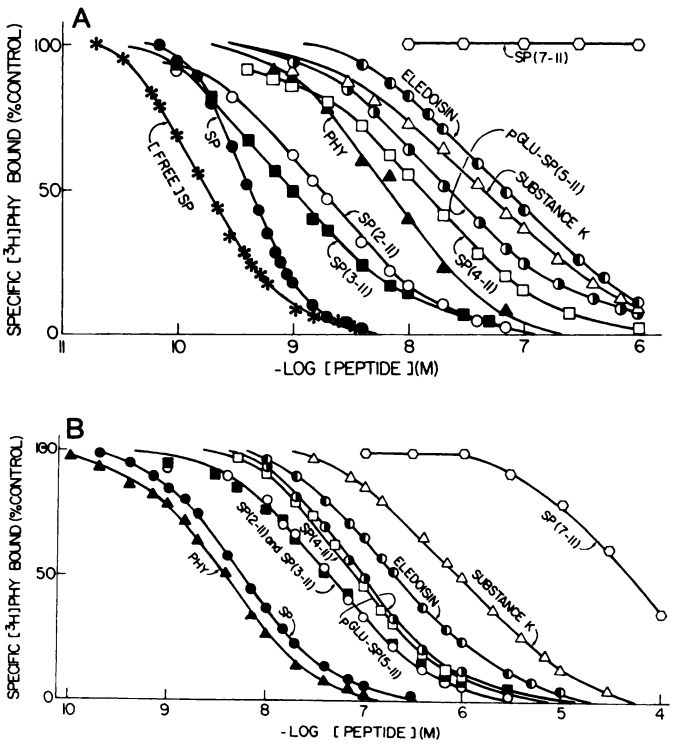

FIG. 2. *Inhibition of [^{3}H]PHY binding to rat submaxillary gland membranes*

[^{3}H]PHY (2.5 nM) was displaced by increasing concentrations of SP, and its fragments and related peptides. Each experiment was performed at least twice in triplicate with the data varying by less than 10%. The figure represents the averaged results of these experiments as calculated by the EBDA computer program. A: membranes suspended in 0.3 M sucrose, 20 mM HEPES, pH 7.4. *SP*: inhibition curve obtained by plotting the concentration of SP added. *[FREE]SP*: inhibition curve obtained by plotting the actual concentration of SP as determined in a parallel experiment using [^{3}H]SP. B: membranes suspended in 0.2 M Na_2SO_4, 20 mM HEPES, pH 7.4.

petition of [^{3}H]SP (1 nM) by SP and PHY resulted in an IC_{50} of 0.5 and 37 nM, respectively (20). To obtain more information about the binding parameters of both ligands, the competition for [^{3}H]PHY by SP and PHY was conducted under the same conditions. The results of the inhibition of [^{3}H]SP and [^{3}H]PHY binding were analyzed by the LIGAND (22) program to derive the best statistical model that could fit the data into a working hypothesis. The statistically preferred model was a single site model with a B_{max} of 335 ± 16 fmol/mg of protein and a K_D for SP and PHY of 0.06 and 4.4 nM, respectively (Table 2). The results indicated that, in 0.3 M sucrose, the affinity of SP for the binding site was 70-fold higher than that of PHY, in agreement with the ratio of their IC_{50}.

Influence of divalent cations and guanine nucleotides on [^{3}H]PHY binding in low ionic strength media. Several groups have reported that divalent cations increase the binding of [^{3}H]SP to rat submaxillary gland membranes (6, 8) and [^{125}I]BH-SP to rat brain membranes (23). Since [^{3}H]PHY unlike [^{3}H]SP, binds to the submaxillary gland membranes with the same affinity in low and high ionic strength media (see below), we studied the influence

TABLE 1

Inhibition of specific [^{3}H]PHY binding to rat submaxillary gland membranes in 0.3 M sucrose and 0.2 M sodium sulfate, 20 mM HEPES, pH 7.4

The inhibition of the binding of 2.2–2.8 nM of [^{3}H]PHY by increasing concentrations of unlabeled peptides. IC_{50} is defined as the concentration of competitor that inhibits 50% of specific [^{3}H]PHY binding.

Peptides	Inhibition of specific [^{3}H]PHY binding to membranes[a]						Salivation[b] Relative Potency
	Low ionic strength			High ionic strength		K_{IH}[c]/K_{IL}	
	IC_{50}	Hill[c]	Relative[d] potency	IC_{50}	Relative[d] potency		
	nM			*nM*			
Substance P	0.14	−0.94	1.0	6.0	1.0	50	1.0
SP(2-11)	0.42	−0.9	0.06	51	0.09	24	0.068
SP(3-11)	1.8	−0.9	0.3	44	0.125	47	0.17
SP(4-11)	0.45	−0.84	0.01	75	0.27	6	0.064
pGlu-SP(5-11)	15	−0.87	0.006	182	0.04	5.3	0.25
SP(7-11)	>50,000		ND[f]	50,000	0.0001	ND	0.00016
SP(1-7), SP(1-8)	>50,000			>50,000			
Physalaemin	7.0	−1.05	0.024	4	1.5	1.0	2.42
Eledoisin	51	−0.65	0.003	180	0.03	5.8	0.62
Substance K	52	−0.7	0.003	930	0.006	21	ND

[a] Values are the mean of two or three independent triplicate determinations which varied by less than 15%.
[b] Data from Liang and Cascieri (5).
[c] Hill slope.
[d] Ratio of SP IC_{50} divided by peptide IC_{50}.
[e] Inhibition constant of labeled ligand in high (K_{IH}) and low (K_{IL}) ionic strength calculated by the EBDA program as described by Cheng and Prusoff (16).
[f] ND, not determined.

TABLE 2

[^{3}H]SP- and [^{3}H]PHY-binding parameters determined by simultaneously fitting the data of homologous and heterogeneous displacement curves in 0.3 M sucrose and 0.2 M Na_2SO_4

The inhibition of the binding of 1 nM [^{3}H]SP and 2.5 nM [^{3}H]PHY by increasing concentrations of SP and PHY were simultaneously conducted in 0.3 M sucrose, 20 mM HEPES, pH 7.4. Similarly, the inhibition of the binding of 4 nM [^{3}H]SP and 2.5 nM [^{3}H]PHY by SP and PHY were conducted in 0.2 M sodium sulfate, 20 mM HEPES, pH 7.4. Pooled data of the inhibition of the specific binding of each ligand in either low or high ionic strength were analyzed by the SCAFIT program. The data represent the affinity (K_D) and receptor concentration (B_{max}) of both ligands in low or high ionic strength. Runs test (14) for all the experiments ($p > 0.05$).

Peptide	Incubation medium							
	0.3 M sucrose				0.2 M sodium sulfate			
	K_D[a]	B_{max}[b]	*df*[c]	MS[d]	K_D	B_{max}	*df*	MS
	nM	*fmol/mg*			*nM*	*fmol/mg*		
Substance P	0.06 ± 0.006	335 ± 16	39	56	4.2 ± 0.27	248 ± 13	39	28
Physalaemin	4.4 ± 0.05	335 ± 16			2.8 ± 0.17	248 ± 13		

[a] Affinity ± SE of each ligand to the binding site.
[b] Maximal receptor concentration (B_{max}) ± SE of both ligands.
[c] Degrees of freedom.
[d] Mean square.

of divalent cations and guanine nucleotides on [^{3}H]PHY binding in both media.

The divalent cations Mg^{2+} and Ca^{2+} decreased slightly but not significantly the binding of [^{3}H]PHY over the range tested (1 μM–10 mM), but Mn^{2+} markedly decreased the binding in a concentration-dependent manner with an IC_{50} between 1 and 3 mM (Fig. 3A). Manganese produced its effect by decreasing the affinity of [^{3}H]PHY from a control vaue of 3.5 to 13 nM without altering the B_{max} (Table 3). Guanine nucleotides did not inhibit the binding of [^{3}H]PHY in the low ionic strength medium (Table 3).

[^{3}H]PHY binding in 0.2 M Na_2SO_4. We reported (8) that, in the presence of 0.2 M Na_2SO_4, the relative potency of the various SP fragments in competing with [^{3}H]SP correlated with their relative salivation potency. Therefore, we studied the effects of this binding medium on [^{3}H]PHY binding and found that the binding of [^{3}H]PHY in 0.2 M Na_2SO_4 was saturable within 30 min at 20° and linear with tissue concentration. Typically, the addition of 2.4 nM [^{3}H]PHY resulted in a total of 1200 cpm bound, of which 100 cpm were nonspecific as determined by the addition of 1 μM PHY. Saturation of the receptor with increasing concentrations of [^{3}H]PHY (0.5–25 nM) resulted in a half-maximal saturation between 2.3 and 3 nM (Fig. 1B). Scatchard analysis of the data indicated that [^{3}H]PHY was binding to a single class of noninteracting sites ($n_H = 1.0$) with a K_D of 2.7 ± 0.6 nM and $B_{max} = 14.3 \pm 1.5$ pmol/g of tissue (240 ± 25 fmol/mg of protein; Fig. 1C). Thus, the K_D of [^{3}H]

TABLE 3

Effect of guanyl nucleotides and divalent cations on the binding constants of [^{3}H]PHY to rat submaxillary gland membranes suspended in low and high ionic strength media

Increasing concentrations of [^{3}H]PHY (0.5–20 nM) were added to membranes suspended in either 0.3 M sucrose or 0.2 M Na_2SO_4 in the absence or presence of either nucleotide, divalent cation, or both. Data represent specific [^{3}H]PHY binding (± SD).

Condition	Affinity (K_D)	B_{max}[a]
	nM	*pmol/g*
0.3 M sucrose	3.5 ± 0.3	17.5 ± 2 (280)
0.3 M sucrose, 5 mM $MnCl_2$	13.0 ± 2[b]	18.0 ± 3 (290)
0.3 M sucrose, 50 μM GTP	4.6 ± 0.5	14.0 ± 2 (224)
0.2 M Na_2SO_4	2.6 ± 0.3	13.5 ± 2 (210)
0.2 M Na_2SO_4, 10 mM $MgCl_2$	2.4 ± 0.3	16.0 ± 2 (256)
0.2 M Na_2SO_4, 10 μM Gpp(NH)p	1.9 ± 0.5	9.6 ± 1 (154)[b]
0.2 M Na_2SO_4, 10 mM $MgCl_2$, 10 μM Gpp(NH)p	3.4 ± 0.4[b]	10.0 ± 1 (160)[b]

[a] Data in parentheses represents the corresponding B_{max} in femtomoles/mg of protein.

[b] $p < 0.05$ compared to control without divalent cation or guanyl nucleotide.

PHY in 0.2 M Na_2SO_4 was equal to its K_D in 0.3 M sucrose ($p > 0.05$).

Inhibition of [^{3}H]PHY binding by the SP fragments and related peptides in 0.2 M Na_2SO_4. PHY was the most potent competitor of [^{3}H]PHY with an IC_{50} of 4 nM (Fig. 2B). SP was slightly less potent than PHY and the potency of its fragments correlated with their relative salivation potency (5). In contrast, eledoisin, which is the prototype ligand for the SP-E subclass of tachykinin receptors (4, 24) had an IC_{50} of 180 nM (Table 1), even though it is a powerful sialogogue with an *in vivo* potency 60% that of SP (5). Substance K (25) had an IC_{50} of 930 nM (Fig. 2B). All the SP fragments had a Hill slope of unity in this binding medium, except eledoisin and substance K, which had Hill slopes of −0.8 and −0.73, respectively.

To compare the change in affinity of the different peptides in 0.2 M Na_2SO_4 and in 0.3 M sucrose, we used the following method: we calculated the inhibition constant (K_I) of each unlabeled peptide in inhibiting [^{3}H]PHY binding in 0.3 M sucrose and 0.2 M Na_2SO_4 (16) and computed the ratio of the K_I of each peptide in high to that in low ionic strength. Peptides that underwent minimal or no change would have a ratio close or equal to unity, but peptides whose affinity decreased (i.e., K_I value increased) in high ionic strength, will have a ratio greater than unity (Table 1). The results show that SP, SP(2-11), and SP(3-11) underwent a major decrease in their affinities when sucrose was replaced by 0.2 M Na_2SO_4. Of this group, SP and SP(3-11), which contain a basic residue in their amino terminus, underwent the greatest reduction in affinities, 50- and 47-fold, respectively. Additionally, SP(2-11), which still retained the charged Lys^3 (now in position 2), underwent a 20-fold decrease in affinity. In contrast, the shorter fragments, namely SP(4-11) and pGlu-SP(5-11), underwent a 5-fold decrease in their affinity, and PHY did not change (Table 1).

Influence of divalent cations and guanine nucleotides on [^{3}H]PHY binding in 0.2 M Na_2SO_4. We tested the divalent cations that increased the binding of [^{3}H]SP (6, 8) on [^{3}H]PHY binding. Although we observed a slight increase in the binding of [^{3}H]PHY, it was not as marked as the one seen with [^{3}H]SP. The order of potency of the divalent cations was $Mg^{2+} > Ca^{2+} > Mn^{2+}$, with a maximal increase of 25 ± 5, 15 ± 5 and 10 ± 3% at 10 mM for Mg^{2+} and Ca^{2+} and 5 mM for Mn^{2+} (Fig. 3B). Magnesium increased the binding of [^{3}H]PHY by increasing the B_{max}, from 13.5 to 16 pmol/g, without changing the affinity (Table 3).

Guanine and adenine nucleotides inhibited [^{3}H]PHY binding with a rank order of GTP > GDP > Gpp(NH)p > GMP > ATP = ADP. GTP had an apparent IC_{50} of 10 μM, but it was completely degraded to GDP and GMP at the end of the incubation period. Thus, the IC_{50} reported here represents only the inhibitory effect produced by the initial concentration. Gpp(NH)p produced a sigmoidal inhibition, with an IC_{50} of 30 μM and a pseudo-Hill coefficient of unity (Fig. 4), and reduced the B_{max} of [^{3}H]PHY to 9.6 pmol/g without changing the K_D (Table 3).

The addition of magnesium (5 mM) did not change the apparent IC_{50} of GTP, but increased its degradation since all the nucleotide was recovered as guanosine. Gpp(NH)p underwent a 30-fold increase in potency in the presence of magnesium with an IC_{50} of 1 μM (Fig. 4), but 20–25% of the nucleotide was degraded to guanosine during the incubation period. Saturation analysis with [^{3}H]PHY in the presence of magnesium and Gpp(NH)p resulted in a K_D of 3.4 nM (increase by 30% above control, $p < 0.05$) and the B_{max} was reduced to 10 pmol/g (Table 3). Therefore, guanine nucleotides reduced the B_{max} to approximately 10 pmol/g or 160 fmol/mg of protein, either in the presence or in the absence of magnesium.

Influence of protein-modifying reagents. PCMB abolished, while NEM reduced by 50%, the specific [^{3}H]PHY binding to the rat submaxillary gland membranes suspended in either low or high ionic strength media (Table 4).

DISCUSSION

[^{3}H]PHY binding to rat submaxillary gland membranes suspended in 0.2 M sodium sulfate, 20 mM HEPES (pH 7.4) is specific, saturable, and reversible with a K_D of 2.7 nM. The affinity of [^{3}H]PHY in this tissue is similar to the K_I of PHY in competing with [^{3}H]SP and [^{125}I-Tyr^1,Nle^{11}]SP (7, 9). Similarly, the K_D of [^{125}I]PHY in dispersed rat parotid cells was 1.4 nM and the K_I of PHY in inhibiting [^{125}I]BH-SP binding was 2.4 nM (5, 26). The identification of the [^{3}H]PHY-binding site with that of a physiologically relevant SP receptor was inferred from the results shown in Table 1, where the relative potencies of SP and its fragments in competing with [^{3}H]PHY in 0.2 M sodium sulfate parallel their relative salivation potencies. The results of this as well as the previously cited studies indicate that [^{3}H]PHY labels a SP-P receptor subtype. Eledoisin deviates from its expected potency, probably because it interacts preferentially with the SP-E receptor subtype to produce salivation, and has a low affinity for the SP-P subtype

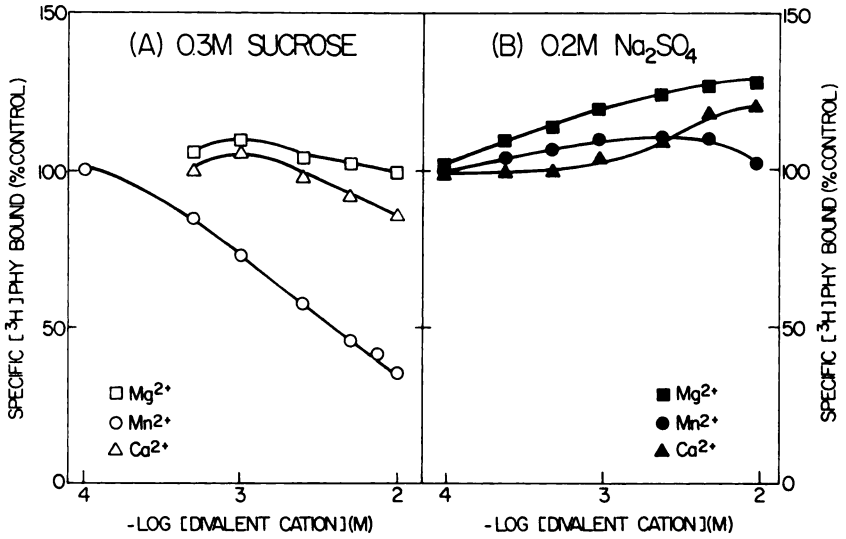

FIG. 3. *Influence of divalent cations on specific [^{3}H]PHY (2.5 nM) binding to rat submaxillary gland membranes suspended in either low (A) or high ionic strength (B) media*

The specifically bound [^{3}H]PHY at each divalent cation concentration is expressed as the percentage of the control samples. The concentration of each divalent cation is shown in the abscissa, and the data represent the means of three experiments, each in triplicate, that varied by less than 15%. In sucrose (A), Mg^{2+} (□) and Ca^{2+} (△) had little effect, but Mn^{2+} (○) inhibited the binding with an IC_{50} of 3 mM. In 0.2 M Na_2SO_4 (B), maximal stimulation of binding was obtained at 2.5 mM Mn^{2+} and 10 mM Mg^{2+} and Ca^{2+}.

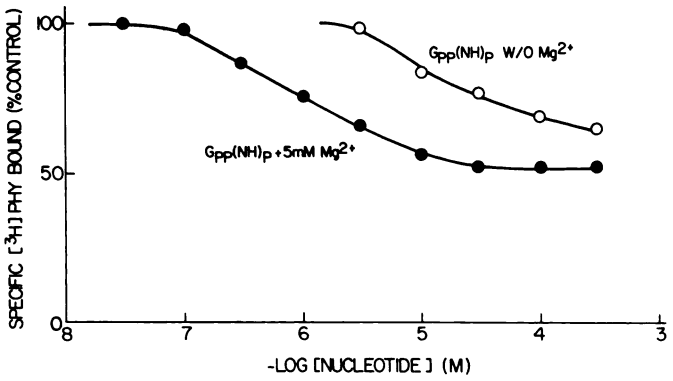

FIG. 4. *Inhibition of specific [^{3}H]PHY binding to rat submaxillary gland membranes suspended in 0.2 M Na_2SO_4 by Gpp(NH)p in the absence (W/OMg^{2+}) and in the presence of 5 mM $MgCl_2$*

Submaxillary gland membranes were incubated with 2.5 nM [^{3}H] PHY in the absence (○) and in the presence of 5 mM $MgCl_2$ (●) with the concentration of Gpp(NH)p indicated in the abscissa. The results are the average of three experiments, each in triplicate, that varied by less than 15%.

TABLE 4

Effect of protein-modifying reagents on specific [^{3}H]PHY binding

The membranes were incubated with the reagents for 30 min at 25°, as described in Materials and Methods. The treated membranes were incubated with 2.5 nM [^{3}H]PHY in the absence or presence of 1 μM PHY for 30 min at 20° and the specifically bound [^{3}H]PHY was determined as described. The results are the average of three experiments.

Reagent	Concentration	Binding medium	Specific [^{3}H]PHY bound[a]
	mM		%
NEM	2.0	0.3 M sucrose	58
PCMB	0.1	0.3 M sucrose	5
Iodoacetamide	10.0	0.3 M sucrose	100
NEM	2.0	0.2 M Na_2SO_4	42
PCMB	0.1	0.2 M Na_2SO_4	4
Iodoacetamide	10.0	0.2 M Na_2SO_4	79

[a] Percentage of control, untreated membranes.

labeled in this study. Preliminary experiments using ^{3}H-labeled substance K (neurokinin A) indicate that the rat submaxillary gland may not contain substance K-binding sites.[2] The results of the labeling of the SP receptor on rat submaxillary gland and brain (23, 27, 28) and the binding of [^{3}H]PHY and its competition by the various SP fragments and analogues in these tissues indicate that the brain and salivary gland SP-P receptors are similar (29).[3]

The affinity of [^{3}H]SP to rat submaxillary gland membranes suspended in low ionic strength was 0.15 nM (6, 8). The competition of [^{3}H]SP by SP and PHY resulted in an IC_{50} of PHY 70-fold higher than SP, even though PHY is more potent than SP as a sialogogue. This study confirms and documents that the affinity of PHY in competing with [^{3}H]PHY in low ionic strength media is 70-fold less than SP. Furthermore, the K_D of [^{3}H]PHY in low ionic strength media is similar to the K_I of PHY in inhibiting [^{3}H]SP binding. The low potency of PHY in competing with [^{3}H]SP in low ionic strength media may be attributed to the presence of two subtypes of the SP-P receptor, with each ligand interacting preferentially with a different one. We ruled out this possibility by showing that, in low ionic strength media, both ligands interact with the same site, but with a 70-fold difference in affinity (Table 2). Furthermore, the inhibition of [^{3}H]PHY binding by SP and PHY results in a Hill slope of

[2] S. W. Bahouth, D. M. Lazaro, D. E. Brundish, and J. M. Musacchio, unpublished observation.

[3] P. M. Narang, S. W. Bahouth, D. E. Brundish, and J. M. Musacchio, submitted for publication.

unity (Table 1) and a linear Hofstee plot (data not shown).

This paper demonstrates that the addition of sodium sulfate decreased the affinity of SP and some of its fragments in competing with [^{3}H]PHY without changing the affinity of PHY. Preliminary evidence in this direction was obtained using indirect competition binding studies with [^{3}H]SP (1, 8) and [^{125}I-Tyr^1,Nleu^{11}]SP (7). To gain some information about the amino acids in the SP molecule responsible for this change, we divided the K_I value of each unlabeled peptide in high by that in low ionic strength. Peptides that behave like PHY would have a ratio close to unity, while peptides that undergo a decrease in their affinity in high ionic strength media (i.e., K_I increased), would have a ratio greater than unity. As shown in Table 1, the peptides with the highest ratio are SP, SP(2-11), and SP(3-11). These peptides have one or two basic amino acids in positions 1 and 3, while SP(4-11) and the shorter fragments have a ratio closer to unity. We speculate that basic amino acids namely, Arg^1 and Lys^3 in the amino terminus of SP, are responsible for the hyperaffinity of these peptides in low ionic strength media. Nevertheless, the first step in the binding of the peptide involves the interaction of its carboxy-terminal recognition site with the receptor, since peptides which lack the recognition site did not inhibit [^{3}H]PHY binding (Table 1). Following the initial binding, the interaction between the peptide containing the basic amino acids and the receptor decrease the dissociation rate of the peptide (K_{off}), consequently decreasing the value of the equilibrium dissociation constant.[4] Therefore, the addition of monovalent cations to the binding medium is necessary to block these interactions and to normalize the binding of SP and other peptides. The use of low ionic strength media may result in erroneous measurements of the relative affinities of tachykinins for the SP-P receptor.

This study further establishes that [^{3}H]PHY binding, like that of [^{3}H]SP is modulated by divalent cations and guanine nucleotides (6, 8). The pattern of the divalent cation effect on [^{3}H]PHY binding in 0.2 M Na_2SO_4 is different from that on [^{3}H]SP binding. First, the rank order in increasing [^{3}H]PHY binding was $Mg^{2+} > Ca^{2+} > Mn^{2+}$, as opposed to $Mn^{2+} > Mg^{2+} > Ca^{2+}$ for [^{3}H]SP binding. Second, the optimal concentration of Mg^{2+} increases the specific binding of [^{3}H]PHY by 20 ± 5% above control, while optimal Mg^{2+} and Mn^{2+} concentrations increase specific [^{3}H]SP binding by 35% in one study (8) and 60% in another (6). The reasons for these discrepancies are not clear at the present time.

Guanine nucleotides reduce the binding of [^{3}H]PHY in high ionic strength media. However, the inhibition is not complete, and reaches a plateau, indicating that the binding sites are heterogeneous, either because there are two receptor subtypes, or because only a fraction of the recognition sites are associated with a GTP-binding regulatory protein. It is possible that a fraction of the GTP-binding regulatory protein is lost or inactivated during the tissue homogenization. This last possibility is likely because the GTP-binding regulatory protein associated with the rat brain SP-P receptor is easily inactivated by sulfhydryl reagents (29) and may be sensitive to oxidation during the preparation of the membranes.

The IC_{50} of Gpp(NH)p to inhibit [^{3}H]PHY binding is decreased about 30-fold by 5 mM $MgCl_2$, as observed with [^{3}H]SP (6, 8), but the maximal inhibition is not increased. These observations indicate that Mg^{2+} increases the affinity of guanine nucleotides for its binding site. The addition of Gpp(NH)p with or without Mg^{2+} produces the same B_{max} of approximately 10 pmol/g, in accordance with our previous results with [^{3}H]SP (1, 20).

The underlying mechanisms involved in the actions of divalent cations and guanine nucleotides are not understood. The information we currently have indicates that the divalent cations and guanine nucleotides interact with the same population of [^{3}H]PHY-binding sites. The possibility that both divalent cations and guanine nucleotides act on the GTP-binding regulatory protein component of the adenylate cyclase system in submaxillary gland membranes was analyzed by Lee *et al.* (6). SP alone did not alter the basal or inhibit the increase in cAMP produced by epinephrine or fluoride, suggesting that the mechanism of action of divalent cations and guanine nucleotides does not involve a GTP-binding protein associated with adenylate cyclase.

In conclusion, we have characterized the binding properties of [^{3}H]PHY in the rat salivary gland and we have documented that the [^{3}H]PHY binding in high ionic strength media accurately reflects the pharmacological potencies of agonists on the SP-P tachykinin receptor. In addition, the nonspecific binding is negligible, even at concentrations severalfold its K_D.

[4] S. W. Bahouth and J. M. Musacchio, in preparation.

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