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The Determination of Dissociation Constants for Substance P and Substance P Analogues in the Guinea Pig Ileum by Pharmacological Procedures

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SUMMARY

The dissociation constants (K_d values) of substance P (SP), physalaemin, kassinin, and SP analogues acting on SP receptors in guinea pig ileal longitudinal muscle strips were determined by the pharmacological procedures of Furchgott [*Adv. Drug Res.* 3:21–55 (1966)]. This method involves analysis of the concentration-response data before and after fractional inactivation of receptors with phenoxybenzamine (2×10^{-5} M). Estimations of the K_d values for SP were similar when phenoxybenzamine was incubated for 10, 13, or 15 min. Coincubation with high concentrations of SP protected against receptor inactivation with phenoxybenzamine, but bradykinin and serotonin did not cross-protect SP receptors. K_d values for SP were similar when trypsin was substituted for phenoxybenzamine [$K_d = 8.1 \pm 4$ nM ($n = 9$) versus 10 ± 6 nM ($n = 5$)]. In atropinized preparations the K_d values obtained for physalaemin were similar to those obtained for untreated preparations [$K_d = 8.0 \pm 3.6$ nM ($n = 5$) and 12.6 ± 3 nM ($n = 4$), respectively]. The effects of phenoxybenzamine on concentration-response curves for kassinin showed greater shifts to the right with phenoxybenzamine. This indicated that kassinin may interact with another population of receptors, in addition to the sites that SP and other analogues bind. A direct correlation was found between EC_{50} values and K_d values for SP and SP analogues. It was estimated that, for SP, a 20% receptor occupancy is required to elicit a 50% response.

INTRODUCTION

SP[3] (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) is an undecapeptide partially purified by von Euler and Gaddum (1) and subsequently isolated and sequenced by Chang and Leeman (2). Several studies have indicated that it may serve as a neurotransmitter: it excites neurons in brain and periphery (3, 4), it has an uneven distribution in brain (5, 6), and radioligand binding studies in the central nervous system have revealed the existence of [^{3}H]SP binding sites with characteristics which are consistent with those expected for SP receptors (7). SP is concentrated in dorsal root ganglia cells (6); it contracts various smooth muscles and causes the release of saliva from salivary glands (1, 2). In addition, it is a potent vasodilator (1).

Despite extensive studies on the structure-activity relationship of SP and SP analogues in guinea pig ileum (8), there is as yet no study that estimates the dissociation constants (K_d values) of these substances. Such studies would be helpful in elucidating the quantitative relationship between the affinity and the physiological response of SP at its receptor sites. In addition, a knowledge of the relative affinities of structurally related compounds in one receptor system can help to identify different receptor types in another tissue. To investigate the affinity of SP and SP analogues, we have employed the pharmacological procedures of Furchgott (9–11).

This method involves analysis of the concentration-response data before and after fractional inactivation of receptor sites by alkylating agents and has been previously applied in studies on the muscarinic and *alpha*-noradrenergic receptors (11–13).

EXPERIMENTAL PROCEDURES

Methods. Isolated longitudinal muscle strips (approximately 3 cm long) from ilea of male guinea pigs were suspended in a 10-ml chamber at 37° with Krebs-Ringer buffer (millimolar: NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; choline chloride, 0.03) and bubbled with 95% O_2-5% CO_2. Isometric contractions were recorded with a Grass Model 79 polygraph. Baseline tension was set at 0.3 g. After equilibration for approximately 40 min, a high concentration of an agonist (approximately 20-fold EC_{50}) was introduced and after

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[3] The abbreviations used are: SP, substance P; (Tyr[1],NLeu[11])-SP, (tyrosine[1], norleucine[11])-SP; SP4–11, SP analogue fragments 4–11.

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the development of the initial phasic contraction (<10 sec), the drug was washed out by overflow. This procedure was repeated twice or more until the response to the high agonist concentration was constant. Thereafter, a concentration-response curve for the test agonist was established. The interval between doses was set at 3–5 min.

To establish a concentration-response curve for the partially inactivated strips, phenoxybenzamine (20 μM) was introduced for 10 min and washed out. The strips were then allowed to reequilibrate for 20–30 min, with repeated washing every 5 min. A dose of agonist was then applied to the strip and washed out after the development of the phasic response. This procedure was repeated until the response to the agonist was stable. Thereafter, a concentration-response curve was re-established. The time interval between doses for the phenoxybenzamine-treated strips was set at 5–7 min. In the majority of experiments, one test agonist was used on each muscle strip. This procedure decreased the time-dependent changes in the contractility of the muscle strip.

To estimate the dissociation constant of each agonist, six or seven equiactive concentrations of agonist, between 30% and 50% maximal response, were selected from the fitted concentration-response data obtained before and after addition of phenoxybenzamine. The K_d was calculated from the double-reciprocal plot of $1/[A]$ versus $1/[A']$, from the equation:

$$\frac{1}{[A]} = \frac{1}{q}\frac{1}{[A']} + \frac{1-q}{q}\frac{1}{K_d}$$

where $[A]$ is the concentration of agonist that produced a certain response before phenoxybenzamine treatment, $[A']$ is the concentration of the agonist that produced the same response, and q is the fraction of active receptor sites remaining after phenoxybenzamine treatment. Two of the assumptions made by Furchgott (9–11) were that receptor sites for the agonist are homogeneous and that the addition of agonist does not result in desensitization of the receptor. Because the tonic contractile response to SP may have a small cholinergic component (14–16), we designed experiments to compare the K_d values determined in atropinized and untreated preparations. To avoid the development of receptor desensitization, we measured only the phasic contraction and washed the preparation thoroughly after each peptide addition. To rule out the possibility of any artifact introduced by phenoxybenzamine, we also used trypsin to inactivate SP receptors and to obtain an independent measurement of the K_d. Unless specified otherwise, the 100% response for each agonist was the response obtained from 50–100 times the EC_{50} of each drug. All peptides tested produced a similar maximal response, between 3 and 4 g of tension.

Materials. SP and physalaemin were purchased from Penisula Laboratories (San Calos, Calif.). Trypsin (L-1-tosylamide-2-phenylethylchloromethyl ketone-treated) was obtained from Millipore Corporation (Freehold, N. J.). Serotonin was purchased from Sigma Chemical Company (St. Louis, Mo.). Phenoxybenzamine was a gift from Smith Kline & French (Philadelphia, Pa.). (Tyr^1,$NLeu^{11}$)-SP and SP4–11 were synthesized and donated by Dr. John Stewart, University of Colorado Health Sciences Center (Denver, Colo.). The purity of each peptide was checked by a reverse-phase, high-pressure liquid chromatographic system with a 20–40% acetonitrile linear gradient in 10 mM NaH_2PO_4. All peptides were dissolved in 10 mM acetic acid and stored as aliquots of a 1 mM solution at −20°. Phenoxybenzamine was dissolved in 0.05 M HCl, and dilutions were made with distilled water to obtain a 10 mM stock solution.

RESULTS

Effects of phenoxybenzamine on the contraction induced by SP. Figure 1 illustrates that the addition of phenoxybenzamine (20 μM) caused a time-dependent loss of the contractile response induced with SP. The ileal muscle strip lost about 20% of its response after 4.5-min incubation with phenoxybenzamine, and an additional 5-min incubation further reduced the contractile response by 15%. In other words, there was a time-dependent shift

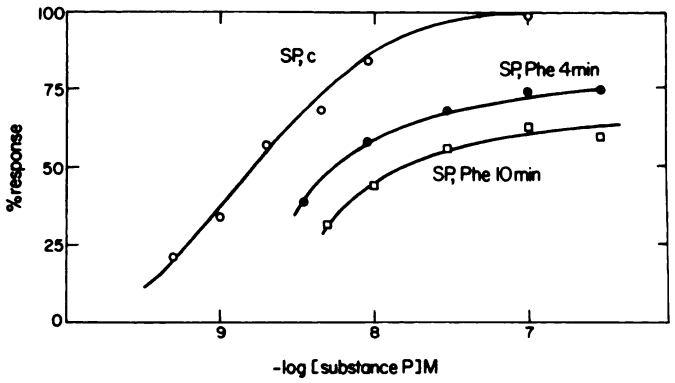

FIG. 1. *Inhibition of SP-induced contractions of guinea pig ileal longitudinal muscle after 4- and 10-min incubations with 20 μM phenoxybenzamine*

Procedures for obtaining the concentration-response curves are described under Methods.

to the right of the concentration-response curve. In the majority of experiments, the shifts in the EC_{50} of SP were 5-fold after a 10-min incubation (from 2.5 to 12 nM). The dissociation constant of SP, calculated from 10-min-treated preparation with the double-reciprocal plot method (Fig. 2), was 8.1 ± 4 nM (n = 9). Similar K_d values were found when phenoxybenzamine was left in contact with the strip for 13 or 15 min. The loss of SP contraction by phenoxybenzamine was not caused by temporal changes in the contractile mechanism. In parallel studies, the strips were tested for contractility after 40 min, with the same wash procedures, and no subsequent changes in contractile response were found. When a high concentration of SP (1 μM) was added before the phenoxybenzamine, the loss of contractile response was only 10–15% (Fig. 3), instead of 30–40% with phenoxybenzamine alone. Pretreatment with high concentrations of bradykinin (1.5 μM) or serotonin (0.1 mM) did not

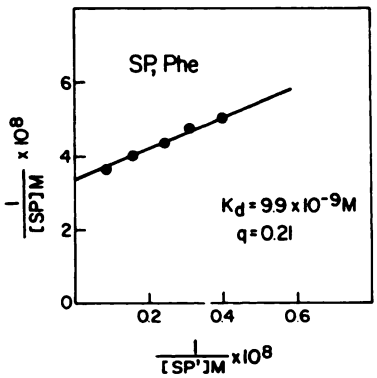

FIG. 2. *Double-reciprocal plot of equiactive concentrations of the control strip and the strip treated with phenoxybenzamine (20 μM) for 10 min, as shown in Fig. 1*

The dissociation constant (K_d) of SP and q, the fraction of active receptors remaining, were determined from the equation:

$$\frac{1}{[A]} = \frac{1}{q}\frac{1}{[A']} + \frac{1-q}{q}\frac{1}{K_d}$$

where $[A]$ is the concentration of SP in the control strip and $[A']$ is the concentration of SP producing the same response as $[A]$ in the phenoxybenzamine-treated strip.

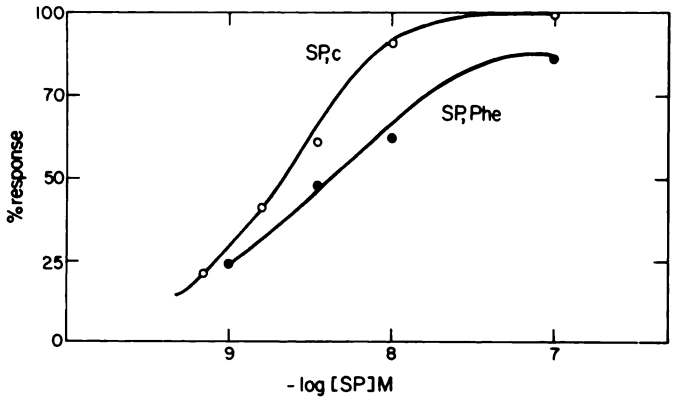

FIG. 3. *SP protection experiment*

A control dose-response curve for SP was first established. Afterward, SP (1 μM) was added and immediately followed by a 10-min incubation with 20 μM phenoxybenzamine. A second dose-response curve was generated after washout and re-equilibration.

protect against the loss of contractile response to SP. Interestingly, when a high concentration of carbachol was added prior to phenoxybenzamine, recovery of the SP response was similar to that after pretreatment with SP (1 μM). Treatment with phenoxybenzamine (20 μM) reduced by more than 60% the response to 10 μM carbachol. SP (1 μM) pretreatment did not protect against the phenoxybenzamine effect of the carbachol response. Pretreatment with carbachol resulted in a 90% recovery of the carbachol response. These experiments demonstrate that the reduced contractile response to SP caused by phenoxybenzamine treatment can be distinguished from the reduced response to other neurotransmitters produced by the same agent.

Comparison of the K_d of SP obtained by phenoxybenzamine and trypsin treatment. To establish that the K_d obtained was the same when different agents were used to inactivate SP receptors, we compared the K_d values obtained from the phenoxybenzamine (20 μM) treatment with those obtained with trypsin (200 μg/ml) treatment. Figure 4 shows that a 5-min treatment with trypsin caused a 50% reduction of the SP contraction. When the K_d (10 ± 6 nM; $n = 5$) was calculated from the double-reciprocal plot (Fig. 5), it was similar to the K_d (8.1 ± 4 nM; $n = 9$) obtained from the phenoxybenzamine-treated preparation (Fig. 2; Table 1).

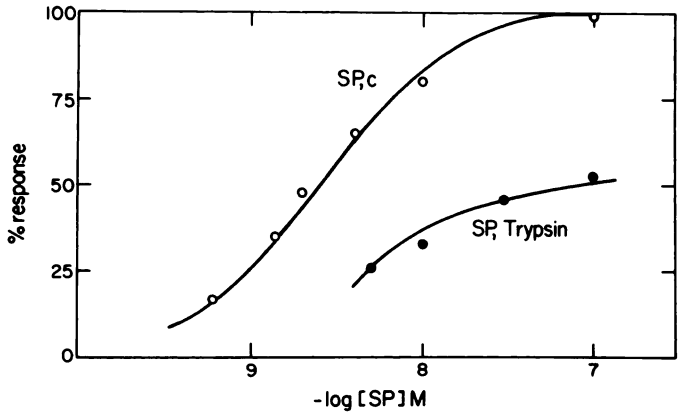

FIG. 4. *Effect of 5-min trypsin (200 μg/ml) treatment on SP-induced contraction*

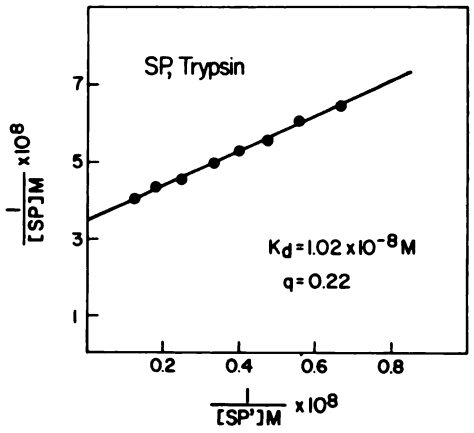

FIG. 5. *Double-reciprocal plot for the trypsin-treatment experiments*

K_d and q values were determined as in Fig. 2.

Comparison of the K_d values of the atropinized and nonatropinized preparations. Although the phasic response recorded in the present study was shown to be primarily due to the interaction of SP with receptor sites in the muscle, several studies have indicated that the tonic response of SP has some cholinergic components (14–16). To establish that the K_d values obtained in the present study were primarily the values of muscle SP receptor sites and that the interaction of peptides with the cholinergic system would not affect the estimated K_d of SP or physalaemin, we studied the effect of atropine pretreatment on the calculated K_d for physalaemin. As illustrated in Table 1, the K_d value of physalaemin, obtained in the presence of atropine (8 ± 3.6 nM; $n = 5$), was similar to that obtained in the absence of atropine (12.6 ± 3 nM; $n = 4$).

Dissociation constants of SP and SP analogues acting on the SP receptors in guinea pig ileum. Table 2 shows the dissociation constants (K_d values) of SP, physalaemin, SP4–11, (Tyr[1]-NLeu[11])SP, and kassinin. There was a direct relationship between the EC_{50} of an analogue and its K_d value; i.e., the more potent a compound in eliciting contraction, the more affinity the compound had for the SP receptor sites. It is of interest to note that kassinin, an SP E-type agonist (17), produced an anomalous shift of the concentration-response curve to the right by phenoxybenzamine (Fig. 6). Whereas the shift of EC_{50} for SP and physalaemin was about 5- to 10-fold, the shift of the concentration-response curve of kassinin was more than 20-fold. In addition, the slope of the reciprocal plot (Fig. 7) was greater than 10, and the fraction of

TABLE 1

Dissociation constants (K_d values) of SP and physalaemin determined by different methods

Values are means ± standard error of the mean. The number of determinations is indicated in parentheses.

Method	K_d	
	SP	Physalaemin
	nM	
Phenoxybenzamine (20 μM)		
Nonatropinized tissue	8.1 ± 4 (9)	12.6 ± 3.0 (4)
Atropinized tissue	—	8.0 ± 3.6 (5)
Trypsin (200 μg/ml)	10.0 ± 6.0 (5)	—

TABLE 2

Comparison of EC_{50} and K_d values of SP analogues acting on the guinea pig ileum

Values are means ± standard error of the means. The number of determinations is indicated in parentheses.

Peptide	EC_{50}	K_d
	nM	nM
SP	2.2 ± 1.04 (9)	8.1 ± 4 (9)
Physalaemin	2.8 ± 0.8 (3)	12.6 ± 3 (4)
SP4–11	3.3 ± 0.49 (4)	21 ± 7.4 (4)
(Tyr^1-NLeu^{11})-SP	6.7 ± 2.5 (7)	71 ± 25 (5)
Kassinin	6.3 ± 1.8 (5)	120 ± 60 (3)

receptors remaining, q, was 0.098 ± 0.013 (n = 4). These values were quite different from those obtained from SP. The q value of SP, determined from the double-reciprocal plot, was 0.281 ± 0.015 (n = 6), and the slope of the double-reciprocal plot was always between 4 and 3. In other words, the fraction of receptors remaining active after an identical treatment with phenoxybenzamine is significantly different ($p < 0.005$) for SP and kassinin receptors.

DISCUSSION

We have used the methods of Furchgott (9–11) to estimate the dissociation constants of SP, physalaemin, and the SP analogues kassinin and (Tyr^1-NLeu^{11})-SP for the peptidergic receptor sites in guinea pig ileum longitudinal muscle. We chose phenoxybenzamine as the alkylating agent to block irreversibly the SP receptor sites because it produces a reliable blockade of the SP response. There are several reasons which indicate that the effect of phenoxybenzamine is on the receptor site and not on the contractile mechanism. When the ileal strips were pretreated with a high concentration of SP, SP effectively prevented the blockade by phenoxybenzamine. On the other hand, when the strip was pretreated with bradykinin or serotonin, the response to SP after phenoxybenzamine treatment was unaltered. The concentration of phenoxybenzamine used in the present study (20 μM) produced greater than 60% reduction in the response to carbachol and serotonin (10 mM), as compared with 30–40% reduction in the response to SP. It appears, therefore, that the phenoxybenzamine effect

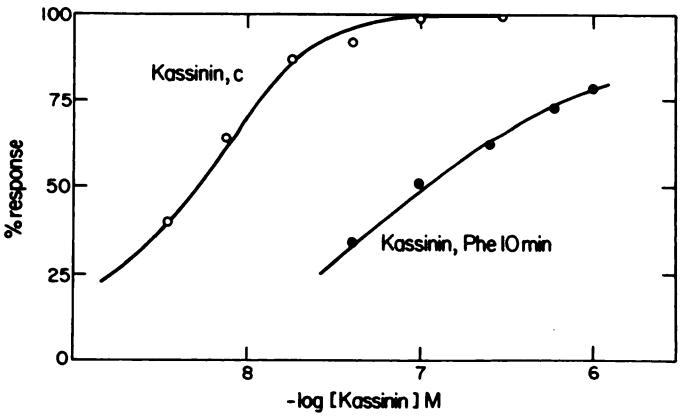

FIG. 6. *Effect of phenoxybenzamine (20 μM) on kassinin-induced contraction*

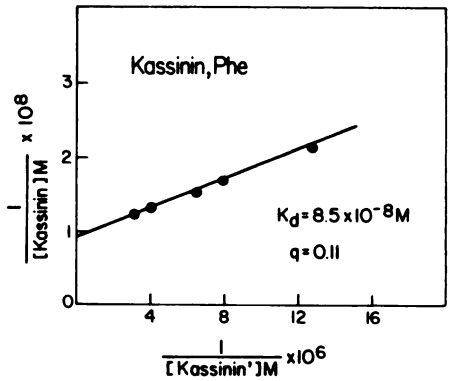

FIG. 7. *Double-reciprocal plot of the results illustrated in Fig. 6* K_d and q values were determined as in Fig. 2.

on the response of the ileum to SP is different from its effects on other classes of neurotransmitters. At present we are unclear as to the cross-protection of SP receptor by carbachol pretreatment. The measured K_d for SP was the same when the duration of the phenoxybenzamine treatment was 10, 13, or 15 min. Also, the K_d values for physalaemin were similar in atropinized and nonatropinized preparations.

We have previously studied SP-induced desensitization of SP receptors (15). It was shown that pretreatment with high concentrations of SP produced a reduced sensitivity to subsequent doses of the peptide. In addition, it was possible to shift the dose-response curve by more than 2 orders of magnitude without affecting the maximal response. In contrast, in the present study with phenoxybenzamine, a reduced maximal response was observed when the sensitivity of the receptor was reduced by 5-fold. Therefore, there are marked qualitative differences between the desensitization of SP receptors and the alkylation by phenoxybenzamine. Desensitization seems to reduce markedly the apparent affinity of SP receptor sites without reducing the maximal response, whereas phenoxybenzamine treatment reduces the apparent affinity of SP receptors and reduces its maximal response. These results are consistent with the idea that phenoxybenzamine irreversibly inactivates SP receptors.

Our results also indicate that kassinin, an SP E-type agonist (17), in contrast to SP, SP4–11, and physalaemin, may interact with sites different from those peptides. Whereas phenoxybenzamine causes a 5- to 10-fold shift in the dose-response curves for SP and SP analogues, there is a greater shift to the right for kassinin. In addition, the q value, which represents the fraction of active receptors remaining after phenoxybenzamine treatment, was 0.281 ± 0.015 for SP, but for kassinin this value was 0.098 ± 0.013. Although kassinin and SP cross-desensitize each other, our results suggest that some kassinin binding sites are not identical with SP receptors. Recently, Lee *et al.* (17) reported that, in contrast to SP, the contractile response to eledoisin (another SP E-type agonist) was partially reduced by atropine pretreatment and showed a different rate of recovery from SP-induced desensitization. They suggested that eledoisin may interact with distinct SP receptors. This is consistent with our results suggesting a distinct SP receptor subtype. However, additional evidence is needed to establish unequivocally the existence of multiple SP receptor subtypes in

the guinea pig ileum. Another possibility is that the COOH-terminal portion of kassinin (which is the active part) may bind to the SP receptor in a fashion similar to that of SP and the other peptides studied, but the NH_2 terminus may bind to a different subsite that is very sensitive to alkylation by phenoxybenzamine. This may explain why the same exposure to phenoxybenzamine inhibits the effects of kassinin more than the effects of SP. Additional experiments are needed to determine the reasons for this discrepancy. It should be pointed out that, if kassinin indeed binds to more than one receptor site, its K_d value as reported in Table 2 can only be viewed as the average, or apparent, K_d of kassinin, because the analysis method described by Furchgott (9–11) requires a homogeneous class of receptors.

In Table 2 the EC_{50} and the K_d value of SP are listed. Using the relationship $[RA]/R_t = A/(A + K_d)$, it can be calculated that for SP a 20% receptor occupation by SP is required to elicit a 50% response in this tissue.

To our knowledge, our study represents the first attempt to estimate the dissociation constants of SP and SP analogues in guinea pig ileum. As there are more than 20 peptides which may function as neurotransmitters in the central nervous system and periphery, our approach with phenoxybenzamine could also be used for estimating the dissociation constants of peptides which can elicit a graded concentration-dependent response. These studies are of interest because they could provide quantitative information about the affinity and physiological response of an agonist. In addition, knowing the relative affinity of an agonist series can help to identify the receptor type of another tissue. Recently, Tallarida and Cowan (18) have reported the affinity of morphine for its receptors *in vivo*, using a similar approach.

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