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Mediation by Prostaglandins of the Stimulatory Effect of Substance P on Cyclic AMP Production in Dog Iris Sphincter Smooth Muscle

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ABSTRACT. The purpose of the present study was to examine the mechanism of the stimulatory effect of substance P (SP) on cyclic AMP (cAMP) accumulation in dog iris sphincter. We found that: (1) SP increased cAMP accumulation in a time- and concentration-dependent manner, the $T_{1/2}$ and EC_{50} values being 1.2 min and 44 nM, respectively. SP has no effect on inositol trisphosphate and muscle contraction in this tissue. (2) SP-stimulated cAMP formation was inhibited by quinacrine, a non-specific phospholipase A_2 inhibitor (IC_{50} = 9.5 μM), and by indomethacin (Indo), a cyclooxygenase inhibitor (IC_{50} = 3.5 nM), in a concentration-dependent manner, suggesting that SP induces cAMP accumulation via an Indo-sensitive pathway. (3) SP-induced arachidonic acid release and SP-induced prostaglandin E_2 (PGE_2) release were inhibited concentration dependently by quinacrine and Indo, with IC_{50} values of 11 μM and 0.8 nM, respectively. (4) PGE_2 (1 μM) increased cAMP formation in the sphincter muscle by 94%, and, furthermore, the PG, but not SP, stimulated the activity of adenylyl cyclase in membrane fractions isolated from this tissue. (5) Indo (1 μM) blocked the relaxing effect of SP (1 μM) in iris sphincter precontracted with carbachol (1 μM). (6) The inhibitory effect of Indo on SP-induced cAMP accumulation was species specific. Increases in cAMP represent a mechanism by which extracellular SP can regulate smooth muscle function. Thus, we conclude from these studies that in dog iris sphincter SP-induced cAMP accumulation is mediated through PGs, and that in this cholinergically innervated muscle SP via cAMP could function, in part, to modulate the physiological responses to muscarinic receptor stimulation. BIOCHEM PHARMACOL 52;8:1261–1269, 1996.

KEY WORDS. substance P; dog iris sphincter; phospholipase A_2; arachidonic acid release; prostaglandin E_2 release; indomethacin; quinacrine; cAMP

SP,† which is currently the best characterized of the tachykinins, is an 11 amino acid peptide that belongs to the tachykinin family of neuropeptides. It is produced by neurons in the central and peripheral nervous system where it is mostly localized in the nerve terminals [1, 2]. Extensive research has been performed on the pharmacological effects of tachykinins, especially SP. Neurotransmitter, neuromodulator, and other reported functions of SP include contraction of smooth muscle, stimulation of secretion, neurotransmitter effects, pain transmission (nociception), vascular effects, immunomodulation, and cell proliferation [3, 4].

In the past few years, the molecular cloning of complementary DNAs encoding rat and human SP receptors has been accomplished [5, 6], and it is well established now that this receptor is a member of the GTP binding protein-coupled receptor family. Studies on the SP receptor stably expressed by DNA transfection into CHO cells and examined for its ability to stimulate IP_3 and cAMP formation revealed that SP activates the production of both second messenger molecules in a concentration-dependent manner [7]. In a similar study Nakajima *et al.* [8] reported that in CHO cells expressing three different tachykinin receptors the stimulation of cAMP accumulation is much less efficient in all three receptors and requires about one order of magnitude higher of the peptides than that required for the IP_3 production. SP-induced cAMP accumulation is not a consistent observation in various cells and tissues where the SP receptor is expressed, although it has been observed in certain tissues such as brain [9], iris sphincter smooth muscle, depending on the species [10], and CHO cells expressing SP receptors [7, 8]. In this connection, several years ago Maggio [2] concluded that a direct adenylate cyclase response to a tachykinin had not yet been convincingly demonstrated; however, this conclusion should now be modified to take into account the more recent work done with the cloned receptors [7, 8].

In the eye, SP is ubiquitous in all ocular tissues, and its

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† *Abbreviations:* SP, substance P; IP_3, inositol 1,4,5-trisphosphate; cAMP, cyclic AMP; AA, arachidonic acid; PG, prostaglandin; Indo, indomethacin; IBMX, 3-isobutyl-1-methyl-xanthine; TCA, trichloroacetic acid; and RIA, radioimmunoassay.

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distribution varies among different species [4, 11]. In rabbit eye, it is probably a peripheral sensory transmitter affecting a miosis [12], and it is a strong miotic, both when infused intracamerally or when applied to an isolated iris sphincter preparation [10, 13–15]. In iris sphincter, SP induces IP_3 production and contraction in a concentration-dependent manner in rabbit, bovine, and pig, and it induces cAMP accumulation in a concentration-dependent manner in dog, cat, and human but has no effect on IP_3 and contraction in these species [10]. In addition, SP induces relaxation in dog iris sphincter precontracted with carbachol, suggesting the involvement of cAMP [10]. Very recently, we found that endothelin-1-stimulated cAMP accumulation in iris sphincter [16] and in ciliary muscle [17] isolated from different mammalian species is mediated by the enhanced release of endogenous PGs. In the present study, we report that SP induced cAMP formation in dog iris sphincter, and that this activation of adenylyl cyclase by the neuropeptide was mediated by the enhanced production of endogenous PGs.

MATERIALS AND METHODS

Materials

In general, the methodology used here is the same as described previously [16–19]. Chemicals used were obtained from the following sources: SP, Indo, quinacrine (mepacrine), and IBMX (Sigma Chemical Co., St. Louis, MO); PGs (Cayman Corp., Ann Arbor, MI); ^{125}I-PGE_2 and ^{125}I-cAMP RIA kits (Advanced Magnetics, Boston, MA); and [^{3}H]AA (100 Ci/mmol) (New England Nuclear, Boston, MA). All other chemicals were of reagent grade.

Animals and Preparation of Iris Sphincter

Dog eyes were obtained through the courtesy of the Richmond County Animal Control (Augusta, GA). Generally, we obtained the eyes within 1 hr after the animals were killed. Eyes were enucleated immediately after death and were transported to the laboratory packed in ice. The iris sphincter was dissected out and placed in modified Krebs–Ringer bicarbonate buffer of the following composition (mM): NaCl, 118; $NaHCO_3$, 25; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.25; D-glucose, 10. The oxygenated Krebs–Ringer bicarbonate buffer was used as the incubation medium in the following studies. The pH of the buffer was adjusted and maintained at 7.4 with 97% O_2/3% CO_2. In general, each muscle was cut into four equal strips, one strip served as a control and the other as experimental. We routinely tested the contractility of the muscle with carbachol before the effects of SP on the pharmacological responses were investigated.

Incubation and Assay of cAMP

Muscles were preincubated in 1 mL buffer containing 0.1 mM IBMX for 10 min at 37°. Then SP was added and incubation continued for another 5 min or as indicated. Indo, a cyclooxygenase inhibitor, and quinacrine, a nonspecific phospholipase A_2 inhibitor, were added 10 min prior to the addition of SP. The incubations were stopped by the addition of 1 mL of 10% (w/v) TCA. After appropriate dilutions of the supernatant, cAMP in the sample was succinylated and then assayed by RIA [20].

Assay of Release of Endogenous PGE_2

Muscles were incubated in 1 mL of Krebs–Ringer bicarbonate buffer (pH 7.4) for 15 min at 37°. Then SP was added as indicated and after 5 min of incubation PGE_2 was assayed in the medium by RIA as described previously [21]. The amounts of PGE_2 in each sample were determined by interpolation from the standard curve. The rate of PGE_2 release is presented as the amount of PG/mg protein/5 min.

Assay of [^{3}H]AA Release

Muscles were first prelabeled with [^{3}H]AA; then the effects of SP on AA release were investigated. The muscles were incubated in 1 mL buffer containing 1 μCi [^{3}H]AA/mL for 90 min at 37°. The tissues were washed five times with nonradioactive buffer, then incubated for an additional 15 min in the same buffer. Incubations with quinacrine were for 10 min. Then SP (1 μM) was added as indicated, and incubations were continued for an additional 5 min. The reactions were terminated by the addition of 10% formic acid. Then the acidified medium was extracted with ethyl acetate, and [^{3}H]AA in the extract was analyzed by radiochromatography as previously described [22].

Assay of Adenylyl Cyclase Activity in Dog Iris Sphincter Membranes

The methods of homogenization, and subcellular fractionation of microsomal preparations were essentially as described previously [19]. Adenylyl cyclase activity in the membrane fraction was assayed according to the procedure of Mittag *et al.* [23]. Briefly, the enzyme activity was determined in a total incubation medium of 250 μL that contained 60 mM sucrose, 80 mM Tris buffer (pH 7.4), 2 mM $MgCl_2$, 1 mM EDTA, 0.2 mM EGTA, 5 mM creatine phosphate, 125 μg creatine phosphokinase, 20 μM GTP, 2 mM IBMX, 0.2 mM ATP, 2.5 μM Indo, and membrane suspension (15–25 μg protein). Incubations were carried out at 37° for 10 min, and the reactions were stopped by the addition of 10% TCA. cAMP formation was determined in the TCA extract by RIA.

Measurement of Contraction Response in the Sphincter Muscle

For measurements of the contraction response, the muscles were mounted individually in separate organ baths (10 mL) containing oxygenated Krebs–Ringer bicarbonate buffer,

which was maintained at 37°. The tissue was allowed to equilibrate for 90 min under a resting tension of 50 mg. During this period, the medium was replaced with fresh buffer every 30 min. After equilibration of the tissue, the agonist was added, and changes in tension were monitored continuously with a Grass FT-03 force transducer connected to a Grass d.c. amplifier.

Determination of Proteins

Protein content was determined by the method of Lowry *et al.* [24] with bovine serum albumin as a standard.

Calculation of Data and Statistics

Results are expressed as means ± SEM. Values for cAMP are reported as picomoles per milligram of protein and for PGE_2 as nanograms per milligram of protein. The EC_{50} value is defined as that concentration of the agonist that produces 50% of maximum response. Statistical differences between the two means were determined by a paired Student's *t*-test. When P was <0.05, the values were considered to be significantly different.

RESULTS

Time Course of SP-induced cAMP Formation

Addition of 1 μM SP increased cAMP formation in dog iris sphincter in a time-dependent manner with a $T_{1/2}$ value of 1.2 min (Fig. 1). The peptide induced a 30% increase in cAMP accumulation by 1 min and reached about 83% increase within 5 min. Based on these observations, in the experiments described below we used 5-min incubations with the agonist.

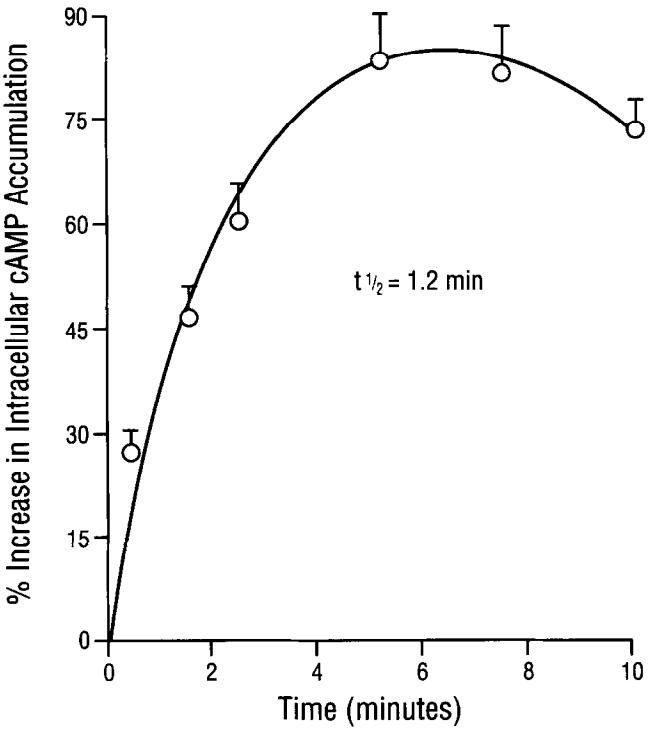

FIG. 1. Time–course of SP-induced cAMP formation in dog iris sphincter. Muscles were preincubated in Krebs–Ringer bicarbonate buffer (pH 7.4) that contained 0.1 mM IBMX for 10 min at 37°. Then SP (1 μM) added, and incubations were continued for various time intervals as indicated. cAMP was determined in the TCA-soluble extracts by RIA as described in Materials and Methods. The basal value for cAMP formation in the dog sphincter was 268 ± 30 pmol/mg protein. The results are means ± SEM, N = 12.

Concentration–Response Effect of SP on Intracellular cAMP Formation

SP-stimulated cAMP formation was concentration dependent (10^{-9}–10^{-5} M) with an EC_{50} of 4.4×10^{-8} M (Fig. 2). Maximal increase in cAMP accumulation was obtained at 1–10 μM concentrations of the peptide.

Concentration–Response Effect of Indo on SP-Induced cAMP Formation

Indo, a cyclooxygenase inhibitor, inhibited SP-stimulated cAMP formation in a concentration-dependent manner (10^{-9}–10^{-6} M) with an IC_{50} of 3.5 nM (Fig. 3). This finding suggests involvement of endogenous PGs in the SP-induced cAMP formation.

Concentration-Response Effect of SP on PGE_2 Release

SP increased PGE_2 release in a concentration-dependent manner (10^{-9}–10^{-5} M) with an EC_{50} of 80 nM (Fig. 4). Maximal increase in PGE_2 release was seen at 1–10 μM

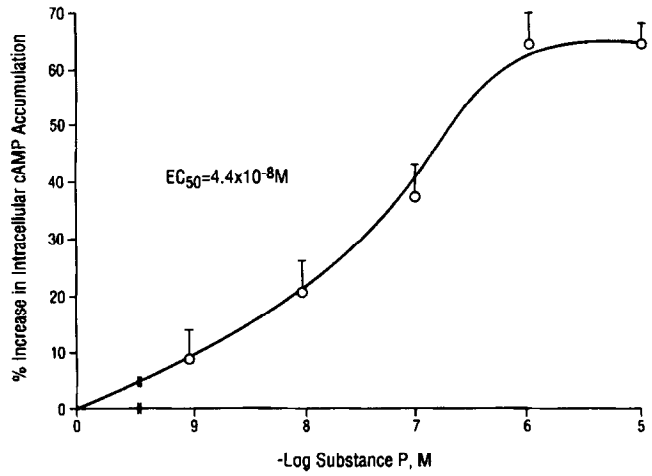

FIG. 2. Concentration–response effects of SP on intracellular cAMP formation. Incubation conditions were the same as described under Fig. 1 except that different concentrations of SP were added as indicated and the time of incubation with SP was 5 min. The basal value for cAMP formation in the iris sphincter was 312 ± 32 pmol/mg protein. The results are means ± SEM, N = 13.

concentrations of the neuropeptide. SP also increased $PGF_{2\alpha}$ release (data not shown).

Concentration–Response Effect of Indo on SP-Induced PGE_2 Release

Indo inhibited SP-induced PGE_2 release in a concentration-dependent manner with an IC_{50} of 0.8 nM (Fig. 5). At 10 nM, Indo completely abolished the stimulatory effect of the neuropeptide on PGE_2 release.

Effects of Various PGs on cAMP Formation

If PGs do mediate the stimulatory effects of SP on cAMP formation in dog iris sphincter, then one must assume that this occurs through activation of adenylyl cyclase by the eicosanoids. As can be seen from Table 1, PGE_2 and PGD_2 increased cAMP formation in a concentration-dependent manner. At 1 μM, PGE_2 and PGD_2 increased cAMP formation by 94 and 66%, respectively. In contrast, $PGF_{2\alpha}$ had little effect on cAMP formation in this tissue.

Concentration–Response Effect of Quinacrine on SP-Induced cAMP Formation

Quinacrine, a non-specific phospholipase A_2 inhibitor, inhibited SP-induced cAMP formation in a concentration-dependent manner with an IC_{50} of 9.5 μM (Fig. 6). These results suggest involvement of phospholipase A_2 in the SP-induced cAMP formation.

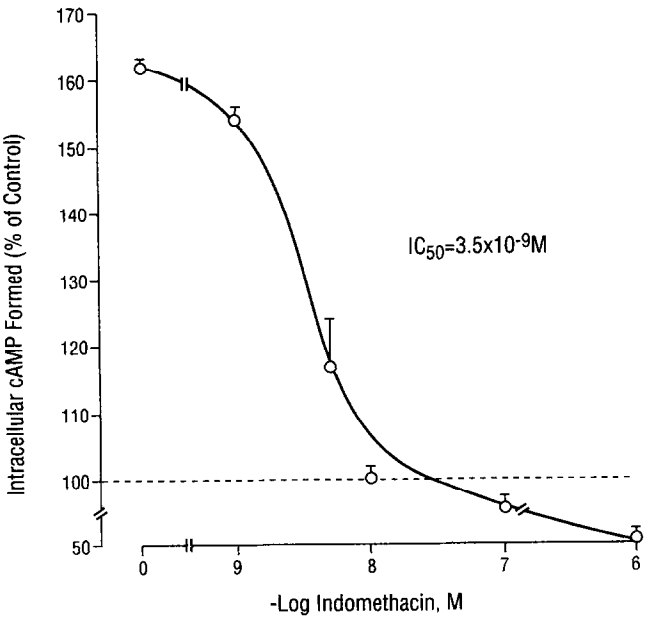

FIG. 3. Concentration–response effect of Indo on SP-induced cAMP formation. Incubation conditions were the same as described under Fig. 2 except that different concentrations of Indo were added 10 min before the addition of SP (1 μM). The time of incubation with SP was 5 min. The control value for cAMP (in the presence of 1 μM SP) was 465 ± 32 pmol/mg protein. The results are means ± SEM, N = 12.

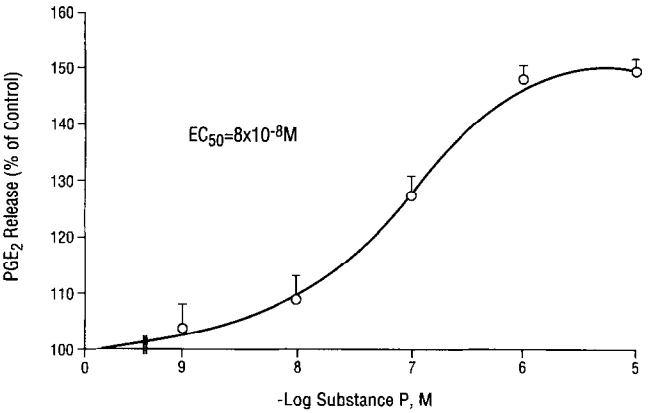

FIG. 4. Concentration–response effect of SP on PGE_2 release in dog iris sphincter. Muscles were incubated in 1 mL buffer for 15 min at 37°. SP was then added as indicated, and after 5 min of incubation PGE_2 was assayed in the medium by RIA as described in Materials and Methods. The basal value for PGE_2 release was 25 ± 2 ng/mg protein. The results are means ± SEM, N = 7.

Concentration–Response Effect of Quinacrine on SP-Induced AA Release

It is well established that PG formation is preceded by a lipolytic process to release free AA from membrane phospholipids. To investigate the mechanism of action of SP on AA release, the muscle was prelabeled with [^{3}H]AA, and the effects of SP and quinacrine on the release of radioac-

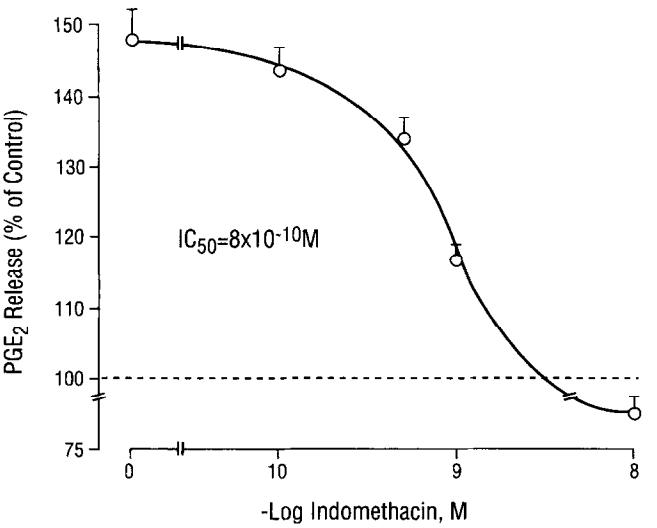

FIG. 5. Concentration–response effect of Indo on SP-induced PGE_2 release in dog iris sphincter. Incubation conditions were the same as described under Fig. 4 except that the tissue was preincubated with different concentrations of Indo as indicated for 10 min and then incubated with SP (1 μM) for 5 min. The control value for PGE_2 (in the presence of 1 μM SP) was 37 ± 2.5 ng/mg protein. The results are means ± SEM, N = 10.

TABLE 1. Effects of PGs on intracellular cAMP formation in dog iris sphincter

Additions	Concentration (μM)	cAMP formation (% of control)
PGE_2	0.1	156 ± 9*
	1	194 ± 6*
	10	221 ± 17*
PGD_2	0.1	152 ± 11*
	1	166 ± 8*
	10	190 ± 8*
$PGF_{2\alpha}$	0.1	102 ± 2
	1	109 ± 4
	10	124 ± 8*

Dog iris sphincters were incubated in the presence of different concentrations of PGs for 5 min at 37°. cAMP was determined in the TCA-soluble extracts by RIA as described in Materials and Methods. The basal value for cAMP formation was 300 ± 25 pmol/mg protein. The results are means ± SEM, N = 12.

* Significantly increased by PGs compared with the control incubation, $P < 0.05$.

tivity were determined by radiochromatography. As can be seen from Fig. 7, SP (1 μM) increased the release of [^{3}H]AA by about 31%, and this increase was blocked by quinacrine in a concentration-dependent manner with an IC_{50} of 11 μM. While these results could suggest the involvement of phospholipase A_2 in SP-induced AA release, caution should be exercised in the interpretation of results obtained using quinacrine, a non-specific phospholipase A_2 inhibitor [25, 26].

Effect of SP on Adenylyl Cyclase Activity in Membranes Isolated from Dog Iris Sphincter

To elucidate the mechanism by which SP stimulates cAMP formation in dog iris sphincter, we investigated the effects

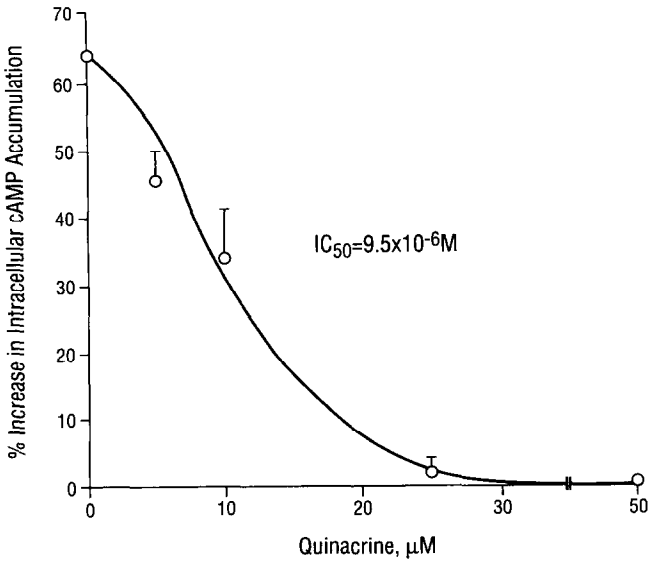

FIG. 6. Concentration–response effect of quinacrine on SP-induced cAMP formation in dog iris sphincter. Incubation conditions were as described under Fig. 2 except that different concentrations of quinacrine were added 10 min before the addition of SP (1.0 μM). Time of incubation with SP was 5 min. The basal value for cAMP formation in iris sphincter was 300 ± 25 pmol/mg protein. The results are means ± SEM, N = 12.

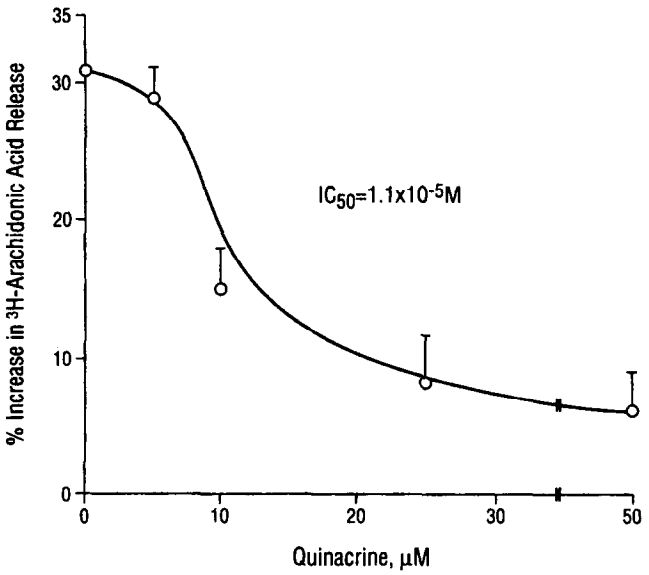

FIG. 7. Concentration–response effect of quinacrine on SP-induced [^{3}H]AA release by dog iris sphincter. Muscles were incubated in 1 mL Krebs–Ringer bicarbonate buffer (pH 7.4) that contained 1 μCi [^{3}H]AA for 90 min at 37°. The tissues were washed five times with nonradioactive buffer, then incubated for another 15 min in the same buffer. Different concentrations of quinacrine were added as indicated, and incubations were continued for 10 min. Then SP (1 μM) added, and incubations were continued for an additional 5 min. The incubations were terminated by the addition of 10% formic acid, and [^{3}H]AA released into the medium was analyzed by radiochromatography. The basal value for the release of [^{3}H]AA by iris sphincter was 3840 ± 90 dpm/mg protein. The results are means ± SEM, N = 12.

of this neuropeptide on adenylyl cyclase activity in microsomal fractions (Table 2). The activity of the enzyme was assayed in the presence of GTP (20 μM) and Indo (2.5 μM). SP was ineffective in stimulating the enzyme at both concentrations (1 and 5 μM) tested. On the other hand, the PGs and isoproterenol, which served as positive controls, stimulated significantly the activity of the enzyme. These results could suggest that SP receptors are not directly coupled to adenylyl cyclase in this tissue.

TABLE 2. Effects of substance P and other cAMP elevating agents on adenylyl cyclase activity in membranes isolated from dog iris sphincter

Additions	Adenylyl cyclase activity (% of control)
SP (1 μM)	100 ± 3
SP (5 μM)	110 ± 9
PGE_2 (1 μM)	142 ± 6*
PGD_2 (1 μM)	124 ± 8*
Isoproterenol (1 μM)	155 ± 14*

Adenylyl cyclase assay was performed as described under Materials and Methods. The basal value for the cyclase activity was 66 ± 8 pmol of cAMP formed/μg protein/10 min. The results are means ± SEM, N = 9.

* Significantly increased by the agonists compared with the control incubation, $P < 0.05$.

Effects of SP in the Absence and Presence of Indo on Carbachol-Induced Muscle Contraction

Since in smooth muscle cAMP is involved in muscle relaxation, it was of interest to determine whether SP could function to induce relaxation in the dog iris sphincter. Addition of SP (1 μM) in the absence of Indo inhibited carbachol-induced contraction by 25% (from 21.2 to 15.9 mg tension/mg wet weight tissue) (Fig. 8A). However, preincubation of the muscle with Indo (1 μM) inhibited the relaxing effect of SP on carbachol-induced muscle contraction (Fig. 8B). This observation suggests that SP-induced muscle relaxation may be mediated through the release of AA metabolites. Further studies on a concentration–response of Indo on SP-induced muscle relaxation were unsuccessful (data not shown). We also found that exogenous PGE_2 (1 μM) had little effect on carbachol-induced muscle contraction, whereas $PGF_{2\alpha}$ potentiated the contractile response in a concentration-dependent manner in this tissue (data not shown).

DISCUSSION

In dog iris sphincter, SP markedly stimulated the release of AA and PG synthesis, enhanced significantly the intracellular concentration of cAMP, and induced muscle relaxation. SP has no effect on IP_3 production and contraction in this smooth muscle [10]. The stimulatory effects of the neuropeptide on cAMP accumulation were blocked by quinacrine, a non-specific phospholipase A_2 inhibitor, and Indo, a cyclooxygenase inhibitor, implying that SP-induced cAMP formation in this tissue may be mediated through AA metabolites. This conclusion is supported by the following findings in the present study. First, SP increased cAMP accumulation in a time- and concentration-dependent manner, the $T_{1/2}$ and EC_{50} values being 1.2 min and 44 nM, respectively (Figs. 1 and 2). This EC_{50} value is more than 7-fold higher than the EC_{50} value reported from this laboratory for endothelin-1-induced cAMP accumulation in cat iris sphincter [16]. Second, the SP-stimulated cAMP formation was inhibited by quinacrine and by Indo in a concentration-dependent manner with IC_{50} values of 9.5 μM and 3.5 nM, respectively (Figs. 6 and 3). This IC_{50} value for Indo inhibition is more than 45-fold lower than the IC_{50} reported from this laboratory for Indo inhibition of endothelin-1-induced cAMP accumulation in cat iris sphincter [16]. The inhibitory effect of Indo on the peptide-induced cAMP production in iris sphincter was species specific (Table 3). In general, the IC_{50} values for Indo inhibition of SP- and endothelin-induced cAMP production were considerably lower in dog sphincters than cat sphincters, and furthermore they were lower for SP-induced cAMP production than for endothelin-induced cAMP production in both species (Table 3). Third, SP increased PGE_2 release in a concentration-dependent manner (EC_{50} = 80 nM) (Fig. 4), and this was inhibited by Indo in a concentration-dependent manner (IC_{50} = 0.8 nM) (Fig. 5). In addition, quinacrine inhibited SP-induced AA release in a concentration-dependent manner with an IC_{50} of 11 μM (Fig. 7). Many inhibitors of phospholipase A_2 have been reported in the literature [27]; however, none, including quinacrine, is a specific inhibitor of this enzyme. Eistetter *et al.* [28], working with recombinant bovine neurokinin-2 receptor stably expressed in CHO cells, reported that addition of neurokinin A to these cells stimulates both IP_3 production and cAMP accumulation. These authors concluded that the increased cAMP production is largely a secondary response and can be at least partially attributed to autocrine stimulation by endogenously generated eicosanoids, particularly PGE_2. Fourth, at 1 μM concentrations, PGE_2 and PGD_2, but not $PGF_{2\alpha}$, increased cAMP accumulation in the sphincter muscle by 94 and 66%, respectively (Table 1), and both PGE_2 and PGD_2, but not SP, stimulated the activity of adenylyl cyclase in membrane fractions isolated from the dog iris sphincter (Table 2), implying that the SP receptor is indirectly coupled to the activation of adenylyl cyclase. The observed differences in potencies of the PGs may not be due to the degradation of the PGs, although we

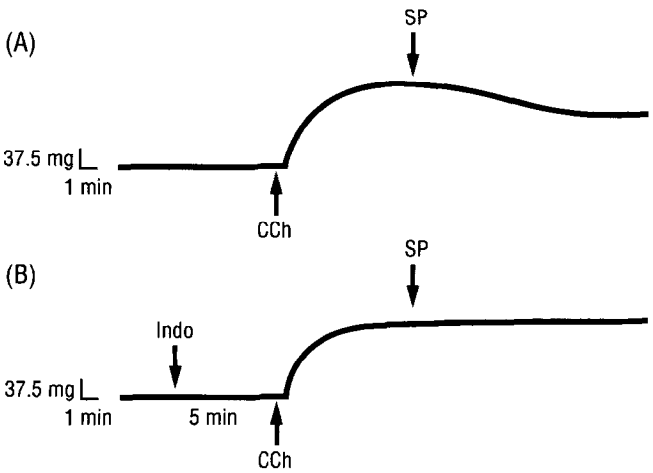

FIG. 8. Tracings showing the effects of SP in the absence (A) and presence (B) of Indo on carbachol (CCh)-induced contraction in isolated dog iris sphincter. The muscles were pre-equilibrated in buffer for 90 min. In (B) 1 μM Indo was added for 5 min; then the muscles were contracted by CCh (1 μM) for 4 min, followed by the addition of 1 μM SP.

TABLE 3. Summary of IC_{50} values for Indo inhibition of SP- and endothelin-induced cAMP production in dog and cat iris sphincters

	IC_{50} values for Indo inhibition of SP- and endothelin-induced cAMP production	
Additions	**Dog**	**Cat**
None	0.6 μM	1.1 μM
SP (1 μM)	3.5 nM	6 nM
Endothelin-1 (0.1 μM)	16 nM	160 nM*

* This value was taken from Ref. 16.

have not monitored this degradation. Thus, both PGE_2 and $PGF_{2\alpha}$ are released by SP. $PGF_{2\alpha}$, which has little effect on cAMP accumulation, is a potent agonist for muscle contraction (EC_{50} = 7.0×10^{-10} M) in this tissue [29], whereas PGE_2, which stimulates cAMP accumulation appreciably (Table 1), has a weak effect on muscle contraction (EC_{50} = 7.3×10^{-7} M). Fifth, Indo blocked the relaxing effect of SP in sphincter muscle precontracted with carbachol (Fig. 8), suggesting the involvement of PGs and cAMP in SP-induced muscle relaxation. The inability to obtain a concentration–response of Indo on SP-induced muscle relaxation in dog iris sphincter could be explained by the following findings. (a) The relaxing effect of SP is relatively small (Fig. 8A). This could be due to the fact that SP induces the release of several AA metabolites whose combined effects seem to relax the muscle. (b) SP-induced PGE_2 release and cAMP accumulation are extremely sensitive to Indo in this species (Table 3).

The data presented may be explained by the scheme given in Fig. 9. The physiological effects of PGE_2 are mediated through EP receptors. There are four EP receptor subtypes: EP_1, which is coupled to IP_3 production, Ca^{2+} mobilization and contraction; EP_2, which is coupled to an increased intracellular cAMP; EP_3, which is coupled to a decreased intracellular cAMP and inhibition of neurotransmitter release [30]; and EP_4, which is coupled to an increased intracellular cAMP [31]. SP interacts with the neurokinin receptor to activate phospholipase A_2, via a G protein, and releases AA and subsequently AA metabolites. The released PGE_2 then interacts with the EP_2 and EP_4 receptor subtypes to activate adenylyl cyclase and increase intracellular cAMP concentration, resulting in modulation of muscle responses. Prostanoid agonists known to be relatively selective for the EP_2 receptor subtype stimulate cAMP formation in the smooth muscles of the iris-ciliary body and induce relaxation [16, 22, 32, 33]. In dog iris sphincter, addition of PGE_2 had little effect on carbachol-induced muscle contraction; this could suggest other modulatory functions for SP-induced cAMP accumulation in this tissue. In smooth muscle, activation of various receptors, such as β-adrenergic and PGs, by cAMP-elevating agents results in a rise in intracellular cAMP concentration and subsequently leads to muscle relaxation. The mechanism of cAMP inhibition of agonist-induced smooth muscle contraction remains unclear [34–36].

In the present study, we used Indo to investigate the involvement of PGs in the observed effects of SP. We found that SP can exert its function in this smooth muscle through an Indo-sensitive pathway. Indo, in addition to cyclooxygenase inhibition, may inhibit cartilage cAMP phosphodiesterase [37], may interfere with the participation of Ca^{2+} in biological processes [38], inhibit cAMP-dependent protein kinases and endogenous phosphorylation [39], and inhibit [^{3}H]PGE_2 binding to human myometrial membranes [40], thromboxane binding to its receptor sites [41], and dilator prostanoid binding to prostacyclin receptors in cerebral microvascular smooth muscle cells [42]. Although in these studies higher concentrations of Indo were used (10^{-7} to 10^{-3} M) than in the present study (10^{-8} to 10^{-6} M), we cannot rule out other effects of the inhibitor in the iris sphincter in addition to its inhibition of the cyclooxygenase pathway.

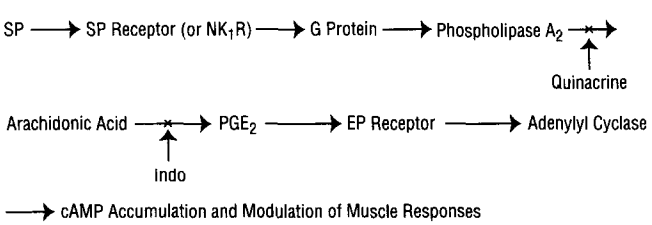

FIG. 9. Scheme showing the possible role of PGE_2 in mediating the stimulatory effects of SP on cAMP accumulation in dog iris sphincter smooth muscle.

There is little information about neurokinin receptors in ocular tissues. The presence of SP receptors has been reported in membrane fractions isolated from rabbit iris sphincter [10, 43]. The density of SP receptors in rabbit, bovine, and dog sphincters was found to be 227, 110.9, and 13.6 fmol/mg protein, respectively, and the K_d values were 1.9, 1.8, and 1.3 nM, respectively [10]. In human astrocytoma cells, which have a high density of functional SP receptors, saturation binding experiments yielded average values of K_d = 447 ± 103 pM, B_{max} = 862 ± 93 fmol/mg protein [44]. SP induced a robust inositol phosphate formation in these cells. In the iris sphincter there are species differences in the effects of SP, endothelins, and PGs on the generation of second messengers and contraction [10, 45, 46]. Thus, in contrast to endothelin-1 which activates phospholipases A_2, C and D in iris sphincters from different mammalian species [45, 46], in dog sphincter SP activated only phospholipase A_2 [the present work, and unpublished work of G. Marathe, S. Y. K. Yousufzai and A. A. Abdel-Latif]. Activation of phospholipase A_2, the cyclooxygenase, or PGE_2 synthase by the neuropeptide could result in an increase in PGE_2 formation.

To summarize, in dog iris sphincter SP binds to the neurokinin receptor to activate phospholipase A_2 and release AA; the latter is then converted into PGs that activate the EP receptor to stimulate adenylyl cyclase. Thus, increases in cAMP represent a mechanism by which extracellular SP can regulate smooth muscle function. SP-induced cAMP formation may function in this cholinergically enriched muscle to modulate the physiological responses to muscarinic receptor stimulation. However, further studies are needed in order to establish the precise role SP-induced PG release and cAMP accumulation play in the modulation of muscle responses in the iris of the eye. It must be emphasized that smooth muscle constitutes only a small percentage of the mass of the iris sphincter, thus leaving the possibility that these responses to SP may be occurring in some non-smooth muscle component of the preparation. Very recently, we succeeded in immortalizing with SV_{40} virus the cat iris sphincter smooth muscle cells [47], and studies

are underway to determine if the effects of SP on second-messenger production in these cells are comparable to those we observed with the intact tissue.

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