Neurokinin A-Stimulated Phosphoinositide Breakdown in Rabbit Iris Sphincter Muscle

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ABSTRACT — Specific [3H]-substance P binding was saturable and of high affinity (K_D = 2.5 nM) with a B_max of 725 fmol/mg protein in the isolated rabbit iris sphincter muscle. The competition for [3H]-substance P binding was in the order of eledoisin > substance P > kassinin > neurokinin B > neurokinin A > physalaemin. In the same preparation, neurokinin A, as well as substance P induced a concentration-related accumulation of [3H]-inositol phosphates (IPs), and the maximum increase was about 200% of the control at 10^-4 M. [D-Arg^1, D-Trp^{7,9}, Leu^{11}]-substance P (SP) and [D-Pro^2, D-Trp^{7,9}]-SP (10^-3 M) inhibited substance P or neurokinin A (10^-4 M)-induced phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis significantly. [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP (10^-3 M) also inhibited neurokinin A (10^-4 M)-induced PIP_2 hydrolysis significantly. Neurokinin A and substance P produced concentration-related contractions in normal Ca^{2+}-containing medium. The contractile response was weaker in Ca^{2+}-free medium, and there was no response in 0.2 mM EGTA medium. In Ca^{2+}-free medium, the basal level of [3H]-IPs accumulation was smaller than that in normal medium, and neurokinin A and substance P significantly increased PIP_2 hydrolysis. In the 0.2 mM EGTA containing medium, neurokinin A and substance P did not stimulate the PIP_2 hydrolysis. These results suggest that in the rabbit iris sphincter muscle, there are tachykinin receptors linking to PIP_2 hydrolysis and Ca^{2+} mobilization and that these mechanisms underlie the mechanism for the neurokinin A-induced contractile response, as well as the substance P-induced one.

Keywords: Neurokinin A-stimulated PI turnover, Neurokinin A-induced contractile response, Iris sphincter muscle (rabbit), substance P antagonists, [3H]-substance P binding

The rabbit iris sphincter muscle, which is highly sensitive to tachykinins, is innervated by substance P-like immunoreactive, trigeminal nerves (1), and transmural electrical stimulation of this preparation causes a nonadrenergic, noncholinergic contraction (2, 3). This nonadrenergic and noncholinergic response was inhibited by substance P-antagonists, and we suggested that the response was mediated by substance P or substance P-like peptide released from the trigeminal nerve (4–6). Substance P and neurokinin A which both exist in the rabbit iris sphincter muscle (7) produced concentration-related contraction in the same muscle (6); however, the contractile response to neurokinin A was more effectively inhibited by [D-Pro^2, D-Trp^{7,9}]-substance P (SP) (10^-5 M) than the response to substance P, and [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}]-SP (10^-5 M) inhibited the contractile response to neurokinin A without affecting the response to substance P (6). Other pharmacological data indicate that the neurokinin A-induced contraction, rather than the substance P-induced one, is similar to the transmural, electrical stimulation-induced one (8).

Substance P and related tachykinins stimulate phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis in a variety of tissues, including rat parotid gland (9), guinea pig ileum longitudinal muscle, rat hypothalamus (10), rat ileum (11), hamster urinary bladder (12) and a guinea pig ileum longitudinal muscle-myenteric plexus preparation (13). Substance P stimulates PIP_2 hydrolysis and induces the contractile response in rabbit iris
sphincter muscle, and both are blocked by \([\text{D-Pro}^2, \text{D-Trp}^7, 9]-\text{SP}\) (14).

We have therefore investigated whether rabbit iris sphincter muscle tachykinin receptors which are activated by neurokinin A are coupled to \(\text{PIP}_2\) hydrolysis. We have also done radioligand binding studies using \([\text{H}]\text{-substance P}\) in the same preparation.

**MATERIALS AND METHODS**

**Tissue preparation and phospholipid labeling**

Albino rabbits of either sex weighing 2.0 to 3.0 kg were anesthetized with sodium pentobarbital (25 mg/kg i.v.), exsanguinated and then the eyes were enucleated immediately. One strip of iris sphincter muscle was prepared from each eye according to Fujiwara et al. (15) and then cut into 4 portions. The slices were dispersed in Krebs buffer (120 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 25 mM NaHCO\(_3\), and 11.7 mM glucose) equilibrated to pH 7.4 with 95% O\(_2\)/5% CO\(_2\), and incubated at 37°C for 1 hr in Krebs buffer containing 0.3 \(\mu\)M \([\text{H}]\text{-inositol}\) to label the phospholipid pool.

**Assay of \([\text{H}]\text{-labeled inositol phosphates**

Following phospholipid labeling with 0.3 \(\mu\)M \([\text{H}]\text{-inositol}\) under oxygenated conditions for 60 min, the slices were washed with an excess volume of fresh buffer containing 5 mM \text{myo-inositol} to remove free \([\text{H}]\text{-inositol}\) and then incubated at 37°C in 240 \(\mu\)l of Krebs buffer containing 10 mM LiCl, previously equilibrated with 95% O\(_2\)/5% CO\(_2\). Following addition of 10 \(\mu\)l of agonists (or buffer for determining basal breakdown), the reaction was allowed to continue for various intervals under a continuous gas flow of the appropriate composition. The reaction was stopped by adding 0.9 ml of chloroform/methanol (1:2 vol./vol.) solution. Subsequent processes included phase separation, elution of the inositol phosphate (IP) fraction, and measurements of radioactivity and protein concentrations according to the method described previously (16). In some experiments, the reaction was stopped by adding 1 ml of 10% (wt./vol.) trichloroacetic acid, and \([\text{H}]\text{-IPs}\) were extracted with anhydrous diethyl ether neutralized with NaOH. The data obtained were essentially the same between the two extraction procedures.

**Membrane preparation for binding assay**

The iris sphincter muscles were minced with scissors and homogenized in 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) with a glass homogenizer. The homogenates were filtered through two layers of gauze, rehomogenized at setting 10 on a Polytron with a 20-sec burst and centrifuged at 1,000 \(\times\) g for 10 min; Then the supernatant was carefully removed and centrifuged at 100,000 \(\times\) g for 60 min. The resulting pellet was resuspended in the 50 mM Tris-HCl buffer. Protein concentration was determined by the method of Lowry et al. (17).

**Binding assay**

The \([\text{H}]\text{-substance P}\) binding assay with the membrane fraction was performed as described elsewhere (18). Briefly, crude membrane fractions were suspended in 50 mM Tris-HCl buffer (pH 7.4) containing 40 \(\mu\)g/ml bacitracin, 4 \(\mu\)g/ml leupeptin, 2 \(\mu\)g/ml chymostatin, 10 \(\mu\)M captopril, 10 \(\mu\)M phosphoramidon, 10 mM MgCl\(_2\), and 0.02% bovine serum albumin to a concentration of 2.5 mg protein/ml. \([\text{H}]\text{-Substance P}\) bindings were performed by incubating aliquots of the membrane fractions at a temperature of 25°C for 60 min in 250 \(\mu\)l of the buffer containing \([\text{H}]\text{-substance P}\) in the presence or absence of unlabeled peptides. The assay was terminated by the addition of 3 ml of the ice-cold buffer and the rapid filtration through Whatman GF/C glass fiber filters (presoaked with 0.1% polyethyleneimine), under suction. Nonspecific binding was determined by carrying out incubations in the presence of unlabeled peptide (10\(^{-6}\) M). The specific binding was calculated by subtracting nonspecific binding from total binding.

**Contractile responses**

One strip of the iris sphincter muscle was prepared from each eye according to the description of Fujiwara et al. (15) and then mounted in a 10-ml bath containing Krebs solution gassed with 95% O\(_2\) and 5% CO\(_2\). The temperature of the bath was maintained at 37 ± 0.5°C. The free end of the preparation was connected to a force-displacement transducer, and the isometric tension was recorded on an ink-writing oscillograph. A resting tension of about 150 mg was allowed to equilibrate for about 90 min in Krebs solution before starting the experiments.

**Data analyses**

Data presented in the text are means ± S.E.M. from \(n\) experiments, each performed in duplicate or triplicate. Statistical analyses were done by analysis of variance.

**Materials**

\text{myo-[2-\text{H}]inositol (14 Ci/mmol) and [\text{H}]substance P (55 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). Dowex-1 anion exchange resin (100–200 mesh; X8 in the formate form)}
was obtained from Bio-Rad (Richmond, CA, U.S.A.). Unlabeled peptides used in this study, [D-Pro², D-Trp⁷⁹]-SP, [D-Arg¹, D-Trp⁷⁹, Leu¹¹]-SP, [D-Arg¹, D-Pro², D-Trp⁷⁹, Leu¹¹]-SP, leupeptin and chymostatin were purchased from Peptide Institute, Inc., Osaka, Japan. Bacitracin was purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade and were obtained commercially.

RESULTS

Saturability and specificity of [³H]-substance P binding

The saturability of the [³H]-substance P specific binding on iris sphincter muscle membrane fractions was studied by increasing concentrations of [³H]-substance P (1–20 nM). Specific [³H]-substance P binding was saturable (Fig. 1). The nonspecific binding ranged from 30% to 40% of the total binding at saturation. A Scatchard plot of the data for specific [³H]-substance P binding produced a straight line, indicating that only a single population of specific binding sites was involved (Fig. 1 inset). The apparent dissociation constant (K_D) at equilibrium for substance P at these sites was 2.5 ± 0.3 nM (N = 4), and the maximum amount of [³H]-substance P bound at saturation (B_max) was 725 ± 43 fmol/mg protein (N = 4).

The specificity of [³H]-substance P binding was studied using unlabeled tachykinins (Fig. 2). The inhibition potency for the binding was eledoisin > substance P > kassinin > neurokinin B > neurokinin A > physalaemin. The IC₅₀ values obtained for these tachykinins were 1.2 nM, 2 nM, 4 nM, 5 nM, 13 nM and 30 nM, respectively.

Accumulation of inositol phosphates after tachykinin stimulation

Addition of substance P (10⁻⁴ M) and neurokinin A (10⁻⁴ M) for 60 min to rabbit iris sphincter muscle in the presence of lithium resulted in an accumulation of IPs.

Concentration-response relationships of accumulation of [³H]-IPs are shown in Fig. 3. The maximum increase in response to substance P and neurokinin A was about 200% of the control.

Effects of atropine and substance P-antagonists on agonist-stimulated phosphoinositide breakdown

Although 10⁻⁴ M carbachol stimulated phosphoinositide (PI) breakdown and 10⁻⁵ M atropine inhibited the carbachol-stimulated PI breakdown, 10⁻⁵ M atropine did not inhibit either 10⁻⁴ M substance P- or 10⁻⁴ M neurokinin A-stimulated PI breakdown (Fig. 4).

The substance P (10⁻⁴ M)-stimulated PI breakdown was inhibited significantly by 10⁻³ M [D-Arg¹, D-Trp⁷⁹, Leu¹¹]-SP and [D-Pro², D-Trp⁷⁹]-SP, but not by [D-Arg¹, D-Pro², D-Trp⁷⁹, Leu¹¹]-SP. The neurokinin A (10⁻⁴ M) stimulated-PI breakdown was inhibited significantly by these three antagonists (Fig. 5).

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**Fig. 1.** Saturation curve of [³H]-substance P binding to rabbit iris sphincter muscle. Membranes were incubated with increasing concentrations of [³H]-substance P (1–20 nM) in the presence or absence of 10⁻⁶ M unlabeled substance P, as described in Methods. Results from a typical experiment are shown. Inset shows a Scatchard plot. The regression line indicates a K_D of 2.2 nM and a B_max of 684 fmol/mg protein.
Fig. 2. Displacement of [3H]-substance P binding to rabbit iris sphincter muscle by various tachykinins. The tachykinins added together with [3H]-substance P (6 nM) were eleoodisin (■), substance P (□), kassinin (▲), neurokinin B (∆), neurokinin A (●), and physalaemin (○). Results are means of three experiments.

Fig. 3. Stimulation of accumulation of [3H]-inositol phosphates by 10^{-4} M neurokinin A (○) and 10^{-4} M substance P (●) for 60 min in rabbit iris sphincter muscle. Results are means ± S.E. of three experiments.

Fig. 4. Effects of atropine on carbachol-, neurokinin A- and substance P-stimulated phosphoinositide breakdown in rabbit iris sphincter muscle. Results are means ± S.E. of three experiments. Significantly different from the basal value: *P < 0.01.
**Effects of Ca^{2+} on agonist-stimulated contraction and PI breakdown**

Figure 6 shows that carbachol, substance P and neurokinin A induce the concentration-related contractile response in normal medium (2.5 mM Ca^{2+}). In the case of Ca^{2+}-free medium, the contractile response was smaller than that in normal medium. No contractile response was seen in 0.2 mM EGTA medium.

Figure 7 shows that the basal level of [3H]-IPs accumulation in Ca^{2+}-free or 0.2 mM EGTA medium is much smaller than that in normal medium and that substance P and neurokinin A still enhance PI breakdown significantly in Ca^{2+}-free medium, but not in 0.2 mM EGTA medium.

**Fig. 5.** Effects of substance P-antagonists on neurokinin A- and substance P-stimulated phosphoinositide breakdown in rabbit iris sphincter muscle. Blocker A: [D-Arg¹, D-Trp⁷⁹, Leu¹¹]-SP; blocker B: [D-Pro², D-Trp⁷⁹]-SP; blocker C: [D-Arg¹, D-Pro², D-Trp⁷⁹, Leu¹¹]-SP. Results are means ± S.E. of five experiments. Significantly different from substance P or neurokinin A: *P < 0.05; **P < 0.01.

**Fig. 6.** Effects of Ca^{2+} on contractile responses to carbachol, substance P and neurokinin A in rabbit iris sphincter muscle. ○: normal Krebs buffer, △: Krebs buffer without Ca^{2+}, ■: Krebs buffer containing 0.2 mM EGTA. Results are means ± S.E. of three to six experiments.
DISCUSSION

Specific binding studies for $[^3H]$-substance P in the membrane fraction of rabbit iris sphincter muscle demonstrated the characteristics of substance P receptors. The binding was saturable and of high affinity. Tachykinins examined competed for this binding with order of eledoisin > substance P > kassinin > neurokinin B > neurokinin A > physalaemin, and all these agents completely inhibited the $[^3H]$-substance P binding at concentration of $10^{-6}$ M. These data suggest that there are tachykinin receptors in the rabbit iris sphincter muscle. The rank order of this inhibitory potency does not fit the order of NK₁, NK₂ and NK₃ receptor subtypes detected based on biological activities (19). Tachykinin receptors in the iris sphincter muscle may have a unique selectivity to tachykinins or more than two types of receptors may exist in this muscle. Our previous pharmacological studies on tachykinin receptor characterization in the same preparation showed that the rank order of contraction-inducing potency was eledoisin > neurokinin B = physalaemin > substance P > neurokinin A (8). Although there was some discrepancy for the rank order of potency between ligand binding and pharmacological activities, the most effective and potent tachykinin tested on rabbit iris sphincter muscle was eledoisin, and substance P was more potent than neurokinin A. Further studies on tachykinin receptor subtypes using $[^3H]$-neurokinin A in the same preparation are required.

The mechanism underlying the transduction of neurotransmitter information at muscarinic cholinergic and $\alpha$-adrenergic receptors is thought to be a turnover of membrane phosphoinositides (20, 21). Activation of such receptors is followed by rapid hydrolysis of PIP₂ by phospholipase C, producing at least two putative second messengers, diacylglycerol (DG) and IP₃. The latter induces Ca²⁺ mobilization and an increase in cellular activity (21, 22). The sphincter muscle of rabbit iris is enriched in muscarinic cholinergic receptors (23) and activation of muscarinic cholinergic receptors in this preparation leads to a rapid breakdown of PIP₂ into DG and IP₃ (24). It has been suggested that PIP₂ hydrolysis and the derived second messengers, IP₃ and DG, are involved in excitation-contraction coupling in the rabbit iris sphincter muscle (25, 26). In the present experiments, carbachol induced contraction and PIP₂ hydrolysis, and neither contraction nor PIP₂ hydrolysis occurred in 0.2 mM EGTA medium. These results are in good agreement with the previous reports (25–27).

In rabbit iris sphincter muscle, it has been reported that [D-Pro², D-Trp⁷,⁹]-SP inhibited the substance P-induced (6, 14) or neurokinin A-induced (6) contractile responses and transmural electrical stimulation-produced contraction (5, 6), and that [D-Arg¹, D-Pro², D-Trp⁷,⁹, Leu¹¹]-SP did not inhibit the substance P-
induced contractile response, but inhibited the neurokinin A-induced contractile response and transmural electrical stimulation-induced contraction (6). The biochemical results that [D-Pro^2, D-Trp^7,9]-SP inhibited the substance P-stimulated (ref. 14, and this study) and neurokinin A-stimulated PIP_2  hydrolysis (in this study) and that [D-Arg^1, D-Pro^2, D-Trp^7,9, Leu^11]-SP did not inhibit the substance P-stimulated PIP_2 hydrolysis but inhibited the neurokinin A-stimulated PIP_2 hydrolysis in rabbit iris sphincter muscle (in this study) are in good agreement with the pharmacological results of the above-mentioned reports, although the concentrations of agonists and antagonists in this study were higher than the former reports. [D-Arg^1, D-Trp^7,9, Leu^11]-SP (spantide) which is thought to be a tachykinin antagonist (28) also inhibited the substance P- and neurokinin A-stimulated PIP_2 hydrolysis in the present investigation. These results suggest that there are tachykinin receptors linked to PIP_2 hydrolysis in the rabbit iris sphincter muscle.

Substance P and neurokinin A produced contraction and stimulated PIP_2 hydrolysis in the rabbit iris sphincter muscle, and such contraction and stimulation of PIP_2 hydrolysis did not occur in EGTA-containing medium. Although definite conclusions must await more extensive experiments, the present results support the view that receptor-mediated PIP_2 hydrolysis and the subsequent intracellular Ca^{2+} mobilization are involved in the contractile response to neurokinin A, a probable transmitter in the sensory, trigeminal nerve fibers in the rabbit iris sphincter muscle.

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