Some Characterization of the Responses to Substance P and Other Tachykinins in Rabbit Iris Sphincter Muscle

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Abstract—Contractile responses to substance P, physalaemin and eledoisin, three members of the tachykinin family, were compared and characterized in rabbit iris sphincter smooth muscle. Eledoisin and physalaemin were approximately 5 times more potent than substance P, and the maximum responses to substance P and physalaemin were about 85 percent of those to eledoisin and carbachol. The contractile responses to the three tachykinins were not affected by tetrodotoxin \((3 \times 10^{-6} \text{ M})\) and atropine \((10^{-6} \text{ M})\). The contractions induced by substance P and physalaemin were well sustained even after they were thoroughly washed out, whereas the eledoisin-induced contraction was rapidly ceased by removing the agonist from the bathing medium. The sustained contraction evoked by substance P or physalaemin was strongly dependent on extracellular calcium ions. Phenoxybenzamine \((2 \times 10^{-5} \text{ M}, 10 \text{ min})\) selectively attenuated the response to eledoisin, but not substance P or physalaemin, and concomitant incubation with excess eledoisin \((10^{-7} \text{ M})\) significantly prevented the inhibitory effect of phenoxybenzamine. The difference between responses to eledoisin and to the other peptides, substance P and physalaemin, may suggest the existence of two different receptor subtypes for tachykinins in rabbit iris sphincter smooth muscle.

Nerve fibers containing substance P-like immunoreactive substance(s) are widely distributed in the body and seem to innervate autonomic ganglia, vasculatures and smooth muscles \((1, 2)\). The presence of substance P-like immunoreactive substance(s) in primary sensory neurones has lent support to the view that it is associated with sensory nerve conduction, probably as a neurotransmitter, and that it is a causative factor in the irritative response to antidromic stimulation of sensory neurones \((3–7)\).

On stimulation of the fifth cranial nerve and chemical or mechanical irritation of the eye, some irritative ocular responses occur, such as prolonged miosis, conjunctival and iridial hyperemia, breakdown of the blood-aqueous barrier and an increased intraocular pressure \((8)\). The causative relationships between substance P and these irritative responses have been debated by many investigators \((5, 9)\), and substance P may play an important pathophysiological role in at least miosis induced by some noxious stimuli resulting in trigeminal nerve stimulation in the rabbit eye \((5, 6, 10)\). In this tissue, it has been reported that direct application of substance P or electrical transmural stimulation produced slow contraction which was inhibited by a substance P antagonist, but not atropine, and that pretreatment with capsaicin or tetrodotoxin greatly attenuated the slow contraction produced by electrical transmural stimulation \((11–14)\).

Recently, many investigators have suggested the existence of multiple receptors for substance P which is a member of the tachykinin peptide family \((15–17)\). On the other hand, existences of tachykinin peptides other than substance P, such as substance K, have been shown \((18–20)\).
The receptor(s) for substance P and other tachykinins in rabbit iris sphincter, however, has not been characterized. In the current study, we compared and characterized the responses to three tachykinin peptides, i.e., substance P, physalaemin, and eledoisin, in rabbit iris sphincter muscle, and we have reported the possibility that substance P or physalaemin and eledoisin can elicit differential effects on this tissue through activations of distinct receptor subtypes.

Materials and Methods

Isolated tissue assays: Male albino rabbits weighing 2–3 kg were used. Each animal was allowed free access to food and water, and maintained on a 12 hr light cycle.

After sacrifice by exsanguination, an eyeball was removed and the iris was rapidly dissected in aerated physiological saline solution (PSS) of the following composition in mM: NaCl, 154.0; KCl, 5.6; MgCl₂, 2.1; CaCl₂, 2.2; NaHCO₃, 6.0 and glucose, 2.8 (21). In Ca-free, EGTA-containing solution, there was 1 mM EGTA (pH 7.4 with Tris base) and no added Ca. One strip of iris sphincter cut open from each eye was mounted in a silanized bath of 20 ml capacity which contained aerated PSS. The temperature of the bath was maintained at 32°C. One end of the preparation was tied to a holder made of polyacrylate, and the other was connected to a force-displacement transducer by a light weight silk thread. The preparation was equilibrated for 90 min with an initial passive tension of about 1.5 mN, and after stress-relaxation, a resting load of about 0.5 mN was maintained in the following experimental protocol. The tissue was exposed several times to approximately the ED₅₀ dose (10⁻⁶ M) of carbachol, and then cumulative dose-response curves were determined until stable curves were obtained (usually 3 times). Thereafter, tachykinin peptide, each on one strip, was applied by a cumulative or noncumulative (single dose) technique. Solutions of the peptides were injected into a bath by a Gilson Pipetman P-20 or P-200 with a polypropylene tip.

To alkylate the receptors in these tissues, after the control response to one of the tachykinins was obtained, 2×10⁻⁵ M phenoxybenzamine was added and left in contact with the tissue for 10 or 30 min. The tissues were then washed repeatedly for 60 min every 10 min, after which time the treated response to the tachykinin was observed. In an attempt to protect receptors against alkylation by phenoxybenzamine, eledoisin was added in excess (10⁻² M), 2 min prior to the addition of phenoxybenzamine. Both were left in contact with the tissue for 10 min. Then the tissue was washed repeatedly every 10 min for a further 60 min.

Treatment of data: Relative potencies and intrinsic activities were calculated from the pD₂ (negative logarithm of a concentration of each agonist producing 50% of the maximum obtainable response to the agonist) and the ratio of the maximum response by each agonist to that by 10⁻⁵ M carbachol, respectively. To assess the effects of drugs on the contraction by the peptides, Student's t-test was performed on the percentage of the treated response to the control response.

Drugs: The following peptides and other agents were used: synthetic substance P (Sigma), eledoisin and physalaemin (Penninsula Labs.), carbachol chloride (Sigma), phenoxybenzamine hydrochloride (Tokyo Kasei, Co., Ltd.), atropine sulfate (Sigma), physostigmine salicylate (E. Merck), tetrodotoxin (Sankyo, Co., Ltd.), EGTA (glycoletherdiamine tetraacetic acid, Dojin Laboratory) and Tris (tris(hydroxymethyl)-aminoethane, Sigma). Substance P and physalaemin were prepared in 0.9% (w/v) NaCl solution containing 10 mM acetic acid and stored in a silanized vial at −20°C. Eledoisin was dissolved in 0.9% NaCl solution. Deionized and distilled water was used in all experiments.

Results

The isolated iris sphincter responded with contraction to all three tachykinin peptides used in this study. The cumulative dose-response curves for substance P, physalaemin, eledoisin and carbachol are shown in Fig. 1, and the pD₂ values and intrinsic activities of the agonists were calculated and shown in Table 1. Tension elicited by the maximum dose (10⁻⁵ M) of carbachol above
the resting level was 4.12±0.16 mN (mean±S.E., N=54). Rank order of potencies in the sphincter was eledoisin>physalaemin >substance P, and the three peptides were 200–800 times more potent than carbachol. The order of intrinsic activities was eledoisin (=carbachol) >physalaemin =substance P. As it has been reported (22, 23) that substance P is rapidly degraded by endogenous proteases in many tissues, it is conceivable that the dose-response relationship obtained by cumulative dosings may not represent a true one. Therefore, the dose-response curve for substance P was constructed in a noncumulative manner (single dose), but the noncumulative curve was practically identical with the cumulative one (Fig. 2).

Table 1. Values of pD₂, relative potency and intrinsic activity for substance P, physalaemin, eledoisin and carbachol in rabbit iris sphincter muscle

| Agents     | pD₂     | Relative affinity | i.a.       |
|------------|---------|------------------|------------|
| Carbachol  | 6.10±0.02 | 0.006            | 1          |
| Substance P| 8.31±0.13 | 1                | 0.85±0.01  |
| Physalaemin| 8.88±0.05 | 3.72             | 0.84±0.04  |
| Eledoisin  | 9.02±0.08 | 5.13             | 0.98±0.04  |

Data from Fig. 1.

Fig. 1. Cumulative dose-response curves for substance P (●, N=5), physalaemin (▲, N=5), eledoisin (■, N=5) and carbachol (○, N=15) in rabbit iris sphincter muscle. The contraction induced by 10⁻⁵ M carbachol was taken as 100%. Each symbol and vertical bar represent mean and standard error, respectively. In the absence of bars, standard errors are within the symbols.

Fig. 2. Noncumulative (single dose) dose-response curve for substance P in rabbit iris sphincter muscle. Tension induced by 10⁻⁵ M carbachol was taken as 100%. Mean±standard error of 5 determinations.

Tetrodotoxin (3×10⁻⁶ M), atropine (10⁻⁶ M) and physostigmine (10⁻⁶ M) essentially had no effect on the responses to substance P, physalaemin and eledoisin (Table 2). Physostigmine alone did not cause any tension development, which differs from its effect in guinea-pig ileum. In this experiment, the peptides were applied by single dosing at their respective ED50 concentrations, but not cumulatively, since tension hardly returned to the resting level once maximum responses were obtained with substance P or physalaemin (see below). These results suggest that these tachykinins directly act on the smooth muscle cells, and not on cholinergic nerve elements. It has also been reported (12) that the action of substance P is not mediated via muscarinic or nicotinic acetylcholine receptor and α- or β-adrenergic receptor. As depicted in Fig. 3, contractions by substance P and physalaemin were sustained even after the applied drugs were
thoroughly washed out with PSS. These sustained contractions were dependent on the extracellular Ca ions because by changing the bathing medium from PSS to Ca-free, EGTA-containing solution, the contraction rapidly decreased to a level below the resting one, and upon reapplication of Ca ions, the previous developed tension was recovered. On the other hand, eledoisin could not elicit such a sustained contraction after washing. Figure 4 shows that the half-life of the substance P-induced sustained contractions after washing out of the agonist was longer as the concentration of substance P was increased.

A 10 min treatment with phenoxybenzamine (2 × 10⁻⁵ M) attenuated the response to eledoisin (7 × 10⁻¹⁰ M) selectively, but not that to substance P (5 × 10⁻⁹ M) or physalaemin (1.3 × 10⁻⁹ M) (Table 3). The equieffective concentrations of the peptides were so selected as to allow the adoption of the null hypothesis (24). Excess eledoisin (10⁻⁷ M) was used as a means of protection against phenoxybenzamine. In the presence of eledoisin, it was found that inhibition of

| Peptides   | Tetrodotoxin 3 × 10⁻⁶ M | Atropine 10⁻⁶ M | Physostigmine 10⁻⁶ M |
|------------|-------------------------|---------------|---------------------|
| Substance P| 106.0±4.74 (5)          | 107.9±5.52 (5)| 113.1±4.92 (6)      |
| Physalaemin| 104.0±4.78 (5)          | 103.9±5.87 (8)| 118.0±7.17 (6)      |
| Eledoisin  | 100.1±2.08 (5)          | 100.3±2.91 (5)| 107.8±6.38 (5)      |

Control response was obtained using the ED50 concentration of each peptide and was taken as 100%. The concentrations used were as follows: substance P: 5 × 10⁻⁹ M, physalaemin: 10⁻⁹ M, and eledoisin: 10⁻⁹ M. Tetrodotoxin, atropine and physostigmine were pretreated for 5 min. Values shown are means with standard errors, and number of experiments is shown in parenthesis.

Fig. 3. Typical tracings of tension developments induced by 10⁻⁷ M substance P (A), 3 × 10⁻⁶ M physalaemin (B) and 10⁻⁸ M eledoisin (C) in rabbit iris sphincter muscle. W: wash-out. Horizontal bars below tracings in A and B represent the periods when the preparation was incubated in Ca-free, EGTA (1 mM)-containing solution.
Table 3. Antagonistic effects of phenoxybenzamine on the contractions induced by substance P, physalaemin and eledoisin in rabbit iris sphincter muscle

| Peptides    | PBZ 10 min | PBZ 10 min + Ele 10^{-7} M | PBZ 30 min |
|-------------|------------|----------------------------|------------|
| Substance P | 113.1±11.4 (5) | —                          | 61.2±3.2 (9) |
| Physalaemin | 92.7±10.0 (5)  | —                          | 58.8±9.8 (5)  |
| Eledoisin   | 38.0±3.6* (5)  | 61.9±4.8** (5)             | 6.6±0.9 (5)  |

Control response was obtained using the equieffective concentration of each peptide, which induced about 40% of the maximum tension by 10^{-5} M carbachol, and was taken as 100%. The concentrations used were as follows: substance P: 5×10^{-8} M, physalaemin: 1.3×10^{-8} M and eledoisin: 7.1×10^{-10} M. Phenoxybenzamine (PBZ: 2×10^{-6} M) was treated for 10 min or 30 min, and in the protection experiments, 10^{-7} M eledoisin (Ele) was added 2 min before applying phenoxybenzamine. After washing out phenoxybenzamine or phenoxybenzamine plus eledoisin (in protection experiments), the tissues were washed repeatedly every 10 min for 60 min. The treated response to the tachykinin was then observed. Values given are means with standard errors, and number of experiments is shown in parenthesis. *: Significantly different from the control (100) and the phenoxybenzamine-treated eledoisin-induced response, respectively.

Fig. 4. Half-lives of the substance P-induced sustained contractions after washing of the agonist. Abscissa: Concentration of substance P to elicit a contraction. Time of contact of substance P with the tissue was 5 min, and after removal of substance P from the medium, washouts were repeated every 10 min. Ordinate: Half life in minutes of the sustained contraction. Mean±S.E. of 4 determinations.

Discussion
We have shown in this study that substance P has a potent and direct action on the sphincter smooth muscle in agreement with the previous reports (11–14) and that the developed contraction was sustained for a long time even after removing the agonist from the bathing medium. This fact suggests that the presence of substance P during the longlasting miosis may not be necessary once the muscle is stimulated. The tracing in Fig. 3 further shows that the sustained contraction is strongly dependent on the extracellular Ca ions.

It has been recently suggested by Iversen et al. (15), Lee et al. (16), Gater et al. (25) and Growcott et al. (17) that multiple receptors may exist for substance P. It has been shown that by ranking the potencies of substance P and a variety of its analogue peptides (in the tachykinin family), it was possible to detect two distinct patterns of activity. It has been found that in some assays, the rank order of potencies was eledoisin >> physalaemin >> substance P, whereas in others, it was physalaemin >> substance P >> eledoisin. These differences have been suggested to be attributed to two different subtypes of receptor: namely, the E-subtype receptor where eledoisin is most potent and the P-subtype receptor where physalaemin is most potent. As shown in this study, the rank order of potencies is eledoisin >> physalaemin >> substance P in the rabbit iris sphincter muscle. In the light of the view described above, there were E-
subtype receptors in this muscle. However, the sustained contraction was induced by substance P and physalaemin, but not eledoisin, and from this result, it may be appreciated that there would be differences in the receptor mechanisms (and excitation-contraction coupling) utilized by substance P or physalaemin and eledoisin, as has been demonstrated in guinea-pig urinary bladder (17), although other explanations could be exist (for example, differential susceptibilities to metabolizing enzymes or difference in detaching rates from the biophase, etc.). Experiments using phenoxybenzamine could further lend support to the existence of two subtypes of receptor. It has been reported that a relatively high concentration of phenoxybenzamine irreversibly antagonized the effect of tachykinins in guinea-pig ileum (26) and guinea-pig urinary bladder (17), and the latter authors moreover suggested that phenoxybenzamine selectively attenuated the response via the E-subtype receptor. Our results show that in rabbit iris sphincter smooth muscle, phenoxybenzamine preferentially caused an irreversible blockade of the response to eledoisin, compared to that of substance P or physalaemin. Moreover, this result suggests that this blockade was produced by alkylating the receptor, because when the tissue was pretreated with excess eledoisin, the effect of phenoxybenzamine was effectively prevented. The response to eledoisin, a full agonist, was selectively antagonized by phenoxybenzamine as opposed to substance P or physalaemin, an apparent partial agonist, and this phenomenon could not be explained by assuming that one single type of receptor exists (27). Moreover, excess eledoisin can considerably protect the receptor from the attack of phenoxybenzamine. There may be two receptor subtypes for tachykinins in rabbit sphincter smooth muscle, one specific for substance P and physalaemin and the other for eledoisin. However, the physiological role of the receptor subtypes remains to be clarified. Most recently, tachykinin peptides other than substance P (substance K, neurokinin β) have been found in mammalian tissues (18–20). In addition, Nawa et al. (20) postulated that substance K is a peptide that serves as an endogenous ligand for the E-subtype receptor. Although Stjernschantz et al. (9) suggested that it was highly unlikely that substance P is the only agent from sensory neurones involved in irritative ocular responses, the presence and the role of these new tachykinins in the eye have not been evidenced. At present, the possibility can not be excluded that there is a common receptor containing two binding sites, and that due to conformational arrangements, the binding site for eledoisin may be the one to which phenoxybenzamine can gain access more easily. Further studies are required to elucidate these problems.

In summary, substance P, physalaemin and eledoisin, three members of the tachykinin family, elicited atropine and tetrodotoxin resistant contractions of rabbit iris smooth muscle in nanomolar concentrations. Characteristic sustained tension developments were induced by substance P and physalaemin after they were washed out, but not eledoisin. The 10 min treatment with phenoxybenzamine (2x10^-5 M) selectively attenuated the response to eledoisin, but not substance P or physalaemin. These results may suggest that there are at least two receptors for tachykinin peptides.

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