Pharmacological characterization of tachykinin-stimulated inositol phospholipid hydrolysis in peripheral tissues

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1 Tachykinin-stimulated inositol phospholipid hydrolysis was examined in slices of rat parotid gland, hamster urinary bladder and guinea-pig ileum longitudinal muscle.

2 In the presence of lithium, substance P and other naturally-occurring and synthetic tachykinins induced large, dose-dependent increases in [³H]-inositol monophosphate accumulation.

3 In slices of rat parotid gland, [pGlu⁶,L-Pro⁹]SP(6–11) was considerably more potent in stimulating inositol phospholipid hydrolysis than [pGlu⁶,D-Pro⁹]SP(6–11).

4 In contrast, in slices of hamster urinary bladder, [pGlu⁶,D-Pro⁹]SP(6–11) exhibited greater potency in evoking inositol phospholipid breakdown than [pGlu⁶,L-Pro⁹]SP(6–11).

5 The differential selectivity of these C-terminal fragments of substance P suggests that they may be useful tools for distinguishing between NK₁ and NK₂ receptors.

6 L-659,837 and L-659,874 antagonized eleodisin-stimulated inositol phospholipid hydrolysis in slices of hamster urinary bladder. Neither compound significantly reduced substance-P evoked inositol phospholipid breakdown in slices of rat parotid gland, or senktide-induced inositol phospholipid hydrolysis in slices of guinea-pig ileum.

7 L-659,837 and L-659,874 had no effect on the atropine-sensitive, carbachol-stimulated inositol phospholipid hydrolysis in slices of rat parotid gland.

8 These data further support the notion that L-659,837 and L-659,874 are potent and selective NK₂ receptor antagonists.

Introduction

There is now considerable evidence for the existence of multiple tachykinin receptors (for reviews see Quirion & Dam, 1988; Guard & Watson, 1991). The current classification (Henry *et al.*, 1987) recognises three main types (NK₁, NK₂ and NK₃) characterized according to the rank order of potency of a variety of tachykinin agonists in various bioassay systems.

The tachykinin agonist profile of NK₁ receptors in both the central nervous system (CNS) and periphery is similar, with the preferred endogenous tachykinin, substance P, showing activity at nanomolar concentrations. The non-mammalian tachykinins eleodisin, physalaemin and kassinin are approximately equipotent with substance P, but the mammalian tachykinins neurokinin A (NKA) and neurokinin B (NKB) are slightly less potent than substance P.

In contrast, at NK₂ receptors in rat vas deferens and hamster urinary bladder, NKA, NKB, eleodisin and kassinin are all active in the nanomolar range, whereas substance P and physalaemin are approximately two orders of magnitude less potent. In addition, substance P methyl ester, a selective NK₁ receptor agonist, which is approximately equipotent with substance P at NK₁ receptors, is 100–1000 fold less active than substance P at NK₂ receptors (Watson *et al.*, 1983).

At NK₃ receptors, the preferred tachykinin, NKB (Laufer *et al.*, 1985) is 100 fold more potent than NKA or substance P (Mastrenghelo *et al.*, 1986; McKnight & Maguire, 1987). The demonstration of NK₃ receptors has been much facilitated by the availability of the potent and highly selective NK₃ receptor agonist, senktide (Wormser *et al.*, 1986).

A putative fourth type of tachykinin receptor has recently

been described in guinea-pig tracheal tissue (McKnight *et al.*, 1988a). The rank order of potency of naturally occurring tachykinins at this site, appears to be similar to that of NK₂ sites, although distinct differences are apparent in potencies of certain synthetic tachykinin analogues. Further studies are required to confirm the existence of these receptors.

NK₁, NK₂ and more recently NK₃ receptors have all been shown to be associated with inositol phospholipid hydrolysis in various tissues. Thus, Hanley *et al.* (1980) reported that substance P and related tachykinins induce inositol phospholipid hydrolysis in rat salivary glands. The rank order of potencies in eliciting this response was similar to the rank order of affinities at the NK₁ binding site, suggesting that inositol phospholipid hydrolysis in this tissue is coupled to NK₁ receptors. Similarly, we have previously reported that the same response in hamster urinary bladder is mediated via NK₂ receptors (Bristow *et al.*, 1987). Finally, tachykinin-induced inositol phospholipid hydrolysis in guinea-pig ileum myenteric plexus has been characterized by Guard *et al.* (1988) and shown to be linked to NK₃ receptors.

In the present study, we describe the effects of a pair of synthetic tachykinin agonists, [pGlu⁶,L-Pro⁹]SP(6–11) and [pGlu⁶,D-Pro⁹]SP(6–11), on NK₁ and NK₂ receptor-mediated inositol phospholipid hydrolysis. Radioligand binding studies suggest that [pGlu⁶,L-Pro⁹]SP(6–11) and [pGlu⁶,D-Pro⁹]SP(6–11) have selectivity for NK₁ and NK₂ receptors respectively (Lee *et al.*, 1986). It was therefore of interest to assess these compounds with respect to their ability to elicit a biochemical response in tissues previously characterized as possessing NK₁ and NK₂ receptors. We have also examined the effects of two synthetic tachykinin antagonists L-659,837 and L-659,874, on NK₁, NK₂ and NK₃ receptor-mediated inositol phospholipid hydrolysis. Both compounds show selectivity for NK₂ sites in radioligand binding studies (Williams *et al.*, 1988) and antagonize responses mediated by NK₂ receptors in *in vitro* pharmacological models of tachykinin receptors (McKnight *et al.*, 1988b). Thus, the present studies were undertaken to examine further the selectivity of these compounds for NK₂ receptors. Some of these data have

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been published in preliminary form (Suman-Chauhan *et al.*, 1988).

Methods

Tachykinin-induced inositol phospholipid hydrolysis was examined in slices of rat parotid gland (NK₁), hamster urinary bladder (NK₂) and guinea-pig ileum (NK₃) by previously described methodology (Hanley *et al.*, 1980; Bristow *et al.*, 1987; Guard *et al.*, 1988). Tissues were rapidly removed, cross-chopped (350 × 350 × 350 µm) with a McIlwain tissue chopper, and dispersed in 10 ml Krebs Ringer bicarbonate (KRB) of the following composition (in mM): NaCl 118, KCl 4.7, CaCl₂ 1.25, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11, (pH 7.4) with a Pasteur pipette. Slices were pre-incubated in 250 ml oxygenated KRB at 37°C for 30 min with two changes of buffer. Twenty-five microlitre aliquots (approximately 0.5 mg protein) of gravity-packed slices were then placed in polypropylene tubes containing 2 µCi [³H]-inositol, 10 mM LiCl, bovine serum albumin (1 mg ml⁻¹) and bacitracin (40 µg ml⁻¹) in freshly gassed KRB. Tubes were gassed, capped and incubated for 30 min to allow incorporation of [³H]-inositol into tissue phospholipids. Agonists (10 µl) were added (to give a final assay volume of 250 µl) and tubes incubated at 37°C for a further 45 min. Antagonists (10 µl) were added, where appropriate, 15 min before exposure to agonists.

Reactions were terminated by addition of 1 ml chloroform/methanol (1:2; vol/vol), followed by 300 µl chloroform and 300 µl deionized water. Tubes were vortexed and aqueous and organic phases allowed to separate by standing at room temperature for 30–60 min. Water soluble [³H]-inositol monophosphates were separated by anion exchange chromatography (Berridge *et al.*, 1982). Aliquots (750 µl) of the upper aqueous phase were removed and diluted with 2 ml deionized water before loading onto glass columns containing 1 ml of a 1:1 (vol/vol) suspension of Dowex AG 1-X8 formate resin in deionized water. Free [³H]-inositol and [³H]-glycerophosphoinositol were removed by eluting columns with 10 ml water followed by 15 ml 25 mM ammonium formate solution, respectively. [³H]-inositol monophosphates were eluted into scintillation vials with 10 ml 150 mM ammonium formate solution containing 5 mM sodium tetraborate. Total radioactivity present in this 10 ml fraction was determined by addition of 10 ml Hydrofluor and counting in the gel phase.

Analysis of results

All data points represent the mean of triplicate determinations. Results were plotted as the mean [³H]-inositol

monophosphate accumulation (d.p.m.) versus log tachykinin concentration. EC₅₀ values (concentration of tachykinin inducing 50% of the maximum response, as defined by 30 µM substance P) were determined from individual dose-response curves composed of at least four different tachykinin concentrations (range 0.001 µM to 100 µM). The geometric means of EC₅₀ values (*n* = 4–6 separate experiments) were obtained by calculating the results in logarithmic form and expressing as the antilog of the mean (–s.e.mean; +s.e.mean).

IC₅₀ values (concentration of antagonist resulting in 50% inhibition of the response to a given concentration of agonist) were determined from individual inhibition curves employing at least four different antagonist concentrations (range 0.01 µM to 100 µM). Geometric means of IC₅₀ values (–s.e.mean; +s.e.mean) were calculated from three separate experiments as described for EC₅₀ values.

Materials

[³H]-inositol, specific activity 16.3 Ci mmol⁻¹, was purchased from Amersham International plc. Substance P methyl ester was obtained from Cambridge Research Biochemicals. All other tachykinins were purchased from Bachem Biochemicals. Senktide (succ-[Asp⁶,MePhe⁸]SP(6–11)), [pGlu⁶,D-Pro⁹]SP(6–11), [pGlu⁶,L-Pro⁹]SP(6–11), L-659,837 and L-659,874 were synthesized and characterized in the Medicinal Chemistry Department of the MSDRL Neuroscience Research Centre. Carbachol, atropine, bovine serum albumin and bacitracin were obtained from Sigma Chemical Co, Ltd.

Results

Effects of agonists

Basal accumulation of [³H]-inositol monophosphates was 679 ± 72 d.p.m. (*n* = 38) and 769 ± 69 d.p.m. (*n* = 34) per 25 µl slices in rat parotid gland and hamster urinary bladder, respectively. In the presence of lithium, the addition of substance P and other naturally occurring or synthetic tachykinins (0.001–500 µM) resulted in large, concentration-dependent increases in accumulation of [³H]-inositol monophosphates, giving rise to 4–7 fold maximal increases above basal in slices of rat parotid gland and 10–15 fold maximal increases above basal in hamster urinary bladder. These data reflect the ability of lithium to inhibit inositol monophosphatase activity in these tissues, thus preventing hydrolysis of [³H]-inositol monophosphates to free [³H]-inositol.

A comparison of the EC₅₀ values obtained for a series of tachykinins in these tissues is shown in Table 1. The rank order of potency of tachykinins for evoking inositol phospholipid hydrolysis in rat parotid gland is typical of an NK₁ receptor-mediated response. Thus, substance P and physala-

Table 1 Comparison of EC₅₀ values (µM) for tachykinin-induced [³H]-inositol monophosphate production in slices of rat parotid gland and hamster urinary bladder

Peptide	EC ₅₀ (µM) (–s.e.mean, +s.e.mean)	
	NK ₁ (rat parotid gland)	NK ₂ (hamster urinary bladder)†
Substance P	0.007 (0.005, 0.008)	5.09 (3.95, 6.56)
Physalaemin	0.005 (0.004, 0.007)	3.97 (3.16, 5.00)
Eledoisin	0.031 (0.021, 0.046)	0.045 (0.033, 0.060)
Kassinin	0.045 (0.04, 0.051)	0.007 (0.005, 0.009)
Neurokinin A	0.383 (0.308, 0.475)	0.013 (0.010, 0.018)
Neurokinin B	0.667 (0.430, 1.035)	0.036 (0.030, 0.044)
Substance P methyl ester	1.56 (0.72, 3.37)	106.6 (66.7, 170.6)
[pGlu ⁶ ,D-Pro ⁹]SP(6–11)	40.60 (29.10, 56.70)	0.216 (0.164, 0.285)
[pGlu ⁶ ,L-Pro ⁹]SP(6–11)	1.04 (0.800, 1.340)	29.00 (21.00, 40.00)

Mean EC₅₀ values (concentration required to induce 50% of the maximum response, as defined by 30 µM substance P) were determined from 4–6 separate dose-response curves each composed of at least four different tachykinin concentrations. Results were calculated in logarithmic form and are expressed as the antilog of the mean (–s.e.mean, +s.e.mean).

† Data taken from Bristow *et al.* (1987).

We have also compared the effects of two synthetic C-terminal fragments of substance P, [pGlu⁶,D-Pro⁹]SP(6-11) and [pGlu⁶,L-Pro⁹]SP(6-11) (Figure 1), on inositol phospholipid hydrolysis in rat parotid gland and hamster urinary bladder (Table 1). These C-terminal hexapeptides, originally synthesized by Piercey *et al.* (1985), have been shown to exhibit differential selectivity for NK₁ and NK₂ binding sites with [pGlu⁶,D-Pro⁹]SP(6-11) being selective for NK₂ sites whilst [pGlu⁶,L-Pro⁹]SP(6-11) is selective for NK₁ sites (Lee *et al.*, 1986). In the present study, [pGlu⁶,D-Pro⁹]SP(6-11) was considerably more potent in stimulating inositol phospholipid hydrolysis in slices of hamster urinary bladder (EC₅₀ value 0.216 μM) than in slices of rat parotid gland (EC₅₀ value 40.6 μM). In contrast, [pGlu⁶,L-Pro⁹]SP(6-11) showed greater potency in evoking inositol phospholipid breakdown in slices of rat parotid gland as compared to hamster urinary bladder (EC₅₀ values 1.04 μM and 29.00 μM respectively). Data relating to NK₂ receptor-mediated inositol phospholipid hydrolysis in hamster urinary bladder (Table 1) has been previously published (Bristow *et al.*, 1987).

The effects of two synthetic hexapeptides, L-659,837 and L-659,874 (Figure 1) on tachykinin-induced inositol phospholipid hydrolysis mediated via NK₁, NK₂ and NK₃ receptors have also been studied. Concentrations of substance P, eleodisin, and the NK₃ receptor selective agonist senktide (Wormser *et al.*, 1986) resulting in submaximal accumulation of [³H]-

Figure 1 Structures of synthetic tachykinin agonists and antagonists. pGlu = pyroglutamic acid.

In order to investigate further the selectivity of L-659,837 and L-659,874, their effects on carbachol-induced inositol phospholipid hydrolysis were examined in slices of rat parotid gland. Carbachol ($10\mu\text{M}$) induced a 10 fold increase in the accumulation of [^3H]-inositol monophosphates over basal levels (Figure 3), a response which was almost completely

Figure 3 Effects of atropine (Atr), L-659,837 and L-659,874 on 10 μ M carbachol-stimulated inositol phospholipid hydrolysis in slices of rat parotid gland. Results are expressed as d.p.m. [³H]-inositol monophosphate accumulation above basal levels per 25 μ l slices. The histogram shows data from a single representative experiment performed in triplicate.

abolished by the muscarinic antagonist, atropine (1 μ M). In contrast, concentrations of up to 100 μ M L-659,837 and L-659,874 had little or no effect on carbachol-induced inositol phospholipid hydrolysis (Figure 3).

Discussion

The current classification of tachykinin receptors depends largely on agonist potency profiles in various assay systems. Definitive classification requires the development of potent, highly selective agonists and antagonists. In the present study, we have compared the ability of the two synthetic C-terminal substance P fragments, reported to have differential selectivity for NK₁ and NK₂ receptor binding sites, to elicit a functional response, i.e. inositol phospholipid hydrolysis. Lee *et al.* (1986) reported that [pGlu⁶,L-Pro⁹]SP(6–11) was considerably more potent in displacing [¹²⁵I]-BH-substance P binding to NK₁ sites (e.g. rat parotid gland) than [pGlu⁶,D-Pro⁹]SP(6–11), whereas the latter showed greater potency in displacing [¹²⁵I]-BH-eledoisin binding to NK₂ sites (e.g. hamster urinary bladder). Thus, D-Pro/L-Pro ratios (IC₅₀ value for [pGlu⁶,D-Pro⁹]SP(6–11)/IC₅₀ value for [pGlu⁶,L-Pro⁹]SP(6–11)) for NK₁ tissues fell within the range 87–124, whereas NK₂ tissues yielded a D-Pro/L-Pro ratio of 0.003. The D-Pro/L-Pro ratios (EC₅₀ value for [pGlu⁶,D-Pro⁹]SP(6–11)/EC₅₀ value for [pGlu⁶,L-Pro⁹]SP(6–11)) calculated for induction of inositol phospholipid hydrolysis in slices of rat parotid gland and hamster urinary bladder were 39 and 0.007 respectively. These results are in close agreement with the data of Lee *et al.* (1986). We have not examined the effects of the synthetic agonists [pGlu⁶,D-Pro⁹]SP(6–11) and [pGlu⁶,L-Pro⁹]SP(6–11) on NK₃ receptor-mediated inositol phospholipid hydrolysis because of the inherent problems which would be encountered as a result of the mixed population of NK₁ and NK₃ receptors present in the guinea-pig ileum (Guard *et al.*, 1988). Such studies would need to be performed either in tissues containing predominantly NK₃ receptors, or in the presence of selective antagonists to block NK₁ receptors. McKnight *et al.* (1988a) have reported that [pGlu⁶,D-Pro⁹]SP(6–11) and [pGlu⁶,L-Pro⁹]SP(6–11) are equipotent at the putative NK₄ receptor-mediated contractions of guinea-pig

trachea. There is to date no published evidence for coupling of this receptor to inositol phospholipid hydrolysis, therefore we were unable to investigate the biochemical correlates of this observation. Nevertheless, it is clear that comparison of D-Pro/L-Pro ratios can provide a useful means of distinguishing between tachykinin receptor types.

L-659,837 and L-659,874 are both synthetic hexapeptides derived from the parent compound L-363,851 (Cascieri *et al.*, 1985), which was originally developed within our laboratories as an NK₂ agonist. L-659,837 possesses a cyclic structure, which retains the constraining lactam unit present in the parent compound. L-659,874 is a linear structure with a primary sequence closely related to that of L-659,837. Both compounds have been evaluated in radioligand binding assays for NK₁, NK₂ and NK₃ receptor sites, and shown to be NK₂ selective (Williams *et al.*, 1988). L-659,837 and L-659,874 both potently displaced [¹²⁵I]-BH-eledoisin binding from membranes prepared from hamster urinary bladder with IC₅₀ values of 0.15 μ M and 0.04 μ M respectively. L-659,837 and L-659,874 were essentially inactive in displacing [¹²⁵I]-BH-substance P binding from NK₁ sites and [¹²⁵I]-BH-eledoisin binding from NK₃ sites in membranes prepared from rat cerebral cortex (IC₅₀ values greater than 100 μ M). This selectivity for NK₂ receptors was also reflected in *in vitro* pharmacological models, with L-659,837 and L-659,874 having no significant effects on NK₁-mediated substance P-induced contractions of guinea-pig ileum (pA₂ values less than 4.5) or on NK₃-mediated, eledoisin-induced contractions of rat portal vein (pA₂ values 4.9 and 4.4 respectively), but potently antagonizing the NK₂-mediated eledoisin-induced contractions of rat vas deferens (pA₂ values 6.7 and 6.8 respectively; McKnight *et al.*, 1988b). Our own studies on inositol phospholipid hydrolysis further substantiate these findings that L-659,837 and L-659,874 are potent and selective antagonists at NK₂ receptors, with essentially no activity in antagonizing NK₁ and NK₃ receptor-mediated inositol phospholipid hydrolysis. The selectivity of L-659,837 and L-659,874 for NK₂ receptors is further emphasized by the observation that neither compound showed significant activity against carbachol-stimulated inositol phospholipid hydrolysis. Preliminary studies suggest a competitive mode of action for the effects of L-659,837 and L-659,874 against eledoisin-stimulated phospholipid hydrolysis in slices of hamster urinary bladder. Thus, the presence of 1 μ M L-659,837 or L-659,874 resulted in a rightward shift of the eledoisin dose-response curve in this tissue (unpublished). If competitive antagonism is assumed, pK_i values of 6.6 and 6.8 can be calculated for L-659,837 and L-659,874, respectively, using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). These values are in good agreement with the previously published pA₂ values for these antagonists versus NK₂ receptor-mediated responses to eledoisin in the rat vas deferens preparation (McKnight *et al.*, 1988b).

Multiple tachykinin receptors appear to be involved in nociception (Dourish *et al.*, 1988b), and therefore tachykinin antagonists may possess antinociceptive properties. Intracerebral injection of L-659,837 potently blocked eledoisin-induced reciprocal hindlimb scratching in mice, but was without effect on substance P-induced scratching, again suggesting NK₂ selectivity (Dourish *et al.*, 1988a). L-659,837 also induced significant latency in the mouse hot plate test suggesting analgesic properties in thermal pain (Dourish *et al.*, 1988a).

In conclusion, we have demonstrated that the tachykinin analogues [pGlu⁶,D-Pro⁹]SP(6–11) and [pGlu⁶,L-Pro⁹]SP(6–11) are useful tools for the characterization of tachykinin receptors. We also provide further evidence for the notion that L-659,837 and L-659,874 are novel, potent and selective NK₂ antagonists. The availability of such selective, high affinity compounds should facilitate research into the physiological roles of multiple tachykinin receptors.

The authors would like to thank Mrs E. Brawn and Mrs S. Burton for typing the manuscript, and A. Butler for preparation of the figures.

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(Received February 24, 1990)

Revised June 21, 1990

Accepted August 4, 1990)