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Different tachykinin receptor subtypes are coupled to the phosphoinositide or cyclic AMP signal transduction pathways in rat submandibular cells

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Tachykinins of different classes (NK1, NK2, NK3) caused the concentration-dependent synthesis of IP_3 in rat submandibular acinar cells with the potency rank order of NK1 > NK2 > NK3. Enhancement of IP_3 was not affected by pertussis toxin treatment. The reverse rank order was found in the tachykinin inhibition of isoproterenol-induced cAMP synthesis and this inhibition was abolished by pertussis toxin, an inactivator of the adenylate cyclase G_i regulatory protein. It is suggested that different tachykinin receptor subtypes are preferentially coupled to phospholipase C or adenylate cyclase by separate G regulatory proteins in rat submandibular acinar cells.

Substance P; Tachykinin receptor; Phospholipase C; Adenylate cyclase; G protein; Pertussis toxin

1. INTRODUCTION

In an earlier study, we reported that two calcium-mobilizing agonists of different classes, SP and carbachol, stimulated the secretion of acinar cell mucin by rat submandibular gland [1]. These agonists also caused the hydrolysis of membrane PIP_2 resulting in increased cellular levels of IP_3 [1,2]. In many cell types, Ca^{2+}-mobilizing agonists exert their physiological effect through receptor-linked activation of PLC, leading to the hydrolysis of membrane PIP_2. Both IP_3 and DG breakdown products then act as second messengers in stimulus-response coupling [3,4]. There is strong evidence that a guanine nucleotide-binding, G regulatory protein couples stimulated receptors to PLC activation [5]. In addition to their effects on mucin release and PIP_2 hydrolysis, SP and carbachol reduced the mucosecretory response evoked by the β-adrenergic agonist, IPR [1].

Recent studies in our laboratory showed that carbachol antagonizes the submandibular mucosecretory response to IPR by its inhibition of IPR-induced cAMP synthesis. It was further demonstrated that this muscarinic effect was mediated by the G_i regulatory protein of the adenylate cyclase complex which could be inactivated by treatment with pertussis toxin. Carbachol stimulation of PLC was, in contrast, mediated by a different Gp protein which was PT-insensitive (Fleming et al., submitted). It was therefore proposed that muscarinic receptors are coupled to two signal transduction pathways by discrete G-proteins that may be differentiated by their response to PT.

The tachykinin agonist, SP, has many effects in common with carbachol in the submandibular model, including the inhibition of β-adrenergic-induced secretion and the stimulation of PIP_2

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Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PI, phosphoinositide; PLC, phospholipase C; DG, diacylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; SP, substance P; SP-ME, substance P methyl ester; IPR, isoproterenol; cAMP, cyclic adenosine 3′,5′-monophosphate; PT, pertussis toxin; NKA, neurokinin A; NKB, neurokinin B; TCA, trichloroacetic acid

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hydrolysis [1]. The present investigation was therefore undertaken to examine whether SP actions are also mediated in the phosphoinositide and cAMP pathways by different regulatory G-proteins. Moreover, since the existence of multiple tachykinin receptors – NK1, NK2 and NK3 – has been established [6], we sought to determine whether specific receptor subtypes may be preferentially linked to each signal transduction pathway.

2. MATERIALS AND METHODS

2.1. *Materials*

Substance P, physalaemin, eledoisin, kassinin, NKA, NKB and SP-ME were obtained from Peninsula Laboratories, Belmont, CA. Purified collagenase, CLSPA grade, was obtained from Worthington, Freehold, NJ. *myo*-[2-^{3}H]Inositol was a product of Amersham, Oakville, Ontario, Canada. Purified pertussis toxin was supplied by List Biological Laboratories, Campbell, CA. New England Nuclear, Montreal, Quebec, Canada provided ^{125}I-cAMP radioimmunoassay kits. Hank's balanced salt solution was obtained from Gibco, Burlington, Ontario, Canada. All other reagents were from Sigma, St. Louis, MO.

2.2. *Methods*

Collagenase-dispersed rat submandibular gland acinar cells were prepared as previously described [1]. Dispersed cells were incubated for 60 min in supplemented Hank's balanced salt solution (HBSS) containing 35 μCi *myo*-[2-^{3}H]inositol (12.3 Ci/mmol). The cells were washed once in medium containing 5 mM unlabelled inositol and resuspended in 1 ml aliquots of inositol-free medium. Test preparations were challenged with a range of concentrations of several tachykinin agonists: SP, physalaemin, eledoisin, kassinin, NKA, NKB or SP-ME for 10 min. The reaction was stopped by addition of 1 ml ice-cold TCA (6% final concentration). Inositol phosphates were extracted from the soluble fraction by anion-exchange chromatography on Bio-Rad AG 1 × 8 formate resin as described previously [1] and quantitated by scintillation counting. In some experiments, cells were exposed to pertussis toxin (200 ng/ml, 1 h) before agonist treatment.

For cAMP studies, collagenase-dispersed submandibular cells were exposed to various concentrations of NKA, NKB or SP for 2 min followed by IPR (10^{-5} M) for 10 min. Additional experimental groups received either IPR alone (10 min), tachykinins alone (12 min) or were left untreated as controls. The cells were precipitated with 6% TCA and centrifuged at 2500 × *g* for 15 min. The supernatant fluid was extracted with water-saturated ether and assayed for cAMP by radioimmunoassay as described previously [7]. In some experiments, cells were preincubated with pertussis toxin (200 ng/ml, 1 h) before agonists were added.

Protein content of the acinar cells was determined by the method of Lowry et al. [8].

Results were examined statistically by two-way analysis of variance and Duncan's multiple range test. Values of $p < 0.05$ were considered significant.

3. RESULTS

3.1. *IP_3 studies*

In the absence of added tachykinins, basal ^{3}H-IP_3 accumulation over 10 min was 321 ± 21 dpm/mg protein. Incubation of cells with the tachykinins produced concentration-dependent increases in IP_3 levels (fig.1). Although all the agonists produced a similar maximal stimulation of IP_3 to around three times control level, there were obvious variations in their potencies (table 1). Calculated pD_2 values fell into three groups that were statistically different (table 1). Thus, SP, physalaemin and SP-ME were more potent in enhancing IP_3 levels than were kassinin, eledoisin and NKA; and the latter three tachykinins were more potent than NKB.

Preincubation of cells with PT had no effect on basal or SP-induced IP_3 production (not shown).

3.2. *cAMP studies*

Substance P, NKA and NKB were chosen as representative agonists of tachykinin classes for cAMP studies. These agonists alone, in concentrations up to 10^{-7} M, did not affect cAMP levels in mucus acinar cells. IPR (10^{-5} M) caused an eight-fold increase in cAMP level from 17.5 ±

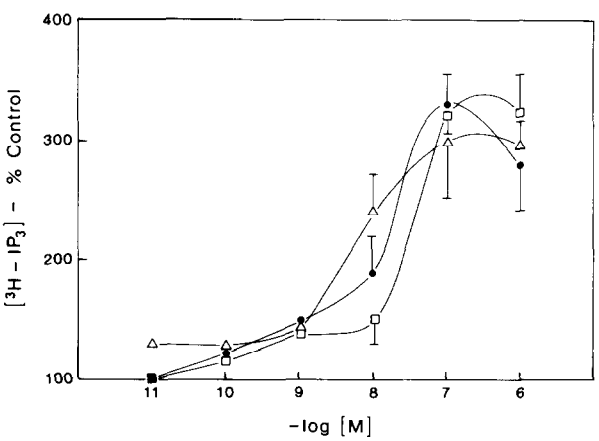

Fig.1. Concentration-response curves for three representative tachykinins in the stimulation of IP_3 levels in rat submandibular acinar cells after 10 min treatment. IP_3 was assayed as described in section 2. Values are means ± SE, $n = 4$. (●) Neurokinin A; (□) neurokinin B; (△) substance P.

Table 1

pD_2 values for tachykinin agonists in the stimulation of IP_3 in dispersed cells of rat submandibular gland

Tachykinin	pD_2
Substance P	8.63 ± 0.06
Physalaemin	8.86 ± 0.13
SP methyl ester	8.10 ± 0.12
Kassinin	7.84 ± 0.02[a]
Eledoisin	7.51 ± 0.03[a]
Neurokinin A	7.66 ± 0.19[a]
Neurokinin B	7.07 ± 0.11[b]

[a] Significantly different from SP, physalaemin or SP methyl ester, $p < 0.05$

[b] Significantly different from all other tachykinin agonists, $p < 0.05$

Dispersed acinar cells were exposed to various agonists for 10 min and IP_3 measured as described in section 2. pD_2(− log EC_{50}) was calculated from four separate concentration response curves for each agonist. Values are means ± SE, $n = 4$

2.9 pm/mg protein in controls to 137 ± 22 pg/mg protein in stimulated cells. Pretreatment of cells with varying concentrations of NKA (10^{-10}–10^{-7} M), NKB (10^{-13}–10^{-9} M) or SP (10^{-9}–10^{-6} M) elicited a concentration-dependent reduction of the IPR-cAMP response in acinar cells (fig.2). Table 2

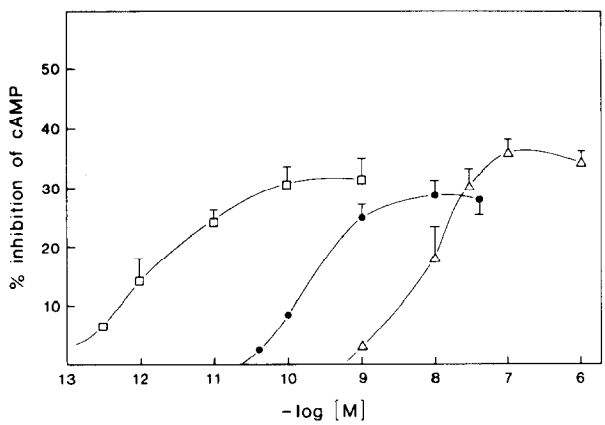

Fig.2. Concentration-response curves for tachykinin inhibition of cAMP levels induced by 10^{-5} M isoproterenol in rat submandibular acinar cells. Cells were incubated with the tachykinins for 2 min, followed by IPR for 10 min. cAMP was assayed by RIA. Results are expressed as percent inhibition of the cAMP levels stimulated by IPR alone. Values are means ± SE, $n = 6$. (●) Neurokinin A; (□) neurokinin B; (△) substance P.

Table 2

pD_2 values for tachykinin agonist inhibition of the isoproterenol-induced cAMP response in dispersed cells of rat submandibular gland; and the maximum inhibition of IPR-cAMP response elicited by these agonists

Tachykinin	pD_2	% inhibition of IPR-cAMP response
Neurokinin B	10.90 ± 0.30	31.0 ± 3.4
Neurokinin A	9.03 ± 0.01	28.3 ± 3.1
Substance P	7.65 ± 0.15	32.0 ± 4.5

Effects of preincubation of acinar cells with three tachykinins on the IPR-induced cAMP response were assessed by RIA as described in section 2. Different optimal concentrations of the agonists inhibited the cAMP response to the same extent but their pD_2 values are significantly different from each other ($p < 0.01$). Values are means ± SE, $n = 6$

shows the pD_2 values of the tachykinins for the cAMP-inhibition response. NKB was significantly more potent than both NKA and SP ($p < 0.01$). At optimally effective concentrations (NKB, 10^{-9} M; NKA, 10^{-8} M; and SP, 10^{-7} M), there was no significant difference in the degree of inhibition elicited by these agonists (table 2).

Pretreatment of cells with PT alone (200 ng/ml) or in combination with SP (10^{-7} M) did not affect basal concentration of cAMP. However, SP-induced reduction of the IPR-cAMP response was abolished after 1 h preincubation with PT (table 3).

Table 3

Effect of pertussis toxin on the SP-induced inhibition of elevated cAMP levels elicited by isoproterenol stimulation in rat submandibular acinar cells

Pre-treatment	Control	SP (10^{-7} M)	Isoproterenol (10^{-5} M)	SP (10^{-7} M) + isoproterenol (10^{-5} M)
− PT	12.0 ± 1.3	14.0 ± 2.0	100	60 ± 5.7[a]
+ PT	14.1 ± 1.2	13.2 ± 0.9	107 ± 2.9	103 ± 11.4

[a] $p < 0.01$ vs isoproterenol

Dispersed acinar cells were preincubated in the presence (+ PT) or absence (− PT) of pertussis toxin (200 ng/ml; 1 h) then exposed to SP (12 min); IPR (12 min); or SP (2 min) followed by IPR (10 min). cAMP was measured by RIA as described in section 2. For normalization of the results of several experiments, values are expressed as percentage of the IPR response. Values are mean ± SE, $n = 6$

4. DISCUSSION

The results of this study extend our earlier observations [1] on the effects of SP on stimulus-response coupling mechanisms in submandibular cells, by confirming that several additional tachykinin/neurokinin agonists stimulate PIP_2 hydrolysis and also inhibit the β-adrenergic-cAMP response in the model.

It has been shown in many studies that Ca^{2+}-mobilizing receptors, including tachykinin and muscarinic, are coupled to PLC by a G regulatory protein [3,4]. We have recently established that a G-protein (G_p) is involved in the phosphoinositide effect in submandibular cells and that the protein is not inactivated by pertussis toxin (Fleming et al., submitted). This PT-resistant property is consistent with that of the PLC-associated G-protein in heart cells [9], pancreas [10] and liver [11]. We have now shown in the present study that tachykinin-induced PIP_2 hydrolysis is also PT-insensitive. In contrast, the tachykinin effect of reducing β-adrenergic-induced cAMP levels is abolished by PT, which is known to inactivate the G_i protein of the adenylate cyclase complex by ADP-ribosylation of the α-subunit. This finding is compatible with our earlier observation that SP inhibition of the IPR mucosecretory response in the model is reversed by PT treatment [2]. Taken together, these results suggest that tachykinin receptors are coupled separately to the phosphoinositide and cAMP pathways by two different regulatory proteins in the same way as are muscarinic receptors in submandibular cells.

Based on the rank order of potency, agonist selectivity and radioligand binding [6,12], the tachykinin receptors have been classified into 3 subtypes – NK1, on which SP is more active than NKA and NKB; NK2, on which NKA > NKB > SP; and NK3, where NKB > NKA > SP. The present study suggests that PIP_2 hydrolysis is coupled to the NK1 receptor subtype, since SP, SP-ME and physalaemin were approximately equipotent but were considerably more potent than NKA and NKB. Hanley et al. [13] have shown that phosphoinositide hydrolysis in rat parotid cells is also mediated via NK1 receptors. However, there is evidence that, depending on the tissue, all the receptor subtypes are capable of mediating PIP_2 hydrolysis. Thus PIP_2 hydrolysis has been observed in NK2-type tissues such as the rat ileum and hamster urinary bladder [14,15]. Substance P and related tachykinins also stimulate inositol phospholipid hydrolysis in guinea pig ileum longitudinal muscle-myenteric plexus preparations, an example of NK3-type tissue [16].

A reverse order of potency was obtained for the tachykinin inhibition of the IPR-cAMP response. Here the rank order was NKB $\gg$ NKA > substance P, indicating an NK3-mediated response. To the best of our knowledge, this is the first classification of the tachykinin receptor modulating the adenylate cyclase system in a salivary gland model. It is also the first report of an NK3-mediated response in rat submandibular gland in vitro.

The results on PIP_2 hydrolysis correlate well with radioligand and autoradiographic evidence that acinar cells of rat submandibular gland contain predominantly NK1-binding sites as well as a smaller number of NK2 or NK3 sites [17]. Our observations are also in agreement with the finding that NK1 agonists, physalaemin and SP stimulate mucus and amylase secretion in the gland [18]. The fact that NK3 receptors modulate adenylate cyclase activity in this study now suggests a physiological function for the small number of this receptor subtype found in radioligand-binding studies. Thus, as indicated by the work of Buck and Burcher [17] and by the present study, the rat submandibular gland may not be a pure NK1 tissue. Murray et al. [19] have recently proposed the presence of three tachykinin receptor subtypes in rat salivary glands.

In conclusion, these results suggest that the effects of tachykinins on the phosphoinositide and cAMP pathways are preferentially mediated by different tachykinin receptor subtypes. Our findings further suggest that these receptor subtypes are coupled to the two effector systems by discrete G regulatory proteins.

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