Homologous desensitization of substance-P-induced inositol polyphosphate formation in rat parotid acinar cells

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Maximal concentrations of substance P and methacholine induced a rapid increase in [^3H]inositol trisphosphate ([^3H]IP $_3$) formation. After about 1 min, the [^3H]IP $_3$ in the substance-P-treated cells ceased to increase further, whereas in the methacholine-treated cells [^3H]IP $_3$ continued to increase. Addition of methacholine to the substance-P-treated cells caused a rapid increase in [^3H]IP $_3$, whereas a second addition of a 10-fold excess of substance P had no effect. Pretreatment of cells with substance P, followed by removal of the substance P by washing, resulted in a decreased response to a second application of substance P. A similar protocol involving pretreatment with methacholine had no effect on subsequent responsiveness to substance P. Analysis of [^3H]substance P binding to substance-P-treated cells indicated that the number of receptors for substance P was decreased, but the affinity of the receptors for substance P was unaffected. After substance P pretreatment, a prolonged incubation (2 h) restored responsiveness of the cells to substance P, measured as [^3H]IP $_3$ formation, and restored the number of binding sites to control values. These findings indicate that, in the rat parotid gland, substance P induces a homologous desensitization of its receptor, which involves a slowly reversible down-regulation or sequestration of substance-P-binding sites.

INTRODUCTION

Substance P is a putative neurotransmitter widely distributed in the mammalian central and peripheral nervous systems (Cuello *et al.*, 1982). In the rat parotid gland, a target organ of substance P, the peptide activates the release of Ca $^{2+}$ from an intracellular pool, and stimulates univalent-ion flux, which may be involved in secretion of water and electrolytes (Putney, 1977). Results from more recent studies in a variety of biological systems indicate that the initial event in Ca $^{2+}$ mobilization involves the breakdown of phosphatidylinositol 4,5-bisphosphate, forming the water-soluble inositol 1,4,5-trisphosphate [(1,4,5)IP $_3$], which then serves as the second messenger to mobilize Ca $^{2+}$ (Berridge, 1984; Berridge & Irvine, 1984). Substance P mediates the breakdown of phosphatidylinositol 4,5-bisphosphate (Weiss *et al.*, 1982) and the accumulation of IP $_3$ in rat parotid cells (Berridge *et al.*, 1983; Aub & Putney, 1985), and (1,4,5)IP $_3$ causes a release of intracellular Ca $^{2+}$ from saponin-permeabilized parotid acinar cells (Putney *et al.*, 1986).

After prolonged incubation of target cells with hormones or neurotransmitters, the cellular responses are often blunted, a phenomenon generally referred to as desensitization. In rat parotid slices, desensitization of substance-P-induced K $^{+}$ release has been shown (Friedman *et al.*, 1985), but the molecular mechanism for this effect is not known.

In the present study, we have evaluated the desensitization of substance-P-induced formation of IP $_3$ in rat parotid acinar cells. The results suggest that a homologous down-regulation or sequestration of the sub-

stance-P receptor is responsible for the desensitization of the IP $_3$ response in rat parotid acinar cells.

METHODS

Preparation of parotid acinar cells

Parotid glands were removed from sodium pentobarbital-anaesthetized male Sprague-Dawley rats (140–200 g), and dispersed acinar cells were prepared as previously described (Putney *et al.*, 1978), except that Krebs–Ringer bicarbonate solution was used instead of Tris-buffered Krebs–Ringer solution. The cells were suspended at a concentration of 1–2 mg of protein/ml in a Ringer medium (composition: NaCl, 116 mM; KCl, 5.4 mM; MgSO $_4$, 0.8 mM; CaCl $_2$, 1.8 mM; NaH $_2$ PO $_4$, 0.96 mM; NaHCO $_3$, 25 mM; glucose, 11.1 mM; 1.0% bovine serum albumin) and incubated at 37 °C under O $_2$ /CO $_2$ (19:1). The preparations were routinely more than 85% viable, as determined by Trypan Blue exclusion.

Measurement of [^3H]IP $_3$

The cells were incubated in medium containing *myo*-[2- ^3H]inositol (10 $\mu\text{Ci/ml}$) for 60 min, at which time they were washed by centrifugation (50 *g* for 5 min) and suspended in non-radioactive medium. After the cells were equilibrated for 10 min, drugs were added and 300 μl portions of the cell suspension were taken for analysis at various times. Cell samples were homogenized with 1.2 ml of 4.5% (w/v) HClO $_4$ and left for 20 min on ice. An additional 0.3 ml of standard medium (without albumin) was added and the samples were centrifuged

Abbreviations used: IP, IP $_2$, IP $_3$, IP $_4$, inositol mono-, bis-, tris- and tetrakis-phosphate.

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(1000 g for 5 min). A sample of the supernatant was then neutralized by a sufficient volume of 0.5 M-KOH/9 mM- $\text{Na}_2\text{B}_4\text{O}_7$ to give pH 8–9. The samples were centrifuged as above and the supernatants were applied to anion-exchange columns containing 1.0 ml of Dowex AG 1-X8 (200–400 mesh; formate form). Labelled inositol phosphates were separated as previously described (Berridge *et al.*, 1983). For each experiment, total lipids were extracted (Weiss & Putney, 1981) and the $[\text{^3H}]\text{IP}_3$ fraction was then expressed as a percentage of total $[\text{^3H}]\text{inositol lipids}$ (generally 20000–30000 c.p.m./300 μl sample). The IP_3 fraction prepared in this way contains, in addition to (1,4,5) IP_3 , (1,3,4) IP_3 (Irvine *et al.*, 1984, 1985) and small amounts of (1,3,4,5) IP_4 , the last two compounds being metabolites of (1,4,5) IP_3 (Irvine *et al.*, 1986).

Separation of inositol polyphosphates by h.p.l.c.

Isolated parotid cells prelabelled with $[\text{^3H}]\text{inositol}$ (500 $\mu\text{Ci}/\text{ml}$) for 90 min were washed twice in $[\text{^3H}]\text{inositol}$ -free medium, equilibrated for 15 min, and then incubated in the presence of methacholine (100 μM) or substance P (0.1 μM) for specified time periods. The reactions were terminated by addition of the samples to ice-cold trichloroacetic acid (final concn. 7%, w/v). The supernatant obtained after centrifugation (10000 g for 5 min) of the samples was extracted three times with diethyl ether and neutralized with 3% NH_4OH . The inositol phosphates formed were analysed by h.p.l.c., by a modification of the method described previously (Irvine *et al.*, 1985; Burgess *et al.*, 1985; Tennes *et al.*, 1987). Inositol phosphates were separated with a Whatman Partisil 10SAX anion-exchange column and ammonium phosphate (pH 3.35) gradients. A convex gradient was run for 25 min from 0 to 0.65 M-ammonium phosphate to elute inositol, IP and IP_2 . The isomers of IP_3 were separated by maintaining the elution buffer at 0.65 M for 15 min. The gradient was then increased linearly in 5 min to 2 M-ammonium phosphate and held there for 10 min to elute IP_4 . Standards of $[\text{^3H}]\text{IP}$, $[\text{^3H}]\text{IP}_2$, $[\text{^3H}](1,4,5)\text{IP}_3$ and $[\text{^3H}]\text{IP}_4$ were separated with this gradient to identify the corresponding labelled materials and to calculate recoveries.

$[\text{^3H}]\text{Substance P}$ binding

Since the available radioligands for the substance-P receptor are agonists, and thus could themselves induce changes in receptor number or affinity, binding of $[\text{^3H}]\text{substance P}$ to parotid acinar cells was examined at 0 °C. Previous studies of the β -adrenergic receptor have shown that quantification of plasma-membrane receptors is best accomplished at 0 °C (Toews *et al.*, 1986). In addition, degradation of radioligand, which occurs to some extent in parotid preparations at higher temperatures, may be suppressed at 0 °C (Liang & Cascieri, 1981).

Binding of $[\text{^3H}]\text{substance P}$ was determined by preincubating cells on ice (0 °C) for 15 min, after which various concentrations of $[\text{^3H}]\text{substance P}$ (0.625–20 μM ; 40–60 Ci/mmol) were added. Non-specific binding was determined by parallel incubations in which 2.5 μM -substance P was added to each concentration of $[\text{^3H}]\text{substance P}$. Non-specific binding at low concentrations of substance P was typically about 10% of total bound in control cells, and about 30% in desensitized cells. Bound radioactivity was separated from free by

10-fold dilution in ice-cold 0.32 M-sucrose and rapid (30 s) centrifugation at 10000 g. The fraction of total radioactivity bound at low concentrations of substance P was typically about 5%.

Despite difficulties reported by others in obtaining equilibrium binding of substance P at low temperatures in other preparations (Womack *et al.*, 1985), in the assays described here equilibrium was achieved within 3 min (see the Results section), which was the standard incubation time used. Furthermore, with this procedure the total and non-specific binding and the affinity and capacity of the substance P receptor agreed well with previous reports by Liang & Cascieri (1980, 1981), in which the parotid substance-P receptor was characterized in considerable detail.

Materials

Collagenase (*Clostridium histolyticum*) was obtained from Boehringer Mannheim. *myo*-[2- $^3\text{H}(n)$]Inositol (10–30 Ci/mmol) was purchased from American Radio-labelled Chemicals (St. Louis, MO, U.S.A.). [*prolyl* $^{2,4,3,4}(n)$ - ^3H]Substance P (40–60 Ci/mmol) was purchased from Amersham. All other reagents were obtained from Sigma.

RESULTS

The time course for formation of $[\text{^3H}]\text{IP}_3$ in response to substance P (0.1 μM) and the muscarinic cholinergic agonist methacholine (100 μM) in rat parotid acinar cells is summarized in Fig. 1. As previously reported (Aub & Putney, 1984, 1985), methacholine (100 μM) stimulated the formation of IP_3 , and the increase was sustained from 30 s up to 5 min. Substance P (0.1 μM) also induced the formation of IP_3 , but the amount IP_3 reached a plateau during the initial 1 min of stimulation. Friedman &

Fig. 1. Effect of substance P and methacholine on $[\text{^3H}]\text{IP}_3$ formation

Parotid cells were incubated in medium containing 0.1 μM -substance P (SP; $\circ$, $\bullet$, $\blacktriangle$) or 100 μM -methacholine (MeC; $\triangle$). At 2 min (arrow), to two of the incubations with substance P either 100 μM -methacholine ($\blacktriangle$) or 1 μM -substance P ($\bullet$) was added. Values are means $\pm$ S.E.M. for three independent experiments.

Fig. 2. Effect of substance P and methacholine on $[^3\text{H}]\text{IP}_3$ formation during the initial 60 s

Parotid cells were incubated in medium containing $0.1 \mu\text{M}$ -substance P (SP; $\circ$), $100 \mu\text{M}$ -methacholine (MeC; $\triangle$), or $0.1 \mu\text{M}$ -substance P plus $100 \mu\text{M}$ -methacholine ($\blacktriangle$). Values are means $\pm$ S.E.M. for three or four independent experiments, except the value at 60 s, which is the mean of two experiments.

Selinger (1978) and Friedman *et al.* (1985) reported that substance P caused the transient release of K^+ in parotid slices, and suggested that one mechanism for the termination of the response to substance P was degradation of the peptide. Therefore, to determine whether the termination of IP_3 formation by substance P in these experiments was caused by degradation of the peptide, a 10-fold excess of substance P ($1.0 \mu\text{M}$) was added to the medium after the concentration of IP_3 reached a plateau (at 2 min) in the presence of substance P (Fig. 1). However, this second addition of substance P had no effect on IP_3 formation. This result suggests that the decline in response to substance P is not due to degradation of the peptide. The effect is specific for substance P, since the addition of $100 \mu\text{M}$ -methacholine to the cells 2 min after substance P caused a rapid increase in $[^3\text{H}]\text{IP}_3$, which paralleled the rate of $[^3\text{H}]\text{IP}_3$ formation with methacholine alone.

The formation of IP_3 involves the phosphodiesteratic breakdown of phosphatidylinositol 4,5-bisphosphate by phospholipase C, which is activated by receptor occupation (Berridge, 1984; Berridge & Irvine, 1984). Previous studies on phosphatidylinositol labelling demonstrated that responses to muscarinic agonists and substance P were not additive, indicating that, in the parotid gland, the muscarinic-cholinergic receptor and the substance-P receptor regulate a common phospholipase C mechanism (Weiss & Putney, 1981). This would suggest that the substance-P-specific desensitization was unlikely to result from alterations in the phospholipase C enzyme. These earlier findings were

confirmed by the results of experiments shown in Fig. 2, in which the initial IP_3 -forming responses to methacholine and substance P were found not to be additive. Surprisingly, examination of the IP_3 response at these short times of stimulation indicates that, in contrast with previous results obtained by less sensitive techniques, substance P actually induces a more rapid initial response than methacholine. These results confirm that activation of both the substance-P and the muscarinic receptors induces the formation of IP_3 through a common pathway, and the desensitization of IP_3 formation by substance P appears to involve steps proximal to the activation of phospholipase C. Thus the substance-P-induced IP_3 formation appears to undergo homologous desensitization.

In order to ensure that the time courses seen in Figs. 1 and 2 were not due to the differences in the metabolism of $(1,4,5)\text{IP}_3$ in the substance-P-treated cells as compared with the methacholine-treated cells, samples taken at various times after the addition of the agonists were analysed for $(1,4,5)\text{IP}_3$, $(1,3,4)\text{IP}_3$ and $(1,3,4,5)\text{IP}_4$ by h.p.l.c. These results are summarized in Fig. 3. When the time course of $(1,4,5)\text{IP}_3$ formation was analysed, the results qualitatively confirmed those with the total $[^3\text{H}]\text{IP}_3$ fraction: at 5 s the response to substance P is greater than for methacholine, but after 30 s the response to substance P is less. The increase in $(1,4,5)\text{IP}_3$ at 5 s was to about 20-fold the initial resting value, whereas at 5 min the increase was to only about twice the initial value, but still significantly elevated. A similar pattern was seen for $(1,3,4,5)\text{IP}_4$. For $(1,3,4)\text{IP}_3$, the rapid increase with substance P was not so evident, but by 5 min there was considerably less of this isomer formed in the substance-P-treated cells than in the methacholine-treated cells. Previously Downes *et al.* (1986) reported that both $(1,4,5)\text{IP}_3$ and $(1,3,4)\text{IP}_3$ were decreased in parotid slices treated with substance P for 10 min as compared with slices treated with carbachol. Thus the diminished response seen in the substance-P-treated cells is not due to alterations in the metabolism of $(1,4,5)\text{IP}_3$, since this isomer, as well as $(1,3,4)\text{IP}_3$ and $(1,3,4,5)\text{IP}_4$, are all uniformly decreased relative to the methacholine-treated cells.

In the experiments summarized in Fig. 4, cells were exposed to substance P ($0.1 \mu\text{M}$), methacholine ($100 \mu\text{M}$) or no drug (control) for 5 min, then washed twice by gentle centrifugation, resuspended and incubated at 37°C for 10 min. When the substance-P-treated cells were then stimulated by substance P ($0.1 \mu\text{M}$), the IP_3 response was substantially decreased as compared with control cells (Fig. 4). However, for cells previously exposed to methacholine ($100 \mu\text{M}$) instead of substance P, the response to substance P was not changed (Fig. 4). Note that the desensitization is most evident at 5 s after substance P addition. Since there was also a decreased accumulation of $[^3\text{H}]\text{IP}_2$ (results not shown), and since at 5 s most of the material in the $[^3\text{H}]\text{IP}_3$ fraction is $(1,4,5)\text{IP}_3$, this must indicate a diminished production of this primary breakdown product.

A similar pretreatment of cells with substance P decreased the specific binding of $[^3\text{H}]\text{substance P}$ to the cells when measured at 0°C (Fig. 5). In the control cells, specific binding of $[^3\text{H}]\text{substance P}$ reached a plateau within 3 min. In the cells pretreated with $0.1 \mu\text{M}$ -substance P, specific binding at 0°C also reached equilibrium within 3 min, but the extent of binding was

Fig. 3. Time course of changes in [3H]inositol polyphosphates in parotid acinar cells

Cells were incubated with [3H]inositol to label inositol lipids as described in the Methods section, and samples were taken just before ($t = 0$) and 5, 30, 120 and 300 s after the addition of 0.1 μM -substance P (●) or 100 μM -methacholine (○). The samples were quenched with trichloroacetic acid and analysed for the indicated inositol phosphates by h.p.l.c. as described in the Methods section. The data represent means $\pm$ S.E.M. for three independent experiments, with replicate determinations in each experiment.

Fig. 4. Effect of substance P on [3H]IP₃ formation in cells pretreated with substance P or methacholine

After incubation for 5 min without agonist (control, ●), with 0.1 μM -substance P (○) or 100 μM -methacholine (10 min experiment only; △), parotid acinar cells were washed twice by gentle centrifugation, resuspended in fresh medium, and incubated for 10, 30, 60 or 120 min. After this second incubation, cells were stimulated by substance P (0.1 μM). For each experiment, the [3H]IP₃ content at zero time was subtracted. Values are means $\pm$ S.E.M. for three or four independent experiments.

substantially decreased. Scatchard analysis of the binding indicated that prior exposure to substance P decreased the number of sites (B_{max}) to about 44% of

control, without significantly affecting the binding affinity (K_d) (Figs. 6 and 7). The lack of effect on apparent affinity argues against the possibility of

Fig. 5. Time course of binding of $[^3\text{H}]$ substance P to control and desensitized parotid cells

The protocol was similar to that for Fig. 4; cells were incubated for 5 min in the absence (control, ●) or presence (○) of $0.1 \mu\text{M}$ -substance P, washed twice by centrifugation, and incubated for an additional 10 min, at which time the cells were cooled to 0°C , and specific binding of $[^3\text{H}]$ substance P (10 nM) was determined at the times indicated as described in the Methods section. Results are means $\pm$ S.E.M. from three independent experiments.

artefacts resulting from carry-over of substance P from the pretreatment incubation.

After prolonged incubation of desensitized cells in the fresh medium, a gradual recovery of the IP_3 -forming

response to substance P was observed (Fig. 4). The increase in $[^3\text{H}]\text{IP}_3$ at 30 s after stimulation had recovered by 30 min, probably because in the controls the test stimulus had induced some desensitization by this time. However, the increase in $[^3\text{H}]\text{IP}_3$ at 5–15 s after stimulation was not fully recovered until 120 min.

Recovery of $[^3\text{H}]$ substance P binding paralleled the recovery of the IP_3 response, with gradual recovery of B_{max} , as shown in Figs. 6 and 7. After incubation for 60 and 120 min, B_{max} was 73% and 88% of control respectively. By comparison, IP_3 formation at 5 s after stimulation was 65% and 93% of control at 60 and 120 min incubation respectively (Fig. 4).

DISCUSSION

Substance P induces various pharmacological actions in target organs, including intestinal contraction (Tregear *et al.*, 1971; Bury & Mashford, 1977), vasodilation (Tregear *et al.*, 1971), depolarization of certain neurons (Konishi & Otsuka, 1974; Phillis & Limacher, 1974; Ogata, 1979), and salivation (Leeman & Hammerschlag, 1967; Liang & Cascieri, 1979; Gallacher, 1983). However, in only a few cases have the biochemical signals which are generated by activation of substance-P receptors been investigated. The rat parotid gland provides an excellent model for such studies. In this gland, substance-P receptors have been demonstrated and characterized biochemically (Putney *et al.*, 1980; Liang & Cascieri, 1981). Activation of these receptors induces univalent-ion fluxes which are secondary to cellular Ca^{2+} mobilization (Putney, 1977). The second messenger for the initial phase of Ca^{2+} mobilization is believed to be $(1,4,5)\text{IP}_3$, derived from phospholipase-C-mediated breakdown of phosphatidylinositol 4,5-

Fig. 6. Scatchard analysis of $[^3\text{H}]$ substance P binding to control and desensitized parotid cells

The protocol was similar to that for Fig. 5; cells were incubated for 5 min in the absence (control, ●) or presence (○) of $0.1 \mu\text{M}$ -substance P, washed twice by centrifugation, and incubated for an additional 10, 60 or 120 min, at which time the cells were cooled to 0°C , and $[^3\text{H}]$ substance P binding was determined as described in the Methods section. Results from a typical experiment are shown. Average B_{max} and K_d values from three to five experiments are summarized in Fig. 7.

Fig. 7. Average B_{max} and K_d values (means $\pm$ S.E.M.) for three to five experiments carried out as described for Fig. 6

□, Control; ▨, substance-P-treated.

bisphosphate (Berridge, 1984; Berridge & Irvine, 1984). The breakdown of phosphoinositides and the accumulation of inositol phosphates in response to substance P have been reported in intestinal muscle (Watson & Downes, 1983; Watson, 1984; Holzer *et al.*, 1985), sympathetic ganglia (Bone & Michell, 1985) and parotid gland (Hanley *et al.*, 1980; Weiss *et al.*, 1982; Berridge *et al.*, 1983; Aub & Putney, 1985). In this study, the response to substance P of IP_3 formation was investigated in detail, specifically with regard to the desensitization of the substance-P response in rat parotid acinar cells.

Desensitization is a widespread phenomenon in biological systems, and is thought to be an important means by which cellular responsiveness is regulated (Sibley & Lefkowitz, 1985). Desensitization is generally divided into two major categories. One form is homologous, which means that the response to the desensitizing agent only is decreased. On the other hand, heterologous desensitization is characterized by a diminished response which is induced by, and to, activation of different receptors. In the present study, desensitization of IP_3 formation induced by substance P did not affect stimulation through muscarinic-cholinergic receptors, nor did muscarinic activation induce desensitization to substance P. Since the muscarinic and substance-P receptors are believed to activate similar biochemical pathways in these cells, this would suggest that the substance-P receptor desensitization is homologous in nature. Furthermore, the desensitization is persistent, but not irreversible, and is correlated with a decreased number of substance-P binding sites. This indicates that the homologous desensitization of the substance-P response is due in part, and perhaps wholly, to the down-regulation or sequestration of substance-P receptors. Evidence for internalization of substance-P receptors in pancreatic acinar cells has also been reported by Sjödin (1984).

The most common mechanism of desensitization of phospholipase-C-linked receptors is believed to be a feedback inhibition mediated by protein kinase C (Lynch *et al.*, 1985; Leeb-Lundberg *et al.*, 1985; Vincenti *et al.*, 1985; Watson & Lapetina, 1985). Such a mechanism is by definition heterologous, since at least in theory

diacylglycerol formation and subsequent protein kinase C activation could be achieved through any phospholipase-C-linked receptor. There is good evidence that protein kinase C is involved in secretory responses in the parotid gland (Putney *et al.*, 1984). However, activation of this pathway with phorbol ester did not cause persistent inhibition of substance-P-induced IP_3 formation (H. Sugiya & J. W. Putney, Jr., unpublished work). This finding further underscores the novelty and potential utility of the parotid substance-P receptor in that it represents to our knowledge the only documented example of a phospholipase-C-linked receptor regulated primarily by a homologous desensitization mechanism.

The mechanisms of receptor desensitization have been extensively studied for the adenylate cyclase-linked β -adrenoceptor (Sibley & Lefkowitz, 1985). For this receptor, homologous desensitization is also the result of receptor sequestration, and is believed to be signalled by the phosphorylation of the receptor by a specific β -adrenergic-receptor kinase (Strasser *et al.*, 1986). Such a mechanism should provide a reasonable working hypothesis for future studies on homologous desensitization of phospholipase-C-linked receptors such as the substance-P receptor.

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