Two modes of regulation of the phospholipase C-linked substance-P receptor in rat parotid acinar cells

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In rat parotid acinar cells prelabelled with [3H]inositol, substance P (100 nm) induced the formation of [3H]inositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]\), 1,4,5-trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]\) reached a maximum 7 s after substance P stimulation, and thereafter decreased and reached a stable value at 60 s. When the cells were exposed to substance P for 10, 30, 60, or 300 s, washed, and re-exposed to this peptide, the formation of [3H]inositol trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]\) was attenuated in a time-dependent manner. In the cells pretreated as described above, the number of [3H]substance-P-binding sites \((B_{\text{max}})\) was also decreased. Possible role(s) of Ca\(^{2+}\) and protein kinase \((\text{PKC})\) control mechanisms in regulating substance P responses were investigated. Desensitization of substance P-induced \(\text{InsP}_3\) was not affected by the Ca\(^{2+}\) ionophore ionomycin, nor was it dependent on Ca\(^{2+}\) mobilization. On the other hand, in the presence of 4\(\beta\)-phorbol 12,13-dibutyrate (PDBu) and 12-O-tetradecanoyl-4\(\beta\)-phorbol 13-acetate, known activators of protein kinase C, substance P-induced \(\text{InsP}_3\) formation was inhibited. However, PDBu had no effect on [3H]substance P binding, whether present during the assay or when cells were pretreated. The persistent desensitization of \(\text{InsP}_3\) formation induced by substance P was not affected by PDBu. These results suggest that the persistent desensitization of \(\text{InsP}_3\) formation induced by substance P is a homologous process involving down-regulation of the substance P receptor; the mechanism does not appear to involve, or to be affected by, the Ca\(^{2+}\) or protein kinase C signalling systems. Protein kinase C activation can, however, inhibit substance P-induced \(\text{InsP}_3\) formation, which may indicate the presence of a negative-feedback control on the substance P pathway.

INTRODUCTION

Activation of a wide variety of hormone and neurotransmitter receptors results in the phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate and the formation of inositol 1,4,5-trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]\) and diacylglycerol (DG) (Berridge & Irvine, 1984; Nishizuka, 1984). \(\text{Ins}(1,4,5)\text{P}_3\) then interacts with the endoplasmic reticulum to initiate the release of Ca\(^{2+}\) stored in this organelle (Streb et al., 1983; Burgess et al., 1984). DG, on the other hand, stimulates the activity of protein kinase C (Kishimoto et al., 1980).

Substance P is a putative neurotransmitter widely distributed in the mammalian central and peripheral nervous system (Cuello et al., 1982). Activation of substance P receptors induces the breakdown of phosphoinositides and accumulation of inositol phosphates in various tissues (Watson & Downes, 1983; Mantyh et al., 1984), including parotid gland (Hanley et al., 1980; Weiss et al., 1982; Berridge et al., 1983; Aub & Putney, 1985; Sugiya et al., 1987). However, the regulation of the phospholipase C-linked substance P receptor is not well understood. We have previously demonstrated that, in rat parotid acinar cells, substance P induces rapid \(\text{InsP}_3\) formation, and this response undergoes persistent desensitization (Sugiya et al., 1987). This desensitization of substance P-induced \(\text{InsP}_3\) formation appeared to be homologous and involved the loss of substance P-binding sites from the cell surface (Sugiya et al., 1987).

In the present study, the kinetics of changes in \(\text{Ins}(1,4,5)\text{P}_3\) in substance P-activated cells and the time course of development of persistent desensitization were examined. Since contents of \(\text{Ins}(1,4,5)\text{P}_3\) appeared to decline more rapidly than the development of persistent desensitization and the loss of substance P-binding sites, other possible mechanisms of regulation of the substance P receptor pathway were explored. Effects of extracellular Ca\(^{2+}\), intracellular Ca\(^{2+}\) or activators of protein kinase C (phorbol esters) on substance P-induced \(\text{InsP}_3\) formation and on substance P-induced persistent desensitization were examined. The results suggest that the phospholipase C-linked substance P receptor is regulated by two mechanisms: a persistent desensitization unaffected by either the Ca\(^{2+}\) or the protein kinase C signalling systems, owing to the down-regulation of substance P receptors, and feedback inhibition, owing to activation of protein kinase C.

EXPERIMENTAL

Materials and chemicals

\(m\text{yos}[2-\text{H(n)}]\text{inositol (10–20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, * To whom reprint requests should be addressed.}
incubated
Measurement of in a
Laboratories (Belmont, CA, U.S.A.) Substance P was from Peninsula Laboratories (Belmont, CA, U.S.A.). Ionomycin was from Calbiochem (San Diego, CA, U.S.A.). All other reagents were obtained from Sigma.

Preparation of parotid acinar cells
Parotid glands were removed from sodium pentobarbital (50 mg/kg)-anaesthetized male Sprague–Dawley rats (180–240 g). Dispersed acinar cells were prepared as previously described (Sugiya et al., 1987). The cells were suspended at a concentration of 2–10 mg of protein/ml in a Krebs–Ringer bicarbonate (KRB) solution, containing NaCl (116 mM), KCl (5.4 mM), MgSO4 (0.8 mM), CaCl2 (1.8 mM), NaH2PO4 (0.96 mM), NaHCO3 (25 mM), glucose (11.1 mM) and 1.0% bovine serum albumin, and incubated at 37 °C in O2/CO2 (19:1). The cell preparations were over 90% viable, as determined by Trypan Blue exclusion.

Measurement of [3H]InsP3
The cells were incubated in KRB solution containing myo-[2-3H]inositol (30 μCi/ml) for 90 min, at which time they were washed by centrifugation (at 50 g for 5 min) and suspended in non-radioactive KRB solution. After the cells were equilibrated for 15 min, drugs were added and 0.3 ml portions of the cell suspension were taken for analysis at appropriate times. The reaction was stopped by the addition of the portions to 4.5% (w/v) HClO4. The samples were treated and applied to anion-exchange columns by the methods previously described (Berridge et al., 1983). The InsP3 fraction prepared with this method contains, in addition to Ins(1,4,5)P3, Ins(1,3,4)P3 (Irvine et al., 1984) and a small amount of Ins(1,3,4,5)P4, the last two compounds being metabolites of Ins(1,4,5)P3 (Irvine et al., 1986). Samples were also taken and extracted with chloroform/methanol/HCl (Sugiya et al., 1987) and the extracts assayed for [3H] content to determine the radioactivity associated with the inositol lipids (primarily phosphatidylinositol), so that InsP3 content could be expressed as a percentage of [3H] phosphoinositides (% PI).

Separation of Ins(1,4,5)P3 and Ins(1,3,4)P3 by h.p.l.c.
Isolated parotid acinar cells prelabelled with [3H]inositol (0.5 mCi/ml) for 90 min were washed twice by centrifugation. The cells were suspended in [3H]inositol-free KRB solution, equilibrated for 15 min, and then incubated in the presence of substance P (100 nM). The reactions were terminated by addition of ice-cold trichloroacetic acid (final conc. 7%, w/v). The supernatants were treated and analysed by h.p.l.c. as previously described (Sugiya et al., 1987), except that the pH of ammonium phosphate for the h.p.l.c. gradient was 3.8 instead of 3.35.

[3H]Substance P binding assay
Binding of [3H]substance P was determined by the methods previously described (Sugiya et al., 1987), except that the various concentrations of [3H]substance P added were 0.3125–10 nM (25–55 Ci/mmol), and 10 μM substance P was added to each concentration of [3H]substance P for the determination of non-specific binding.

Persistent desensitization induced by substance P
Persistent desensitization of substance P responses and loss of substance P binding sites were measured as described previously (Sugiya et al., 1987). Cells, either [3H]inositol-prelabelled or non-labelled, were incubated with substance P to induce desensitization, and subsequently substance P-induced [3H]InsP3 formation or loss of [3H]substance P binding sites was measured. The cell suspension preincubated with substance P (100 nM) was diluted with 400 vol. of ice-cold KRB solution (4 °C) to dilute and terminate the effect of substance P, and centrifuged at 50 g for 5 min at 4 °C. The cell pellet was rinsed a second time with ice-cold KRB solution (100-fold volume of the first cell suspension) and centrifuged. The cells were resuspended in fresh KRB solution and incubated at 37 °C for 10 min. Then, for the assay of [3H]InsP3, the cells were stimulated by substance P (100 nM) for 5 s, and the reaction was terminated by the addition of HClO4. At 5 s, most of the increased [3H] in the InsP3 fraction after stimulation by substance P was in Ins(1,4,5)P3 (Sugiya et al., 1987). For the assay of [3H]substance P binding, the cells were then cooled to 4 °C for 10 min before the assay.

Removal of extracellular Ca2+ and the agonist-sensitive pool
To examine the effect of extracellular Ca2+ on substance P-induced InsP3 formation and persistent desensitization, cells were incubated in a Ca2+-free KRB solution containing 0.1 mM-EGTA for 10 min. The cells were then stimulated with the muscarinic–cholinergic receptor agonist methacholine (0.1 mM) for 5 min, and then stimulation was terminated by the addition of 0.1 mM-atropine. Prior studies have shown that this protocol depletes the agonist-sensitive Ca2+ pool from parotid cells, such that, on subsequent application of substance P, neither Ca2+ entry nor internal Ca2+ release occurs (Aub et al., 1982). Controls for these experiments were cells which received the same sequence of agonist and antagonist but in the presence of extracellular Ca2+, such that the intracellular pool remained intact (Aub et al., 1982). Desensitization was then induced as described above by the addition of substance P for 1 min, followed by dilution in ice-cold KRB solution (Ca2+-depleted if appropriate), re-equilibration for 10 min, and application of a 5 s test exposure to substance P.

RESULTS
Substance P-induced formation and desensitization of Ins(1,4,5)P3
The time course of substance P-induced formation of Ins(1,4,5)P3 and Ins(1,3,4,5)P4 in rat parotid acinar cells analysed by h.p.l.c. is summarized in Fig. 1. The amount of Ins(1,4,5)P3 was dramatically increased by the exposure of cells to substance P (100 nM) for 2 s, and reached a maximum at 7 s, about 18 times the initial resting value. Thereafter the amount of Ins(1,4,5)P3 decreased, in spite of the presence of substance P, and reached a stable value at 60 s, 4 times the initial amount. In contrast, by 5 s the amount of Ins(1,3,4,5)P4, which is a metabolite produced from Ins(1,4,5)P3 via the intermediate inositol 1,3,4,5-tetrakisphosphate (InsP4) (Irvine et al., 1986), was not as rapidly increased by substance P stimulation during the first 10 s. But thereafter the amount continued to rise,
and reached a plateau at 120 s. As demonstrated in our previous report (Sugiya et al., 1987), these results imply that \( \text{Ins}(1,4,5)P_3 \) formation is rapidly activated and rapidly desensitized by substance P. Furthermore, we reported that substance P induced a persistent desensitization of the \( \text{Ins}P_3 \) response which resulted from a loss of substance P binding sites. Thus we examined the time course of development of this persistent desensitization and loss of receptor sites for comparison with the rate of decrease in \( \text{Ins}(1,4,5)P_3 \) in stimulated cells. In cells pretreated with substance P for 10, 30, 60 or 300 s (see the Experimental section), the \( \text{Ins}P_3 \) formation induced by a second exposure to substance P was decreased to an extent dependent on the duration of the first, or conditioning, stimulus (Fig. 2a). With 60 s of pre-incubation, the \( \text{Ins}P_3 \) response to substance P was 27% of control, and was decreased to 11% (presumably the minimum) by 300 s. In substance P binding studies, pre-incubation with substance P also induced a similarly time-dependent decrease in \( B_{\text{max}} \) (Fig. 2b) without a change in \( K_d \) (Fig. 2b inset). After preincubation with substance P for 60 s, the number of binding sites (\( B_{\text{max}} \)) was decreased to 59% of control. When the cells were preincubated with substance P for 300 s, 30% of the binding sites remained. The radioligand used in these studies would not select between different subtypes of substance P receptors, and this may explain the observation that, at apparent steady state, a greater fraction of sites remain than predicted from the \( \text{Ins}P_3 \) data. Nonetheless, the kinetics for loss of binding and response were similar; when the 300 s value was subtracted from each data point, the \( t_1 \) values for the rate of decline in the \( B_{\text{max}} \) of the substance P receptor and for loss of the substance P-induced \( \text{Ins}P_3 \) formation were approx. 40 s and 30 s respectively. These results demonstrate that the development of persistent desensitization and the loss of substance P binding sites are rapid processes; however, the rate of these processes may be somewhat slower than the decrease in \( \text{Ins}(1,4,5)P_3 \) in substance P-stimulated cells; i.e., by 60 s, the \( \text{Ins}(1,4,5)P_3 \) content had reached a minimum, but the persistent desensitization and loss of binding sites had not (Figs. 1 and 2). Thus the possibility that other mechanisms, involving either the Ca\(^{2+} \) or the protein kinase C messenger systems, might be involved in regulating the substance P pathway was investigated.

![Graph](image_url)

**Fig. 1.** Time course of changes of \([\text{H}]\text{inositol trisphosphates in parotid acinar cells stimulated by substance P (100 nM)}\)

\([\text{H}]\text{inositol-prelabelled parotid acinar cells were stimulated for the indicated intervals and extracted, and a sample of the extract was analysed by h.p.l.c. Results are means ± s.e.m. for three to ten independent experiments: ○, \([\text{H}]\text{Ins}(1,4,5)P_3; } ●, \([\text{H}]\text{Ins}(1,3,4)P_3.}\)

![Graph](image_url)

**Fig. 2.** Time course of desensitization of substance P-induced \([\text{H}]\text{Ins}P_3 \) formation and loss of \([\text{H}]\text{substance P binding sites in cells pretreated with substance P (100 nM).}\)

(a) \([\text{H}]\text{inositol-prelabelled parotid acinar cells were incubated with substance P (100 nM)} \) for 0, 5, 30, 60 or 300 s. After washing and resuspension as described in the Experimental section, the cells were stimulated by substance P (100 nM) for 5 s. In the case of pretreatment with substance P for 0 s, after addition of ice-cold KRB solution to stop the reaction, substance P was added to the diluted cell suspension. For each experiment \([\text{H}]\text{Ins}P_3 \) content is shown as % of control, i.e. \([\text{H}]\text{Ins}P_3 \) in cells treated similarly except that the pretreatment with substance P was omitted. (b) Cells pretreated with substance P (100 nM) for 0, 10, 30, 60 or 300 s as described above were washed and resuspended in fresh KRB solution. Then the cells were cooled to 4 °C, and used for the \([\text{H}]\text{substance P binding assay as described in the Experimental section. Inset shows plot of } K_d \text{ versus time. Results are means ± s.e.m. from three to nine independent experiments.}
Effects of Ca²⁺

Ionomycin is a specific ionophore for Ca²⁺ (Liu & Herman, 1979), and induces Ca²⁺ influx as well as release of Ca²⁺ from an intracellular pool in rat parotid gland (Poggioili et al., 1982). Therefore, to examine possible effects of Ca²⁺ mobilization on the desensitization of substance P-induced InsP₃ formation, cells were pretreated with ionomycin (1 μM) with the same protocol used previously for substance P. However, although this concentration would cause Ca²⁺ mobilization of a magnitude similar to that for substance P (Poggioili et al., 1982), no desensitization of the substance P response occurred (control response to substance P, 0.416 ± 0.012% PI; after pretreatment with ionomycin, 0.408 ± 0.010% PI).

In the next experiments, the effects of Ca²⁺ entry and intracellular Ca²⁺ release on [³H]InsP₃ formation and on the persistent desensitization induced by substance P were examined. To test the role of extracellular Ca²⁺ on substance P-induced InsP₃ formation, cells were stimulated by 100 nM substance P in the presence or absence of extracellular Ca²⁺. To determine whether intracellular Ca²⁺ release might affect the response, cells were treated sequentially with methacholine and atropine in the presence or absence of extracellular Ca²⁺ and stimulated by substance P (100 nM), again in the presence or absence of extracellular Ca²⁺ (see the Experimental section). In the absence of extracellular Ca²⁺, or after depletion of the agonist-sensitive intracellular Ca²⁺ pool, the increase in [³H]InsP₃ was unaffected (Fig. 3). A similar protocol was used to assess the effects of Ca²⁺ mobilization on substance P-receptor desensitization. Cells, again treated with methacholine and atropine in the presence or absence of Ca²⁺, were pretreated with substance P (100 nM) for 1 min, resuspended in fresh KRB solution, and tested for the development of persistent desensitization with a second exposure to substance P (100 nM) for 5 s. However, the prevention of both Ca²⁺ entry and intracellular Ca²⁺ release had no effect on the desensitization of substance P-induced InsP₃ formation (Table 1).

Effect of activation of protein kinase C

The hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C produces both Ins(1,4,5)P₃ and DG (Berridge & Irvine, 1984; Nishizuka, 1984). DG stimulates the activity of protein kinase C (Kishimoto et al., 1980), and this effect is mimicked by phorbol ester drugs (Castagna et al., 1982). Therefore we investigated the effect of activation of protein kinase C with phorbol esters on substance P-induced [³H]InsP₃ formation and on the development of persistent desensitization of InsP₃ formation. First, we studied the effect on substance P-induced InsP₃ formation of phorbol dibutyrate (PDBu); this phorbol ester has been previously shown to activate enzyme secretion in the parotid gland (Putney et al., 1984).

The time course for formation of InsP₃ in response to substance P (100 nM) in the presence or absence of PDBu (1 μM) is summarized in Fig. 4(a). In the control cells, the amount of InsP₃ reached a maximum within 10 s after exposure to substance P. In the cells treated with PDBu, the time course of substance P-induced InsP₃ formation was similar, but the magnitude of the increase was substantially less. When the cells were pretreated with PDBu and subsequently stimulated by substance P, InsP₃ formation was much less inhibited (Fig. 4(b)).

Table 1. Lack of effect of Ca²⁺ on the desensitization of substance P-induced InsP₃ formation

<table>
<thead>
<tr>
<th>Substrate P</th>
<th>Control</th>
<th>Substance P</th>
<th>Mec + Atp pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>No pretreatment</td>
<td>0.848±0.052</td>
<td>0.187±0.027</td>
<td>0.506±0.023</td>
</tr>
<tr>
<td>+Ca²⁺</td>
<td>0.092±0.039</td>
<td>0.173±0.026</td>
<td>0.498±0.033</td>
</tr>
</tbody>
</table>
results suggest that activation of protein kinase C by PDBu may inhibit substance P-induced InsP₃ formation by a mechanism which can be reversed by removal of the phorbol ester.

To ensure that the inhibitory action of PDBu was in fact due to the ability of this phorbol ester to activate protein kinase C, two other phorbol esters with markedly different potencies in activating the enzyme were investigated. Fig. 5 summarizes the dose-dependent inhibition of substance P-induced InsP₃ formation by the phorbol esters PDBu, TPA and PDD. PDBu and TPA inhibited the response to a maximum of about 50% in a concentration-dependent manner, whereas PDD had no effect in the concentration range tested (up to 20 μM); the potency was TPA > PDBu > PDD, as expected for the activation of protein kinase C (Niedel & Blackshear, 1986). Curiously, the dose-response curve for PDBu was biphasic, such that inhibition began to decrease as the concentration was increased beyond 1 μM. The reason for this apparent anomalous behaviour of PDBu, which was not seen with the more potent TPA, is not clear, but could possibly be due to actions of high concentrations of PDBu not related to its ability to activate protein kinase C.

The effect of PDBu on the substance P binding sites was examined. As shown in Table 2, in the presence of PDBu or in cells pretreated with PDBu (1 μM), neither Bₘₐₓ, nor Kₛ of substance P binding was affected.

The effect of PDBu on the induction of persistent desensitization of the InsP₃ response by substance P was examined next (Table 3). The persistent desensitization was neither potentiated nor inhibited by PDBu (1 μM). These results suggest that protein kinase C does not play a role in the persistent desensitization induced by substance P, and that the locus of inhibition of the pathway by phorbol esters (and presumably protein kinase C) is probably distal to the site at which the persistent desensitization is activated.

**DISCUSSION**

After prolonged incubation of target cells with hormones or neurotransmitters, the cellular responses...
Table 3. Lack of effect of PDBu on substance P-induced persistent desensitization

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[H]InsP$_3$ (% PI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (DMSO)</td>
<td>0.418 ± 0.007</td>
</tr>
<tr>
<td>DMSO + substance P</td>
<td>0.124 ± 0.007</td>
</tr>
<tr>
<td>PDBu</td>
<td>0.329 ± 0.025</td>
</tr>
<tr>
<td>PDBu + substance P</td>
<td>0.129 ± 0.041</td>
</tr>
</tbody>
</table>

are often diminished, a phenomenon generally referred to as desensitization. Desensitization is a widespread phenomenon in biological systems (Sibley & Lefkowitz, 1985), and is generally divided into two 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 diminished responsiveness of a receptor pathway which can be induced by an agonist acting on a different receptor type, but linked to the same signalling pathway.

In a previous report (Sugiya et al., 1987), we demonstrated that substance P induced a persistent desensitization of its own ability to activate InsP$_3$ formation, which was associated with a loss of surface membrane-binding sites for substance P. Substance P O-methyl ester activated phospholipase C and induced desensitization with similar potencies (about one-tenth that of substance P itself; H. Sugiya & J. W. Putney, Jr., unpublished work) indicating that both of these phenomena are likely to be due to activation of P-type substance P receptors on the parotid acinar cell (Watson et al., 1983). Also, substance P did not induce desensitization of responsiveness to muscarinic-cholinergic receptor stimulation, and muscarinic-cholinergic stimulation did not induce desensitization of the substance P response; thus it was concluded that the persistent desensitization was of the homologous type (Sugiya et al., 1987).

In the present study, the time courses of development of persistent desensitization of substance P-induced InsP$_3$ formation and of loss of substance P binding sites were examined. These responses occurred extremely rapidly, with half-times of about 30 s and 40 s respectively, and with detectable decreases within 10 s (Fig. 2). This thus appears to be one of the fastest systems for receptor desensitization and loss of binding sites yet described. Even so, it is not clear whether these phenomena occur sufficiently quickly to account for the rapid decrease in Ins(1,4,5)P$_3$ in stimulated cells (Fig. 1). Thus additional studies were carried out to determine whether other mechanisms, possibly heterologous ones involving the protein kinase C or Ca$_{2+}$-signalling systems, might be involved in regulating the substance P stimulus–response pathway.

Experiments with ionomycin and with protocols which eliminated the ability of substance P to activate either Ca$_{2+}$ entry or Ca$_{2+}$ release indicated that Ca$_{2+}$ probably plays no role in regulating the substance P receptor activity. Indeed, the results in Fig. 3 confirm the previous demonstration, by the more indirect procedure of measuring phosphatidylinositol 4,5-bisphosphate breakdown, that the activation of phospholipase C in parotid cells by substance P is unaffected by Ca$_{2+}$ mobilization, whether derived from entry or from intracellular release (Weiss et al., 1982). By similar reasoning, the results in Table 1 indicate that neither Ca$_{2+}$ entry nor Ca$_{2+}$ release mediates or modifies the development of persistent desensitization.

On the other hand, substance P-induced InsP$_3$ formation was inhibited by the active phorbol esters PDBu and TPA. These reagents readily penetrate cells, and they directly stimulate the activity of protein kinase C by acting at the same site as DG (Niedel & Blackshear, 1986; Kikkawa & Nishizuka, 1987). Therefore substance P-induced InsP$_3$ formation can apparently be inhibited by activation of protein kinase C. This inhibitory mechanism, however, does not seem to be related to the development of persistent desensitization. First, the effect was at least somewhat reversible (Fig. 4; the remaining inhibition may be due to an inability to wash out completely all of the PDBu). Second, activation of protein kinase C by PDBu had no effect on the number or apparent affinity of substance P binding sites (Table 2). Therefore, these results suggest that substance P-induced InsP$_3$ formation is regulated by two rather distinct mechanisms. The first is a rapidly developing and slowly reversible (persistent) desensitization, which involves receptor down-regulation, and which appears to be homologous. The second mechanism involves inhibition of the substance P response pathway by one of the signals activated in that pathway, protein kinase C. Such a mechanism would seem by definition to be a heterologous one, since any receptor capable of activating protein kinase C, such as the muscarinic-cholinergic or $\alpha$-adrenergic, would, at least in principle, be capable of producing such an effect. However, this prediction is not easily tested, since this mechanism of regulation does not appear to be a persistent one. We conclude that the inhibition of the substance P pathway induced by protein kinase C may be involved in the minute-to-minute regulation of the response level of the system, functioning more as a negative-feedback signal than as a mechanism of desensitization.

The mechanism of homologous desensitization has been extensively studied in $\beta$-adrenergic receptor systems. For example, evidence indicates that receptor phosphorylation and subsequent sequestration mediates the homologous desensitization of the $\beta$-adrenergic receptor (Strasser et al., 1986; Benovic et al., 1987a,b). Although such a mechanism could also explain the homologous desensitization in the present study, no direct evidence for the existence of such a kinase regulating phospholipase C-linked receptors is available.

On the other hand, the inhibition of agonist-induced phospholipase C reaction by TPA and PDBu, which activate protein kinase C, has been shown in several receptor systems (Orellana et al., 1987; Brown et al., 1987; Smith et al., 1987; Garcia-Sainz & Hernandez-Sotomayor, 1987; Leeb-Lundberg et al., 1987; Cooper et al., 1987; Tohmatsu et al., 1986; Liles et al., 1986;
Sha’afi et al., 1986). Possible mechanisms for the inhibition of agonist-induced phospholipase C pathway by protein kinase C can be suggested. (1) The receptor protein could be phosphorylated by protein kinase C, rendering the receptor less capable of coupling to its appropriate guanine nucleotide-dependent regulatory protein (G-protein). In DDT-MF-2 cells, phorbol esters promoted the phosphorylation of α2-adrenergic receptors in parallel with inhibition of their function (Leeb-Lundberg et al., 1987). In the present study, however, phorbol ester did not affect [H]substrate P binding sites, and neither potentiated nor inhibited the development of persistent homologous desensitization. This would suggest (but does not prove) that the site of interference of protein kinase C is distal to the substance P receptor. (2) Agonist-induced phospholipase C reactions are believed to be regulated through a putative G-protein which couples receptor binding to phospholipase C activation (Taylor & Merritt, 1986). The phosphorylation of this G-protein by protein kinase C could inhibit this coupling. Orellana et al. (1987) suggested the G-protein as the site at which protein kinase C phosphorylates and blocks hormone-stimulated phosphoinoside hydrolysis in 321N1 astrocytoma cells, since the stimulatory effect of guanosine 5'-ly-thioltriphostate on Ins$_3^P$ formation was inhibited by phorbol esters and by purified protein kinase C. In rat parotid acinar cells, a G-protein is probably necessary for the activation of phospholipase C, since guanosine 5'-ly-thioltriphosphate, a non-hydrolysable analogue of GTP, activates Ins$_3^P$ formation by substance P in permeabilized cells (Taylor et al., 1986); thus such a mechanism could conceivably explain some of the results reported here. (3) The 5-phosphomonoesterase that hydrolyses Ins(1,4,5)$_3$P to Ins(1,4)$_2$P could be phosphorylated and activated by protein kinase C, as is known to occur in human platelets (Connolly et al., 1986). However, this mechanism seems highly unlikely in this instance, since substance P-induced Ins$_3^P$ formation was also decreased in desensitized cells and in cells treated with active phorbol esters (H. Sugiyama & J. W. Putney, Jr., unpublished work).

Activation of phospholipase C-linked receptor systems involves the release of Ca$^{2+}$ from an intracellular pool and possibly the activation of Ca$^{2+}$ entry. Therefore the regulation of the amount of Ins(1,4,5)$_3$P in cells may be a critical factor in the regulation of intracellular Ca$^{2+}$ concentrations. To our knowledge, this is the first demonstration of two independent, but apparently simultaneous, regulatory mechanisms for a single phospholipase C-linked receptor. The co-existence in a single cell type of these two regulatory mechanisms for a receptor suggests complex molecular mechanisms underlying these processes. Further studies should lead to a better understanding of the molecular regulation of the phospholipase C-linked substance P receptor pathway, and may serve as a model for better understanding the regulation of the general class of phospholipase C-linked receptors.

REFERENCES


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