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

Hiroshi SUGIYA, Johnny F. OBIE and James W. PUTNEY, JR.*

Section of Calcium Regulation, Laboratory of Cellular and Molecular Pharmacology,
National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709, U.S.A.

In rat parotid acinar cells prelabelled with [^3H]inositol, substance P (100 nM) induced the formation of [^3H]inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$]. $\text{Ins}(1,4,5)\text{P}_3$ 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 (InsP_3) was attenuated in a time-dependent manner. In the cells pretreated as described above, the number of [^3H]substance-P-binding sites (B_{max}) was also decreased. Possible role(s) of Ca^{2+} and protein kinase (protein kinase C) control mechanisms in regulating substance P responses were investigated. Desensitization of substance P-induced 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 4β -phorbol 12,13-dibutyrate (PDBu) and 12-*O*-tetradecanoyl- 4β -phorbol 13-acetate, known activators of protein kinase C, substance P-induced 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 InsP_3 formation induced by substance P was not affected by PDBu. These results suggest that the persistent desensitization of 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 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 InsP_3 formation, and this response undergoes persistent desensitization (Sugiya *et al.*, 1987). This desensitization of substance P-induced InsP_3 for-

mation 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 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

myo-[2- ^3H (n)]Inositol (10–20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO,

Abbreviations used: $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,3,4,5)\text{P}_4$, inositol 1,3,4,5-tetrakisphosphate; $\text{Ins}(1,4)\text{P}_2$, inositol 1,4-bisphosphate; InsP_3 , inositol trisphosphate; InsP_2 , inositol bisphosphate; PtdIns, phosphatidylinositol; PI, phosphoinositides; DG, diacylglycerol; protein kinase C, Ca^{2+} - and phospholipid-dependent protein kinase; PDBu, 4β -phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoyl- 4β -phorbol 13-acetate; PDD, 4α -phorbol 12,13-didecanoate; KRB solution, Krebs–Ringer bicarbonate solution; G-protein, guanine nucleotide-dependent regulatory protein; DMSO, dimethyl sulphoxide.

* To whom reprint requests should be addressed.

U.S.A.). [2-prolyl-3,4- $^3\text{H}(\text{n})$]Substance P (25–55 Ci/mmol) was obtained from New England Nuclear (Wilmington, DE, 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), MgSO_4 (0.8 mM), CaCl_2 (1.8 mM), NaH_2PO_4 (0.96 mM), NaHCO_3 (25 mM), glucose (11.1 mM) and 1.0% bovine serum albumin, and incubated at 37 °C in O_2/CO_2 (19:1). The cell preparations were over 90% viable, as determined by Trypan Blue exclusion.

Measurement of [^3H]Ins P_3

The cells were incubated in KRB solution containing *myo*-[2- ^3H]inositol (30 $\mu\text{Ci}/\text{ml}$) 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) HClO_4 . The samples were treated and applied to anion-exchange columns by the methods previously described (Berridge *et al.*, 1983). The Ins P_3 fraction prepared with this method contains, in addition to Ins(1,4,5) P_3 , Ins(1,3,4) P_3 (Irvine *et al.*, 1984) and a small amount of Ins(1,3,4,5) P_3 , the last two compounds being metabolites of Ins(1,4,5) P_3 (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 Ins P_3 content could be expressed as a percentage of [^3H] phosphoinositides (% PI).

Separation of Ins(1,4,5) P_3 and Ins(1,3,4) P_3 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 concn. 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]Ins P_3 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]Ins P_3 , the cells were stimulated by substance P (100 nM) for 5 s, and the reaction was terminated by the addition of HClO_4 . At 5 s, most of the increased ^3H in the Ins P_3 fraction after stimulation by substance P was in Ins(1,4,5) P_3 (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 Ca^{2+} and the agonist-sensitive pool

To examine the effect of extracellular Ca^{2+} on substance P-induced Ins P_3 formation and persistent desensitization, cells were incubated in a Ca^{2+} -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 Ca^{2+} pool from parotid cells, such that, on subsequent application of substance P, neither Ca^{2+} entry nor internal Ca^{2+} 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 Ca^{2+} , 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 (Ca^{2+} -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) P_3

The time course of substance P-induced formation of Ins(1,4,5) P_3 and Ins(1,3,4) P_3 in rat parotid acinar cells analysed by h.p.l.c. is summarized in Fig. 1. The amount of Ins(1,4,5) P_3 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) P_3 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) P_3 , which is a metabolite produced from Ins(1,4,5) P_3 via the intermediate inositol 1,3,4,5-tetrakisphosphate (Ins P_4) (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,

Fig. 1. Time course of changes of [³H]inositol trisphosphates in parotid acinar cells stimulated by substance P (100 nM)

[³H]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 $\pm$ S.E.M. for three to ten independent experiments: ○, [³H]Ins(1,4,5)P₃; ●, [³H]Ins(1,3,4)P₃.

and reached a plateau at 120 s. As demonstrated in our previous report (Sugiyama *et al.*, 1987), these results imply that Ins(1,4,5)P₃ formation is rapidly activated and rapidly desensitized by substance P. Furthermore, we reported that substance P induced a persistent desensitization of the InsP₃ 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 Ins(1,4,5)P₃ in stimulated cells. In cells pre-

treated with substance P for 10, 30, 60 or 300 s (see the Experimental section), the InsP₃ 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 preincubation, the InsP₃ response to substance P was 27 % of control, and was decreased to 11 % (presumably the minimum) by 300 s. In substance P binding studies, preincubation with substance P also induced a similarly time-dependent decrease in $B_{\max}$ (Fig. 2b) without a change in K_D (Fig. 2b inset). After preincubation with substance P for 60 s, the number of binding sites ($B_{\max}$) 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 InsP₃ data. Nonetheless, the kinetics for loss of binding and response were similar; when the 300 s value was subtracted from each data point, the $t_{1/2}$ values for the rate of decline in the $B_{\max}$ of the substance P receptor and for loss of the substance P-induced InsP₃ 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 Ins(1,4,5)P₃ in substance P-stimulated cells; i.e., by 60 s, the Ins(1,4,5)P₃ 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 Ca²⁺ or the protein kinase C messenger systems, might be involved in regulating the substance P pathway was investigated.

Fig. 2. Time course of desensitization of substance P-induced [³H]InsP₃ formation and loss of [³H]substance P binding sites in cells pretreated with substance P (100 nM)

(a) [³H]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 [³H]InsP₃ content is shown as % of control, i.e. [³H]InsP₃ 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 [³H]substance P binding assay as described in the Experimental section. Inset shows plot of K_D versus time. Results are means $\pm$ S.E.M. from three to nine independent experiments.

Fig. 3. Lack of effect of Ca^{2+} on substance P-induced InsP_3 formation

To investigate the effects of extracellular Ca^{2+} , [^3H]-inositol-prelabelled cells were stimulated with substance P (100 nM) for 5 or 60 s in the presence (●) or absence (○) of extracellular Ca^{2+} . To investigate the roles of both extracellular Ca^{2+} and the agonist-sensitive intracellular Ca^{2+} pool, the cells were preincubated with methacholine (0.1 mM) for 5 min and with atropine (0.1 mM) for another 5 min in the presence (▲) or absence (△) of extracellular Ca^{2+} . The cells were then stimulated with substance P (100 nM) for 5 or 60 s. For each experiment, the radioactivity at zero time was subtracted. Results are means $\pm$ S.E.M. from four independent experiments.

Effects of Ca^{2+}

Ionomycin is a specific ionophore for Ca^{2+} (Liu & Herman, 1979), and induces Ca^{2+} influx as well as release of Ca^{2+} from an intracellular pool in rat parotid gland (Poggioli *et al.*, 1982). Therefore, to examine possible effects of Ca^{2+} mobilization on the desensitization of substance P-induced InsP_3 formation, cells were pretreated with ionomycin (1 μM) with the same protocol used previously for substance P. However, although this concentration would cause Ca^{2+} mobilization of a magnitude similar to that for substance P (Poggioli *et al.*, 1982), no desensitization of the substance P response occurred (control response to substance P, $0.416 \pm 0.012\%$ PI; after pretreatment with ionomycin, $0.408 \pm 0.010\%$ PI).

In the next experiments, the effects of Ca^{2+} entry and intracellular Ca^{2+} release on [^3H] InsP_3 formation and on the persistent desensitization induced by substance P were examined. To test the role of extracellular Ca^{2+} on substance P-induced InsP_3 formation, cells were stimulated by 100 nM substance P in the presence or absence of extracellular Ca^{2+} . To determine whether intracellular Ca^{2+} release might affect the response, cells were treated sequentially with methacholine and atropine in the presence or absence of extracellular Ca^{2+} and stimulated by substance P (100 nM), again in the presence or absence of extracellular Ca^{2+} (see the Experimental section). In the absence of extracellular Ca^{2+} , or after depletion of the agonist-sensitive intracellular Ca^{2+} pool, the increase in [^3H] InsP_3 was unaffected (Fig. 3). A similar protocol was used to assess the effects of Ca^{2+} mobilization on substance P-receptor desensitization. Cells, again treated

Table 1. Lack of effect of Ca^{2+} on the desensitization of substance P-induced InsP_3 formation

To investigate the effects of extracellular Ca^{2+} , [^3H]-inositol-prelabelled cells were stimulated with substance P (100 nM) for 5 or 60 s in the presence (+ Ca^{2+}) or absence (− Ca^{2+}) of extracellular Ca^{2+} . Cells were then washed and re-equilibrated, and [^3H] InsP_3 formation in response to a 5 s substance P stimulus was determined. To investigate the roles of both extracellular Ca^{2+} and the agonist-sensitive intracellular Ca^{2+} pool, the cells were preincubated with methacholine (0.1 mM) for 5 min and with atropine (0.1 mM) for another 5 min (Mec + Atp) in the presence or absence of extracellular Ca^{2+} , before conditioning with 100 nM substance P for 1 min. The cells were then washed, equilibrated and assayed for the development of persistent desensitization as in previous experiments. Controls were treated similarly, but were not pretreated with substance P (control). For each experiment, [^3H] InsP_3 radioactivity at zero time was subtracted. Results are means $\pm$ S.E.M. for three independent experiments.

	InsP_3			
	No pretreatment		Mec + Atp pretreatment	
	Control	Substance P	Control	Substance P
+ Ca^{2+}	0.484 ± 0.052	0.187 ± 0.027	0.506 ± 0.023	0.089 ± 0.016
− Ca^{2+}	0.492 ± 0.039	0.173 ± 0.026	0.498 ± 0.233	0.193 ± 0.010

with methacholine and atropine in the presence or absence of Ca^{2+} , 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 Ca^{2+} entry and intracellular Ca^{2+} release had no effect on the desensitization of substance P-induced InsP_3 formation (Table 1).

Effect of activation of protein kinase C

The hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C produces both $\text{Ins}(1,4,5)\text{P}_3$ 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] InsP_3 formation and on the development of persistent desensitization of InsP_3 formation. First, we studied the effect on substance P-induced InsP_3 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 InsP_3 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 InsP_3 reached a maximum within 10 s after exposure to substance P. In the cells treated with PDBu, the time course of substance P-induced InsP_3 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, InsP_3 formation was much less inhibited (Fig. 4b). These

Fig. 4. Effect of PDBu on the substance P-induced InsP_3 formation

(a) [^3H]Inositol-prelabelled cells were preincubated in the presence (○) or absence (●) of PDBu ($1\ \mu\text{M}$) for 10 min, and in the continuous presence of the phorbol ester, stimulated with substance P ($100\ \text{nM}$) for the indicated intervals. (b) Cells preincubated in the presence (○) or absence (●) of PDBu ($1\ \mu\text{M}$) for 10 min were washed in ice-cold KRB solution, and resuspended in the fresh KRB solution as described for previous experiments on desensitization of substance P-induced InsP_3 formation. The cells were stimulated with substance P ($100\ \text{nM}$) for the indicated intervals. Results are means $\pm$ S.E.M. for three independent experiments.

results suggest that activation of protein kinase C by PDBu may inhibit substance P-induced InsP_3 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 InsP_3 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 $20\ \mu\text{M}$); the potency was $\text{TPA} > \text{PDBu} \gg \text{PDD}$, 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 $1\ \mu\text{M}$. 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 ($1\ \mu\text{M}$), neither B_{max} nor K_D of substance P binding was affected.

The effect of PDBu on the induction of persistent

Fig. 5. Concentration-dependence of phorbol ester effects on substance P-induced InsP_3 formation

[^3H]Inositol-prelabelled cells were pretreated with the indicated concentrations of PDBu (○), TPA (●) or PDD (△) for 10 min. The cells were then stimulated by substance P ($100\ \text{nM}$) for 5 s. For each experiment, the content of [^3H] InsP_3 is shown as % of control, i.e. the content in the cells pretreated for 10 min without PDBu. Results are means $\pm$ S.E.M. for three independent experiments.

Table 2. Lack of effect of PDBu on [^3H]substance P binding

Cells were incubated in the presence of PDBu ($1\ \mu\text{M}$) or DMSO (vehicle) for 10 min, immediately cooled to $4\ ^\circ\text{C}$, and assayed for [^3H]substance P binding ('Presence'), or they were pretreated with PDBu ($1\ \mu\text{M}$) or DMSO for 10 min, washed, and resuspended in fresh KRB solution. After incubation at $37\ ^\circ\text{C}$ for an additional 10 min, the cells were cooled to $4\ ^\circ\text{C}$ and used for the assay of [^3H]substance P binding ('Pretreated'). Results are means $\pm$ S.E.M. from six independent experiments.

Treatment	B_{max} (fmol/mg of protein)	K_D (nM)
Presence		
DMSO	247 ± 13	2.19 ± 0.07
PDBu	251 ± 20	2.12 ± 0.26
Pretreated		
DMSO	240 ± 13	2.40 ± 0.15
PDBu	248 ± 23	2.13 ± 0.21

desensitization of the InsP_3 response by substance P was examined next (Table 3). The persistent desensitization was neither potentiated nor inhibited by PDBu ($1\ \mu\text{M}$). 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

[³H]Inositol-prelabelled cells were preincubated with PDBu (1 μ M) or DMSO (vehicle) for 10 min, and then stimulated with substance P (100 nM) for 1 min. The cells were washed and resuspended in fresh KRB solution. The cells were incubated at 37 °C for 10 min, and then stimulated by substance P (100 nM) for 5 s to assess the development of persistent desensitization. For each experiment, [³H]InsP₃ radioactivity at zero time was subtracted. Results are means $\pm$ S.E.M. for three independent experiments.

Treatment	[³ H]InsP ₃ (% PI)
Control (DMSO)	0.418 $\pm$ 0.007
DMSO + substance P	0.124 $\pm$ 0.007
PDBu	0.329 $\pm$ 0.025
PDBu + substance P	0.129 $\pm$ 0.041

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 InsP₃ 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 InsP₃ 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)P₃ 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 Ca²⁺-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 Ca²⁺ entry or Ca²⁺ release indicated that Ca²⁺ 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 Ca²⁺ mobilization, whether derived from entry or from intracellular release (Weiss *et al.*, 1982). By similar reasoning, the results in Table 1 indicate that neither Ca²⁺ entry nor Ca²⁺ release mediates or modifies the development of persistent desensitization.

On the other hand, substance P-induced InsP₃ 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 InsP₃ 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 Ins(1,4,5)P₃ 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 α -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 β -adrenergic receptor systems. For example, evidence indicates that receptor phosphorylation and subsequent sequestration mediates the homologous desensitization of the β -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; Tohmatu *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 DDT₁MF-2 cells, phorbol esters promoted the phosphorylation of α_1 -adrenergic receptors in parallel with inhibition of their function (Leeb-Lundberg *et al.*, 1987). In the present study, however, phorbol ester did not affect [³H]substance 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 phosphoinositide hydrolysis in 1321N1 astrocytoma cells, since the stimulatory effect of guanosine 5'-[γ -thio]triphosphate on InsP_3 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'-[γ -thio]triphosphate, a non-hydrolysable analogue of GTP, activates InsP_3 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 $\text{Ins}(1,4,5)\text{P}_3$ to $\text{Ins}(1,4)\text{P}_2$ 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 InsP_2 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 $\text{Ins}(1,4,5)\text{P}_3$ 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

- Aub, D. L. & Putney, J. W., Jr. (1985) *Biochem. J.* **225**, 263–266
- Aub, D. L., McKinney, J. S. & Putney, J. W., Jr. (1982) *J. Physiol. (London)* **331**, 557–565
- Benovic, J. L., Mayor, F., Staniszewski, C., Lefkowitz, R. J. & Caron, M. G. (1987a) *J. Biol. Chem.* **262**, 9026–9032
- Benovic, J. L., Strasser, R. H., Caron, M. G. & Lefkowitz, R. J. (1987b) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2797–2801
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berridge, M. J., Dawson, R. M., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Brown, K. D., Blakeley, D. M., Hamon, M. H., Laurie, M. S. & Corps, A. N. (1987) *Biochem. J.* **245**, 631–639
- Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F. & Putney, J. W., Jr. (1984) *Nature (London)* **309**, 63–66
- Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. & Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Connolly, T. M., Lawing, W. J., Jr. & Majerus, P. W. (1986) *Cell* **46**, 951–958
- Cooper, R. H., Coll, K. E. & Williamson, J. R. (1987) *J. Biol. Chem.* **260**, 3281–3288
- Cuello, A. C., Priestly, J. V. & Matthews, M. R. (1982) *Substance P in the Nervous System: Ciba Found. Symp.* **91**, 51–83
- Garcia-Sainz, J. A. & Hernandez-Sotomayor, S. M. T. (1987) *Eur. J. Pharmacol.* **163**, 417–421
- Hanley, M. R., Lee, C. M., Jones, L. M. & Michell, R. H. (1980) *Mol. Pharmacol.* **18**, 78–83
- Irvine, R. F., Letcher, A. J., Lander, D. J. & Downes, C. P. (1984) *Biochem. J.* **223**, 237–243
- Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
- Kikkawa, U. & Nishizuka, Y. (1987) *Annu. Rev. Cell Biol.* **2**, 149–178
- Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 2273–2276
- Leeb-Lundberg, L. M. F., Cotecchia, S., DeBlasi, A., Caron, M. G. & Lefkowitz, R. J. (1987) *J. Biol. Chem.* **262**, 3098–3105
- Liles, W. C., Hunter, D. D., Meier, K. E. & Nathanson, N. M. (1986) *J. Biol. Chem.* **261**, 5307–5313
- Liu, C.-M. & Herman, T. E. (1979) *J. Biol. Chem.* **253**, 5892–5894
- Mantyh, P. W., Pinnock, R. D., Downes, C. P., Goedert, M. & Hunt, S. P. (1984) *Nature (London)* **309**, 795–797
- Niedel, J. E. & Blackshear, P. J. (1986) in *Phosphoinositides and Receptor Mechanisms* (Putney, J. W., Jr., ed.), pp. 47–88, Alan R. Liss, New York
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Orellana, S., Solski, P. A. & Brown, J. H. (1987) *J. Biol. Chem.* **262**, 1638–1643
- Poggioli, J., Leslie, B. A., McKinney, J. S., Weiss, S. J. & Putney, J. W., Jr. (1982) *J. Pharmacol. Exp. Ther.* **221**, 247–253
- Putney, J. W., Jr., McKinney, J. S., Aub, D. L. & Leslie, B. A. (1984) *Mol. Pharmacol.* **26**, 261–266
- Sha'afi, R. I., Molski, T. F. P., Huang, C.-K. & Naccache, P. H. (1986) *Biochem. Biophys. Res. Commun.* **137**, 50–60
- Sibley, D. R. & Lefkowitz, R. J. (1985) *Nature (London)* **317**, 124–129
- Smith, C. D., Uhing, R. J. & Snyderman, R. (1987) *J. Biol. Chem.* **262**, 6121–6127
- Strasser, R. H., Sibley, D. R. & Lefkowitz, R. J. (1986) *Biochemistry* **25**, 1371–1377
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–68
- Sugiyama, H., Tennes, K. A. & Putney, J. W., Jr. (1987) *Biochem. J.* **244**, 647–653

- Taylor, C. W. & Merritt, J. E. (1986) *Trends Pharmacol. Sci.* **7**, 238–242
- Taylor, C. W., Merritt, J. E., Putney, J. W., Jr. & Rubin, R. P. (1986) *Biochem. Biophys. Res. Commun.* **136**, 362–368
- Tohmatsu, T., Hattori, H., Nagao, S., Ohki, K. & Nozawa, Y. (1986) *Biochem. Biophys. Res. Commun.* **134**, 868–875
- Watson, S. P. & Downes, C. P. (1983) *Eur. J. Pharmacol.* **93**, 245–253
- Watson, S. P., Sandberg, B. E. B., Hanley, M. R. & Iversen, L. L. (1983) *Eur. J. Pharmacol.* **87**, 77–84
- Weiss, S. J., McKinney, J. S. & Putney, J. W., Jr. (1982) *Biochem. J.* **206**, 555–560

Received 17 November 1987/23 February 1988; accepted 28 March 1988