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Protein kinase C-dependent and -independent mechanisms regulating the parotid substance P receptor as revealed by differential effects of protein kinase C inhibitors

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Substance P-induced inositol trisphosphate ($InsP_3$) formation was inhibited by 1 μM-4β-phorbol 12,13-dibutyrate (PDBu) in rat parotid acinar cells. The inhibitory effect of PDBu was reversed by the protein kinase C inhibitors H-7 or K252a. Substance P also elicits a persistent desensitization of subsequent substance P-stimulated $InsP_3$ formation. However, this desensitization was not inhibited by H-7. In addition, H-7 had no effect on the time course of substance P-induced $InsP_3$ formation. These results suggest that, although activation of protein kinase C by phorbol esters can inhibit the substance P receptor-linked phospholipase C pathway, this mechanism apparently plays little, if any, role in regulating this system after activation by substance P.

INTRODUCTION

Activation of a family of Ca^{2+}-mobilizing receptors stimulates phospholipase C-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (Berridge, 1984). These products of phosphoinositide hydrolysis have been shown to be important intracellular messengers. Ins(1,4,5)P_3 acts at specific intracellular sites to initiate the release of sequestered Ca^{2+}, and diacylglycerol stimulates the activity of protein kinase C (Berridge, 1984).

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, substance P receptor activation induces the hydrolysis of phosphinositides (Hanley *et al.*, 1980; Weiss *et al.*, 1982) and the formation of Ins(1,4,5)P_3 (Sugiya *et al.*, 1987). Consequently, Ins(1,4,5)P_3 increases cytosolic [Ca^{2+}] (Merritt & Rink, 1987), which in turn stimulates K^+ release (Putney, 1977). Diacylglycerol triggers protein kinase C, which, together with Ca^{2+}, activates protein discharge (Putney *et al.*, 1984). This latter effect can be mimicked by pharmacological activators of protein kinase C, such as phorbol diesters (Putney *et al.*, 1984).

Previous studies have suggested that the parotid substance P receptor may be regulated by a homologous mechanism involving disappearance of surface membrane substance P receptors (Sugiya *et al.*, 1987). In addition, phorbol esters appear to inhibit substance P-induced $InsP_3$ formation at a step after receptor activation. Thus it has been suggested that activation of protein kinase C may also contribute to regulation of the substance P receptor–phospholipase C pathway in parotid acinar cells (Sugiya *et al.*, 1988). In the present work, the effects of inhibitors of protein kinase C on parotid acinar-cell responses to substance P and to 4β-phorbol 12,13-dibutyrate (PDBu) were investigated. The results indicate that, although activation of protein kinase C can inhibit the substance P receptor–phospholipase C pathway, this mechanism probably plays little, if any, role in the regulation of the substance P receptor after agonist activation.

MATERIALS AND METHODS

Materials

myo-[2-^{3}H(n)]Inositol (10–20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). Substance P was from Peninsula Laboratories (Belmont, CA, U.S.A.). 1-(5-Isoquinoline sulphonyl)-2-methylpiperazine (H-7) was from Seikagaku America Inc. (St. Petersburg, FL, U.S.A.). K252a was kindly provided by Dr. H. Kase (Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Tokyo, Japan). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

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).

Measurement of [^{3}H]InsP_3 formation

Substance P-induced [^{3}H]InsP_3 formation was measured as previously described (Sugiya *et al.*, 1987). Briefly, the cells were incubated in Krebs–Ringer bicarbonate (KRB) solution containing *myo*-[2-^{3}H]inositol (30 μCi/ml) for 90 min, after which they were washed by centri-

Abbreviations used: Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; $InsP_3$, inositol trisphosphate; PI, phosphoinositides; protein kinase C, Ca^{2+}- and phospholipid-dependent protein kinase; PDBu, 4β-phorbol 12,13-dibutyrate; KRB, Krebs–Ringer bicarbonate solution.

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fugation (at 50 *g* for 5 min) and suspended in non-radioactive KRB solution. After a further 15 min incubation, drugs were added to the cell suspension, and then 0.3 ml portions of the cell suspension were taken for analysis at appropriate times. The reaction was stopped by addition of the samples to 4.5% (w/v) $HClO_4$. The samples were neutralized and applied to anion-exchange columns by the methods previously described (Berridge *et al.*, 1983; Aub & Putney, 1984). The material eluted with 1.0 M-ammonium formate is designated as InsP_3, since isomers are not separated by this procedure; however, for short periods of stimulation the substance P-induced increase in ^3H in this fraction has been shown to be > 90% Ins(1,4,5)P_3 (Sugiya *et al.*, 1988). Samples were also taken and extracted with chloroform/methanol/HCl (Sugiya *et al.*, 1987), and the extracts were assayed for ^3H content to determine the radioactivity associated with inositol lipids, so that InsP_3 contents could be expressed as a percentage of [^3H]phosphoinositides (%PI). Basal [^3H]InsP_3 calculated in this way was 0.1–0.2 %PI, corresponding to 1000–2000 c.p.m./sample, and this was generally increased 2.5–3-fold by addition of substance P for 5 s. For each experiment, determinations were carried out in duplicate, and the number of replications reported in the Results section refers to the number of independent experiments.

Desensitization induced by substance P

[^3H]Inositol-labelled cells were incubated with substance P (100 nM) for 1 min. The cell suspension was diluted by a 400-fold volume 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 cells were resuspended in fresh KRB solution and incubated at 37 °C for 10 min. Then the cells were stimulated with substance P (100 nM) for 5 s, and the reaction was terminated by addition of $HClO_4$ for determination of [^3H]InsP_3.

RESULTS

When parotid acinar cells were stimulated with substance P (100 mM) for 5 s, cellular [^3H]InsP_3 increased by 2.5–3-fold. Preincubation with PDBu (1 μM) for 10 min inhibited substance P-induced [^3H]InsP_3 by about 50% (Table 1), confirming previous findings (Sugiya *et al.*, 1988).

H-7, an isoquinolinesulphonamide derivative, has been shown to be a potent inhibitor of protein kinase C (Hidaka *et al.*, 1984; Kawamoto & Hidaka, 1984). The inhibitory effect of PDBu on [^3H]InsP_3 formation was reversed by H-7 in a dose-dependent manner (Table 1). Another putative protein kinase C inhibitor, K252a, isolated from culture broth of *Nocardiopsis* sp. (Yamada *et al.*, 1987), also inhibited the effect of PDBu (Table 1). Neither drug affected the basal amount of [^3H]InsP_3 at any of the concentrations tested. These results are consistent with the previous conclusion that the inhibition of substance P-induced InsP_3 formation by phorbol esters is due to activation of protein kinase C (Sugiya *et al.*, 1988).

In cells pre-exposed to substance P (100 nM) for 1 min, the effect of a subsequent challenge with substance P on InsP_3 formation was dimished by 80% (Table 2). As shown previously (Sugiya *et al.*, 1987), this inhibition results from the development of a persistent, apparently homologous, desensitization of the substance P receptor. The ability of substance P to induce desensitization was not inhibited when cells were preincubated with H-7 (50 μM) for 3 min before the initial conditioning incubation with substance P (Table 2). These data thus support the previous conclusion that substance P-induced persistent desensitization of InsP_3 formation does not involve activation of protein kinase C.

An alternative role for protein kinase C-induced inhibition of the substance P response is that it could represent a negative-feedback mechanism which would contribute to the slowing of [^3H]InsP_3 formation seen when the time course of substance P stimulation is followed (Sugiya *et al.*, 1987). Thus the time course of substance P-induced [^3H]InsP_3 formation after preincubation with, and in the continued presence of, H-7 was examined (Fig. 1). H-7 had no effect on either the rapid phase of InsP_3 formation induced by substance P, or on the secondary maintained amount of InsP_3, by which time significant desensitization of the InsP_3-forming capacity of the cells had occurred (Sugiya *et al.*, 1988).

Table 1. Effect of H-7 and K252a on the inhibition of substance P-mediated InsP_3 induced by PDBu

The cells prelabelled with [^3H]inositol were preincubated without or with H-7 or K252a for 3 min and PDBu (1 μM) for another 10 min. The cells were stimulated by substance P (100 nM) for 5 s. The response to substance P without PDBu is indicated as 100%. In each case, paired incubations were used to determine basal InsP_3 formation, and these values were subtracted. Neither H-7 nor K252a had any effect on basal InsP_3 amounts at any of the concentrations tested. Results are means ± S.E.M. for three independent experiments.

H-7		K252a	
Concn. (μM)	Response to substance P (% of control response)	Concn. (μM)	Response to substance P (% of control response)
0	53.0±0.4	0	57.8±6.0
10	70.5±6.1	1	82.1±4.8
25	74.8±6.4	5	87.7±3.4
50	92.7±9.0	10	91.7±2.0

Table 2. Substance P-induced persistent desensitization of InsP_3 formation in the presence or absence of H-7

The [^3H]inositol-labelled cells pretreated without (Control) or with H-7 (50 μM) for 3 min were preincubated without (Control) or with 100 nM-substance P (SP pretreatment) for 1 min. After washing and resuspension as described in the Materials and methods section, the cells were stimulated by substance P (100 nM) for 5 s. Results are means ± S.E.M. for three independent experiments.

	[^3H]InsP_3 (% [^3H]PI)	
	Control	+H-7
Control	0.47±0.03	0.49±0.05
SP pretreatment	0.12±0.00	0.13±0.02

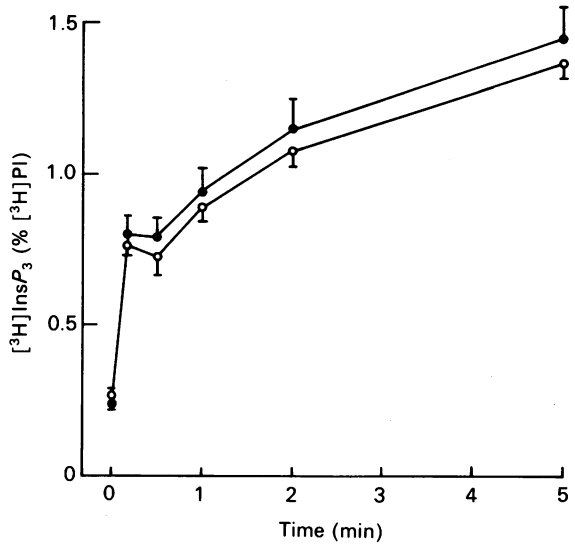

Fig. 1. Time course of substance P-induced InsP_3 formation in the presence or absence of H-7

After preincubation without (●) or with (○) H-7 (50 μM), the cells prelabelled with [^{3}H]inositol were stimulated by substance P (100 nM). Results are means ± S.E.M. for seven independent experiments.

DISCUSSION

Substance P induces both InsP_3 formation and the desensitization of this response in rat parotid acinar cells (Sugiya *et al.*, 1987). In previous studies, the desensitization of substance P-induced InsP_3 formation was correlated with an apparent loss of surface-membrane substance P binding sites (Sugiya *et al.*, 1987). Furthermore, it appears that the persistent densensitization of InsP_3 formation induced by substance P is of the homologous type (Sibley & Lefkowitz, 1985; Sugiya *et al.*, 1987). This suggests that components of this signalling system, common to the different receptor pathways such as protein kinase C, would not be involved. Thus the data reported here with the protein kinase C inhibitors are consistent with this conclusion, since persistent densitization of substance P-induced InsP_3 formation was not inhibited by H-7 (Table 2). This finding also further underscores the concept that substance *P*-induced persistent desensitization occurs by a different mechanism from the densitization seen after treatment with phorbol esters (Sugiya *et al.*, 1988).

However, in this and in previous studies, protein kinase C activators (phorbol esters) have been shown to inhibit partially substance P-induced InsP_3 formation. This inhibitory effect of PDBu was reversed by the protein kinase C inhibitors H-7 or K252a (Table 1); similar results have been reported for other receptor systems (Garcia-Sainz & Hernandez-Sotomayor, 1987; Tohmatsu *et al.*, 1986; Sha'afi *et al.*, 1986). These results have been interpreted to mean that the phospholipase C-linked substance P receptor might be regulated by protein kinase C acting like a negative feedback, rather than as part of the mechanism of persistent desensitization. Since protein kinase C can presumably be activated through any of several receptor types on the parotid acinar cells, this mechanism would by definition appear to be heterologous (Sibley & Lefkowitz, 1985). In a previous report (Sugiya *et al.*, 1988), it was suggested that such a protein kinase C-mediated negative feedback might explain, at least in part, the rapid decrease in inositol phosphate production seen in parotid acinar cells in the continued presence of substance P. However, in the present studies, when cells were pretreated with H-7 under conditions where phorbol ester effects would be almost completely reversed, the time course of InsP_3 formation in response to substance P was unaffected. If the slowing of inositol phosphate production after 30 s to 1 min were due to a negative feedback mediated by protein kinase C, H-7 should have potentiated the rise in InsP_3, especially at later times of stimulation. Thus, despite the clear potential for inhibition of this pathway by protein kinase C, as revealed by the inhibitory effects of phorbol esters, such a mechanism appears to contribute little, if at all, to the regulation of the response when cells are activated by the physiological agonist, substance P. This may indicate that a greater degree of protein kinase C activation is necessary to achieve inhibition than is possible when receptors are activated with substance P.

Studies in a number of laboratories have clearly demonstrated, by use of phorbol diester drugs, the ability of protein kinase C to inhibit receptor signalling pathways (Smith *et al.*, 1987; Orellana *et al.*, 1987; Labarca *et al.*, 1984; Muldoon *et al.*, 1987; Osugi *et al.*, 1987; Cooper *et al.*, 1987; Liles *et al.*, 1986; Kikuchi *et al.*, 1987; Johnson *et al.*, 1986; Toews *et al.*, 1987). The findings reported here suggest that it may be incorrect, or at least premature, to conclude that such a mechanism contributes to regulation of these signalling pathways during agonist stimulation. At least for the parotid substance P receptor, it would appear that a homologous mechanism, not involving protein kinase C, may be the predominant mode of regulation.

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