Role of protein kinase C in the regulation of histamine and bradykinin stimulated inositol polyphosphate turnover in adrenal chromaffin cells

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1 The possibility that bradykinin- or histamine-stimulated inositol polyphosphate accumulation may be regulated by protein kinase C (PKC) in bovine adrenal chromaffin cells has been addressed.

2 Initial experiments confirmed that the phorbol ester 12-O-tetradecanoyl-phorbol 13-acetate (TPA) dramatically inhibited agonist-stimulated [³H]-inositol phosphate accumulations in [³H]-inositol prelabelled cells. In contrast, the PKC inhibitor, Ro 31-8220, did not affect this response.

3 Histamine (100 µM) or bradykinin (100 nM) evoked rapid increases in inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) mass accumulations (maximal accumulations within 10 s and 30 s, respectively) which declined towards basal values over a 10 min incubation period. TPA (1 µM) significantly attenuated the peak Ins(1,4,5)P₃ response to bradykinin and histamine by 30% and 70% respectively. In contrast, TPA did not significantly affect agonist-stimulated Ins(1,3,4,5)P₄ responses.

4 Ro 31-8220 (10 µM) significantly enhanced the maximal Ins(1,4,5)P₃ accumulations elicited by both bradykinin and histamine.

5 The results indicate that the initial Ins(1,4,5)P₃ response to either bradykinin or histamine in bovine adrenal chromaffin cells can be attenuated by PKC activation by phorbol ester and enhanced by PKC inhibition by Ro 31-8220. In contrast, agonist-stimulated Ins(1,3,4,5)P₄ accumulation does not appear to be affected by these manipulations of PKC activity. Possible bases for differential modulation of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ are discussed.

Keywords: Inositol polyphosphates; protein kinase C; histamine; bradykinin; phorbol ester; Ro 31-8220; adrenal chromaffin cell

Introduction

The role and regulation of agonist-stimulated hydrolysis of inositol phospholipids in neuronal function is poorly understood. This is true for one of the most widely studied neurone-like cells in culture, the bovine adrenal chromaffin cell, in which a variety of agonists stimulate both polyphosphoinositide hydrolysis and catecholamine release (Livett & Marley, 1986; Eberhard & Holz, 1987; Plevin & Boarder, 1988; Plevin *et al.*, 1990; Burgoyne, 1991; Challiss *et al.*, 1991). One such agonist, bradykinin, has been shown to stimulate inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) accumulation and to cause an elevation of free cytosolic Ca²⁺, in part, by mobilization of intracellular pools (O'Sullivan & Burgoyne, 1989; Challiss *et al.*, 1991). However, the role this plays in stimulus-secretion coupling is unclear, since release of noradrenaline in response to bradykinin appears to be dependent upon agonist-stimulated Ca²⁺ influx via a cadmium-sensitive, dihydropyridine-insensitive route (Owen *et al.*, 1989).

The role of agonist-stimulated inositol phospholipid-specific phospholipase C (PLC) may be related to protein kinase C (PKC) stimulation via the formation of *sn* 1,2-diacylglycerol (DAG). One of several possible consequences for the activation of this arm of the second messenger pathway is the presence of a short inhibitory feedback loop in which stimulated DAG accumulation and PKC activity cause an attenuation of agonist-stimulated PLC. This mechanism has been proposed for the regulation of agonist-stimulated PLC in a number of non-neuronal cells (Helper *et al.*, 1988; King & Rittenhouse, 1989; Pfeilschifter *et al.*, 1989). We, and others, have previously shown that activation of PKC does attenuate histamine-stimulated formation of total [³H]-

inositol phosphates in [³H]-inositol prelabelled adrenal chromaffin cells (Wan *et al.*, 1989; Jones *et al.*, 1990).

In this study we have investigated the effects of altering PKC activity upon agonist stimulation of inositol polyphosphate accumulation using both the PKC activator 12-O-tetradecanoylphorbol 13-acetate (TPA) and the selective PKC-inhibitor Ro 31-8220 (referred to as compound 3 in Davis *et al.*, 1989). We have measured the response to stimulation by bradykinin and histamine of Ins(1,4,5)P₃ and the putative second messenger inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) concentrations by the mass assays previously described for these inositol polyphosphate isomers (Challiss *et al.*, 1988; Challiss & Nahorski, 1990).

Methods

Cell culture

Bovine adrenal glands were obtained from a local abattoir within 30 min of slaughter. Cells from the medulla were dissociated by digestion with collagenase/protease, and the chromaffin cells purified (routinely to 80% or greater of total cell number) by differential centrifugation and differential plating as described earlier (Owen *et al.*, 1989). Cells were cultured at 0.5 × 10⁶ cells per well on 24-well primary plates at a cell protein concentration of about 50 µg/well. Cells were used after 4–7 days in culture.

[³H]-inositol labelling and [³H]-inositol phosphate determination

For 48 h preceding the experiments, cells were cultured in M199 medium (0.5 ml) containing 37 kBq [³H]-inositol per

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well and supplemented with streptomycin ($50 \mu\text{g ml}^{-1}$), penicillin (50 i.u. ml^{-1}) and cytosine arabinoside ($5 \mu\text{M}$). Cells were then incubated for 10 min in a balanced salt solution (BSS (in mM): NaCl 125, KCl 5.4, NaHCO_3 16.2, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid 15, NaH_2PO_4 1, MgSO_4 0.8, CaCl_2 1.8 and glucose 5.5) pH 7.4 following oxygenation with O_2/CO_2 (19:1). This medium was removed and the same BSS supplemented with 10 mM LiCl used for the incubations (30 min). Reactions were terminated with cold methanol, followed by chloroform extraction and the total [^3H]-inositol phosphate ([^3H]- InsP_x) fraction recovered by use of Dowex-1 (Cl^- form) as described by Rooney & Nahorski (1986).

Measurement of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ accumulations

Cells cultured as described above were washed twice with the BSS solution prewarmed to 37°C . Preincubations were for 15 min, followed by incubation for the time periods stated in the Results section. Drugs present during the preincubation (TPA and/or Ro 31-8220) were also present for the period of stimulation with histamine or bradykinin (or control). Reactions were terminated by rapid removal of the incubation medium followed by immediate replacement with 0.5 ml of ice-cold trichloroacetic acid (0.5 M). Wells were thoroughly scraped and the recovered extract centrifuged (10,000 g, 4 min). The acid extract was washed with 3×3 vol of water-saturated diethylether, and the extract buffered to pH 7 with NaHCO_3 . Radioreceptor-based mass assays for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were performed as described previously (Challiss *et al.*, 1988; Challiss & Nahorski, 1990).

Statistical analysis

Data are expressed as means $\pm$ s.e.mean for the indicated number of experiments performed on different preparations of chromaffin cells. Time-course data were analyzed by unpaired Student's *t* tests. Concentration-response data were analyzed by one-way analysis of variance; where the *F* ratio gave $P < 0.05$, comparisons between individual groups were made by Duncan's multiple range test at significance levels of 0.05 and 0.01.

Materials

Myo-[2- ^3H]-inositol (17 Ci mmol^{-1}) was purchased, whilst [^3H]- $\text{Ins}(1,4,5)\text{P}_3$ (17 Ci mmol^{-1}) and [^3P]- $\text{Ins}(1,3,4,5)\text{P}_4$ ($100\text{--}200 \text{ Ci mmol}^{-1}$) were obtained as gifts, from DuPont (U.K.) Ltd. (Stevenage, Herts.). Bradykinin was from Cambridge Research Biochemicals Ltd. (Northwich, Cheshire). Ro 31-8220, a bis indolylmaleimide derivative whose structure is given by Davis *et al.* (1989), was a kind gift from Dr. G. Lawton, Roche Products Ltd. (Welwyn Garden City, Herts.). Cell culture supplies were from GIBCO (Paisley, Scotland) and other chemicals and drugs were from Sigma Chemical Co. Ltd. (Poole, Dorset) or Fisons plc (Loughborough, Leics.).

Results

Effect of TPA and Ro 31-8220 on agonist-stimulated [^3H]- InsP_x accumulation

In agreement with our previous studies (Plevin & Boarder, 1988; Jones *et al.*, 1990), preliminary experiments demonstrated that exposure of chromaffin cells to 100 nM bradykinin or 100 μM histamine for 30 min resulted in 2–3 fold and 5–7 fold increases in [^3H]- InsP_x accumulations,

Figure 1 Effects of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) and Ro 31-8220 on total [^3H]-inositol phosphate ([^3H]- InsP_x) responses to bradykinin and histamine in chromaffin cells. (a) Concentration-response relationship for the effect of Ro 31-8220 on [^3H]- InsP_x accumulation at 30 min in the absence (O) or presence of bradykinin (100 nM: ●) or histamine (100 nM: ■). (b) Concentration-response relationship for the effect of 30 min exposure to histamine in the absence (O) or presence of TPA (1 μM : ▲) or Ro 31-8220 (10 μM : △). Values are shown as means $\pm$ s.e.mean (vertical bars) for a representative experiment of three separate experiments performed in triplicate.

respectively (Figure 1a).

The [^3H]- InsP_x accumulation elicited in response to either agonist was dramatically attenuated in the presence of 1 μM TPA; in contrast, the protein kinase C inhibitor Ro 31-8220 (at concentrations up to 50 μM) had little effect on basal, histamine- or bradykinin-stimulated [^3H]- InsP_x accumulation (Figure 1a). Time-course studies confirmed these effects of TPA and Ro 31-8220; for example, 1 μM TPA significantly attenuated the histamine-stimulated [^3H]- InsP_x response at the earliest time-point investigated (5 min) and caused an 85–95% inhibition of this response at 30 min after histamine challenge (data not shown). Similarly, concentration-response relationships for histamine-stimulated [^3H]- InsP_x accumulation demonstrated the inhibitory action of TPA at all stimulatory concentrations of histamine (1–100 μM), whilst Ro 31-8220 (10 μM) had no significant effect (Figure 1b).

It should be noted that despite the lack of effect of Ro 31-8220, this agent was able to antagonize completely the inhibitory action of TPA on histamine-stimulated [^3H]- InsP_x accumulation (data expressed as a % of the control response (100 ± 8): 100 μM histamine 724 ± 70 ; histamine + TPA (1 μM) 189 ± 18 ; histamine + TPA + Ro 31-8220 (10 μM) 744 ± 67 , for 2 experiments performed in quadruplicate).

Modification of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ responses

We have previously characterized the rapid, transient changes in both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ mass accumulations

elicited by maximally effective concentrations of bradykinin and histamine in cultured bovine adrenal chromaffin cells (Challiss *et al.*, 1991): since publication of our original data, a number of studies have reported a biphasic accumulation of $\text{Ins}(1,4,5)\text{P}_3$ mass, where the initial, rapid rise and fall in $\text{Ins}(1,4,5)\text{P}_3$ level is followed by a secondary increase in $\text{Ins}(1,4,5)\text{P}_3$ over the subsequent time-course of continued agonist exposure (Roche *et al.*, 1991; Tobin *et al.*, 1992). For example, Roche *et al.* (1991) have reported that gastrin stimulates an initial $\text{Ins}(1,4,5)\text{P}_3$ accumulation in parietal cells which is maximal at 15 s and decreases towards pre-stimulation levels by 30 s; however, over the subsequent 4–5 min period of continued agonist exposure the $\text{Ins}(1,4,5)\text{P}_3$ level increases to, or beyond, the 15 s peak value. Some evidence for this unusual response has also been presented recently by Stauderman & Pruss (1990) for histamine-stimulated $[\text{H}]\text{-Ins}(1,4,5)\text{P}_3$ accumulation in $[\text{H}]\text{-inositol}$ pre-labelled bovine adrenal chromaffin cells. We have therefore carried out preliminary extended time-course studies to ascertain whether a similar biphasic inositol polyphosphate mass accumulation occurs in response to bradykinin or histamine.

The results summarized in Table 1 substantiate our previous data with regard to the relative stimulatory effects of bradykinin and histamine on $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation, and indicate that no secondary increases, subsequent to the detailed 0–120 s time courses reported previously (and see Figures 2 and 3) are observed. Thus, bradykinin (100 nM) elicited an 156% increase in $\text{Ins}(1,4,5)\text{P}_3$ accumulation at 10 s and the accumulation of this metabolite then declined, such that by 10 min after agonist addition the $\text{Ins}(1,4,5)\text{P}_3$ accumulation was not significantly elevated above basal levels. Although histamine (100 μM) evoked a smaller 51% increase in $\text{Ins}(1,4,5)\text{P}_3$, this metabolite was maintained at a significantly elevated level over the 10 min investigation period. The transient nature of the agonist-stimulated $\text{Ins}(1,3,4,5)\text{P}_4$ response was also well-illustrated by these data. Thus, bradykinin and histamine evoked respectively 675% and 250% increases in $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation at 30 s which returned to levels not significantly different from basal values by 5 min post-agonist addition.

For further investigations to evaluate the effects of agents which alter PKC activity, we have concentrated on the initial 2 min period after addition of the agonist. The effect of 1 μM TPA upon the time-course of bradykinin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation is shown in Figure 2. TPA significantly inhibited the $\text{Ins}(1,4,5)\text{P}_3$ response, producing a 30% attenuation of the maximal increase in mass accumulation. In contrast, TPA was without effect on the $\text{Ins}(1,3,4,5)\text{P}_4$ response elicited by bradykinin (Figure 2b). TPA exerted a more profound effect upon histamine-stimulated $\text{Ins}(1,4,5)\text{P}_3$ accumulation producing a 70% attenuation of the maximal response observed 10 s after

Figure 2 Effect of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) on bradykinin-stimulated inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) (a) and inositol 1,3,4,5-tetrakisphosphate ($\text{Ins}(1,3,4,5)\text{P}_4$) (b) mass accumulations in chromaffin cells. Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the absence (○, ●) or presence of 1 μM TPA (□, ■). Additions of 100 nM bradykinin (●, ■) or vehicle (○, □) were made at zero-time. Values are means $\pm$ s.e.mean (vertical bars) from either 3 ($\text{Ins}(1,3,4,5)\text{P}_4$) or 4 ($\text{Ins}(1,4,5)\text{P}_3$) separate experiments performed in triplicate.

Statistically significant differences between cell responses in the presence and absence of TPA are indicated as * $P < 0.05$, ** $P < 0.01$.

agonist addition (Figure 3a); despite this dramatic attenuation of the $\text{Ins}(1,4,5)\text{P}_3$ response, TPA again failed to exert any significant effect on agonist-stimulated $\text{Ins}(1,3,4,5)\text{P}_4$ accumulation (Figure 3b).

The effects of increasing concentrations of TPA on $\text{Ins}(1,4,5)\text{P}_3$ accumulation stimulated by bradykinin, histamine or control additions to chromaffin cells for 10 s are shown in Figure 4. Statistically significant attenuations of the bradykinin-stimulated response were observed in the presence of 100 nM TPA (31%) and 1 μM TPA (38%), whereas 1 μM TPA caused a 64% attenuation of the histamine-stimulated $\text{Ins}(1,4,5)\text{P}_3$ response. In contrast, the relatively inactive phorbol ester 4-methoxy TPA failed to affect bradykinin-stimulated $\text{Ins}(1,4,5)\text{P}_3$ accumulation at concentrations of 100 nM or 1 μM .

Table 1 Initial time-courses of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ accumulations elicited by bradykinin and histamine

	Time (s)				
	10	30	60	300	600
<i>Ins(1,4,5)P₃</i> (pmol/well)					
Control	4.60 $\pm$ 0.43	4.97 $\pm$ 0.46	4.55 $\pm$ 0.36	4.71 $\pm$ 0.16	4.92 $\pm$ 0.26
Bradykinin	11.78 $\pm$ 1.25***	8.40 $\pm$ 0.27***	7.66 $\pm$ 0.55**	7.27 $\pm$ 0.43***	5.31 $\pm$ 0.46
Histamine	6.93 $\pm$ 0.59**	6.83 $\pm$ 0.33**	6.05 $\pm$ 0.45*	6.03 $\pm$ 0.36*	5.87 $\pm$ 0.27*
<i>Ins(1,3,4,5)P₄</i> (pmol/well)					
Control	0.93 $\pm$ 0.31	0.79 $\pm$ 0.13	0.90 $\pm$ 0.15	0.80 $\pm$ 0.16	0.85 $\pm$ 0.18
Bradykinin	4.50 $\pm$ 0.59**	6.12 $\pm$ 0.62***	5.43 $\pm$ 0.41***	1.24 $\pm$ 0.21	0.90 $\pm$ 0.04
Histamine	1.98 $\pm$ 0.24*	2.79 $\pm$ 0.42**	2.74 $\pm$ 0.24***	0.73 $\pm$ 0.16	0.75 $\pm$ 0.11

Cultured bovine adrenal chromaffin cells were exposed to bradykinin (100 nM), histamine (100 μM) or vehicle for 10 s. Values are shown as means $\pm$ s.e.mean for 3 separate experiments performed in triplicate.

Statistically significant differences between agonist-stimulated inositol polyphosphate accumulations compared to respective control values are indicated as * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Figure 3 Effect of 12-O-tetradecanoyl-phorbol 13-acetate (TPA) on histamine-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) (a) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) (b) mass accumulations in chromaffin cells. Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the absence (O, ●) or presence of 1 μM TPA (Δ, ▲). Additions of 100 μM histamine (●, ▲) or vehicle (O, Δ) were made at zero-time. Values are means ± s.e.mean (vertical bars) from either 3 (Ins(1,3,4,5)P₄) or 4 (Ins(1,4,5)P₃) separate experiments performed in triplicate.

Statistically significant differences between cell responses in the presence and absence of TPA are indicated as **P* < 0.05, ***P* < 0.01.

Although Ro 31-8220 was without apparent effect on [³H]-InsP₃ accumulation in response to 30 min stimulation with either bradykinin or histamine, we wished to investigate whether inhibition of PKC would affect the initial response of the cell, the generation of Ins(1,4,5)P₃ during the first few seconds of agonist exposure. The results from experiments where chromaffin cells were pre-incubated in the presence of 0.1 to 10 μM Ro 31-8220 and then stimulated with histamine or bradykinin for 10 s are shown in Table 2. Ro 31-8220 (10 μM) significantly increased the agonist-stimulated Ins(1,4,5)P₃ responses. Furthermore, if the relative increases in Ins(1,4,5)P₃ accumulation are expressed as a % increase over basal values, then PKC inhibition resulted in 55% and 61% greater accumulations respectively for bradykinin- and histamine-stimulated responses (Table 2).

Discussion

In the present study we have assessed the effects of acute modulations of PKC activity on phosphoinositide metabolism stimulated by bradykinin and histamine in chromaffin cells. When considering evidence relating to the possible role of a short inhibitory feedback loop which may regulate the activation of PLC by PKC, it is perhaps helpful to distinguish between two types of experimental design. Firstly, the exogenous activation of PKC, usually by a phorbol ester such as TPA, can show whether the components of the loop, i.e. inhibition of agonist stimulation by PKC, are present. Secondly, however, it is necessary to ask whether this feedback circuit is activated by particular conditions of agonist stimulation, and here it is necessary to look at the consequences of interruption of the loop: this has been achieved by

Figure 4 Concentration-response relationships for 12-O-tetradecanoyl-phorbol 13-acetate (TPA) inhibition of agonist-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) mass accumulations in chromaffin cells. Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the various concentrations of TPA (O, ●, ■) or 4-methoxy TPA (Δ), followed by exposure to bradykinin (100 nM: ●, Δ), histamine (100 μM: ■) or vehicle (O) for 10 s. Values are means ± s.e.mean (vertical bars) for 3 separate experiments performed in triplicate.

Statistically significant differences for the effect of a given concentration of TPA compared to the absence of TPA are shown as **P* < 0.05, ***P* < 0.01.

Table 2 Effect of Ro 31-8220 on bradykinin- or histamine-stimulated Ins(1,4,5)P₃ accumulation in chromaffin cells

Ro 31-8220 (μM)	Ins(1,4,5)P ₃ (pmol/well)		
	Control	Bradykinin	Histamine
0	4.1 ± 0.3	9.6 ± 0.4	7.4 ± 0.4
0.1	4.3 ± 0.3	10.1 ± 0.8	7.4 ± 0.4
1	4.5 ± 0.4	10.9 ± 0.6	8.3 ± 0.5
10	4.4 ± 0.2	13.1 ± 1.1**	9.9 ± 1.1*

Cultured bovine adrenal chromaffin cells were pre-incubated for 10 min in the presence of the indicated concentrations of Ro 31-8220. Additions of bradykinin (100 nM), histamine (100 μM) or vehicle were made for 10 s. Values are shown as means ± s.e.mean for 3 separate experiments performed in triplicate.

Statistically significant differences between agonist-stimulated Ins(1,4,5)P₃ accumulation in the presence compared with the absence of Ro 31-8220 are indicated as **P* < 0.05; ***P* < 0.01.

the down-regulation of PKC following long term (24 h or 48 h) treatment with TPA, or by use of a limited range of PKC inhibitors. With respect to the first experimental design, there are numerous examples of agonist stimulation being attenuated by TPA (Drummond, 1985; Rittenhouse & Sasson, 1985; Aiyar *et al.*, 1986) including studies on cultured chromaffin cells previously referred to (Wan *et al.*, 1989; Jones *et al.*, 1990). The conclusion of this earlier work, that the components of the feedback circuit are present in

chromaffin cells stimulated by histamine and bradykinin, are confirmed by the experiments with TPA on [^3H]-InsP $_x$ accumulation described in this paper. With respect to the second experimental design, there are indications in other cell systems that agonist-initiated PKC stimulation may activate this inhibitory feedback circuit (Helper *et al.*, 1988; King & Rittenhouse, 1989; Pfeilschifter *et al.*, 1989); however, interpretation of such studies are subject to the complexities of long-term treatment with phorbol esters and the recognised limited selectivity of many of the PKC inhibitors used (Ruegg & Burgess, 1989). Our earlier study in this area used down regulation of PKC by 24 h TPA pretreatment (Jones *et al.*, 1990) and showed that agonist-stimulated [^3H]-InsP $_x$ production was reduced, not enhanced as the inhibitory feedback circuit hypothesis would predict. While difficult to interpret, these results offered no support for the agonist activation of such a feedback mechanism. In the present study we have attempted a more direct approach using the selective PKC inhibitor Ro 31-8220 (Davis *et al.*, 1989; Nixon *et al.*, 1992). Preliminary studies measuring histamine- and bradykinin-stimulated [^3H]-InsP $_x$, demonstrated no effect of PKC inhibition and therefore suggested that there is no functional negative-feedback circuit.

We have previously reported that stimulation of chromaffin cells with bradykinin or histamine rapidly increases the levels of Ins(1,4,5)P $_3$ and Ins(1,3,4,5)P $_4$ (Challiss *et al.*, 1991). Considering the observations, discussed above, that short term TPA treatment substantially attenuated agonist-stimulated [^3H]-InsP $_x$ in the presence of lithium, it was to be expected that a similar attenuation of the inositol polyphosphate responses would also be seen. In fact, attenuations of 30% and 70% were observed for the bradykinin and histamine Ins(1,4,5)P $_3$ responses respectively. In contrast, the agonist-stimulated Ins(1,3,4,5)P $_4$ responses were unaffected by the presence of TPA, even in the case of histamine where the Ins(1,4,5)P $_3$ response was almost eliminated by phorbol ester treatment. This action of TPA to affect differentially agonist-stimulated inositol polyphosphate accumulations cannot be categorically interpreted from these experimental data, but a number of possible mechanisms can be suggested. Consideration of the TPA inhibition of agonist-stimulated Ins(1,4,5)P $_3$ accumulation and the dramatic attenuation of [^3H]-InsP $_x$ accumulation (in the presence of Li $^+$) lead to the conclusion that a primary site of action is at the level of PLC activation. Furthermore, the similar increase in agonist-stimulated Ins(1,3,4,5)P $_4$ accumulation observed in the presence and absence of TPA suggests that this response is not dependent upon the magnitude of the initial increase in Ins(1,4,5)P $_3$ concentration, but is likely to be regulated independently of changes in substrate concentration, at least within the variations in Ins(1,4,5)P $_3$ levels observed under basal or agonist-stimulated conditions in chromaffin cells. Certainly, the low K_m values generally reported for Ins(1,4,5)P $_3$ 3-kinase with respect to Ins(1,4,5)P $_3$ (Shears,

1991) would be consistent with this enzyme approaching saturation with its substrate, even under basal conditions, and external regulatory factors (e.g. increases in [Ca $^{2+}$] $_i$) being necessary and sufficient to increase Ins(1,3,4,5)P $_4$ accumulation.

Although PKC-mediated modifications of the activities of 5-phosphatase (Molina y Vedia & Lapetina, 1986; King & Rittenhouse, 1989) and 3-kinase (Sim *et al.*, 1990) have been reported, the findings in the present study are not easily accounted for by the 5-phosphatase activation or 3-kinase inhibition found by these workers.

The experiments on the effects of PKC inhibition on Ins(1,4,5)P $_3$ accumulation in response to stimulation with either histamine and bradykinin show that Ro 31-8220 does enhance this response (Table 2). These data provide support for the existence of a short inhibitory-feedback circuit which can be elicited by agonist stimulation. This is apparently in conflict with our earlier conclusion, based on [^3H]-InsP $_x$ measurements, where no evidence for such feedback was obtained. However, it should be noted that there were significant differences between the two experimental designs (i.e. the presence vs the absence of lithium; the stimulation for 30 min vs 10 s; the measurement of [^3H]-InsP $_x$ as an index of PLC acting on inositol phospholipids vs the direct measurement of Ins(1,4,5)P $_3$ mass changes). Therefore it is possible that activation of PKC does inhibit agonist-stimulated PLC activity, but the extent to which this occurs in response to histamine and bradykinin is time-limited, and is only effective during the first few seconds following agonist addition. Thus, the consequence for [^3H]-InsP $_x$ accumulation at longer incubation times is not discernible. This proposal is consistent with our previous observations that bradykinin stimulation produces no measurable elevation in DAG in chromaffin cells, but does produce an elevation of phosphatidic acid (Owen & Boarder, 1991). Thus, diacylglycerol accumulation and consequent activation of a PKC inhibitory feedback loop may be curtailed by diacylglycerol kinase activity, producing phosphatidic acid. It is curious in this regard that chromaffin cells are unusual in that they express no phorbol ester- or agonist-stimulated phospholipase D (PLD) activity (Purkiss *et al.*, 1991). Thus, they also lack another route for agonist-stimulated DAG formation (synthesis of phosphatidic acid by PLD and subsequent DAG formation by phosphatidate phosphohydrolase) which may play a major role in activating a feedback circuit in other systems.

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