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Influence of phorbol esters, and diacylglycerol kinase and lipase inhibitors on noradrenaline release and phosphoinositide hydrolysis in chromaffin cells

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1 We have investigated the modification of catecholamine efflux and inositol phosphate formation in cultured adrenal chromaffin cells by tetradecanoyl phorbol acetate (TPA) and inhibitors of diacylglycerol kinase (R 59 022) and diacylglycerol lipase (RG 80267), the two principal pathways of diacylglycerol metabolism.

2 TPA (1 nM to 1 μM) elicited a slow, calcium-dependent, sustained release of noradrenaline, which was partially blocked by the dihydropyridine calcium channel blocker (−)-202 791 and potentiated by the channel enhancer (+)-202 791.

3 R 59 022 enhanced noradrenaline efflux at 30 and 50 μM, while the lipase inhibitor RG 80267 failed to elicit release.

4 Neither R 59 022 nor RG 80267 affected bradykinin- or histamine-stimulated release, but both drugs substantially attenuated nicotine- and high K^+-stimulated release.

5 Pretreatment for 10 min with TPA (but not the relatively inactive 4-methoxyTPA) or the non-phorbol protein kinase C stimulator mezerein potently inhibited bradykinin- and histamine-stimulated accumulation of total [^{3}H]-inositol phosphate; inhibition of [^{3}H]-inositol phosphate formation was also seen with 24 h TPA treatment.

6 Neither R 59 022 nor RG 80267, separately or together, affected bradykinin-stimulated [^{3}H]-inositol phosphate formation.

7 Thus while the mechanism exists for inhibition of formation of inositol phosphates by stimulation of protein kinase C, these studies failed to show that this mechanism is activated by agonists acting on phospholipase C linked receptors.

Introduction

Chromaffin cells maintained in primary culture release catecholamines in response to activation of a variety of cell surface receptors. This includes receptors which, in addition to stimulating release of noradrenaline, stimulate hydrolysis of polyphosphoinositides, generating inositol phosphates and diacylglycerol, such as receptors to bradykinin, histamine, angiotensin II and prostaglandin (Livett & Marley, 1986; Zimlichman *et al.*, 1987; Noble *et al.*, 1988; Plevin & Boarder, 1988; Koyama *et al.*, 1988; Owen *et al.*, 1989a; Plevin *et al.*, 1990). The generation of diacylglycerols which may activate protein kinase C (PKC) is likely to be of significance in stimulus-secretion coupling. The clearest indication of this is the demonstration that PKC activating phorbol esters such as tetradecanoyl phorbol acetate (TPA) can themselves stimulate release, in a calcium-dependent manner, both in intact (Brocklehurst *et al.*, 1985; Pocotte *et al.*, 1985) and permeabilised cells (Knight & Baker, 1983; Pocotte *et al.*, 1985; Brocklehurst & Pollard, 1985). The studies with permeabilised cells demonstrate that PKC activation may play a role in exocytosis at the level of fusion of granules with the cell membrane, while Bittner & Holz (1990) have shown that more than one mechanism may be responsible for enhancement of release from permeabilized cells.

A second possible role for PKC in stimulus-secretion coupling may be at the level of the receptor. Activation of phospholipase C (PLC) may cause a diacylglycerol-mediated stimulation of PKC which feeds back to inhibit the agonist-induced activation of PLC. Evidence for such a mechanism falls into two categories. Firstly, the inhibition of agonist-induced stimulation of PLC by exogenous PKC activators such as phorbol esters. Secondly, evidence that inhibitory feedback loop is activated by an agonist, such as the enhancement of agonist-stimulated PLC by prior down-regulation of PKC, or attenuation of agonist-stimulated PLC by blocking diacylglycerol breakdown. Studies in a variety of preparations have shown that phorbol esters can attenuate agonist-stimulated formation of inositol phosphates; in chromaffin cell preparations it has been shown that histamine-induced inositol phosphate accumulation can be attenuated by phorbol ester (Wan *et al.*, 1989). Several recent studies have provided evidence that such a feedback loop is activated by agonists acting on PLC-linked receptors in platelets, smooth muscle and epithelial cells (Helper *et al.*, 1988; King & Rittenhouse, 1989; Pfeilschifter *et al.*, 1989; Crouch & Lapetina, 1989).

In the present study, we used bovine cultured adrenal chromaffin cells to characterize the effect of phorbol esters and inhibitors of diacylglycerol metabolism on release of catecholamines. We demonstrated the inhibition of agonist-stimulated PLC by phorbol esters and, also, sought to provide evidence for the regulation of inositol phosphate responses by agonist-stimulated diacylglycerol production.

Methods

Freshly obtained bovine adrenal medullae were digested with collagenase/protease as described by Marriott *et al.* (1988). Chromaffin cells were purified to about 90% by centrifugation and differential plating and cultured on 24-well 'Primaria' plates in complete medium as described earlier (Owen *et al.*, 1989a). Cells for release studies were washed twice with HEPES-buffered balanced salt solution (BSS) containing (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8 and glucose 5.5, gassed with 95% O_2: 5% CO_2 and buffered to pH 7.4. Release was measured by incubating the cells at 37°C in BSS in the presence or absence of drugs. A preincubation period of 10 min, in the presence or absence of drugs, was introduced where appropriate. At the

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end of the incubation period the medium was removed, centrifuged and the supernatant was acidified with 0.1 M HCl. The cell contents were extracted into 0.1 M HCl and the noradrenaline and adrenaline contents of both supernatant and cell extract were estimated by high pressure liquid chromatography with electrochemical detection. Similar patterns of release were seen for both noradrenaline and adrenaline, but only the release of noradrenaline is shown, expressed as either pmol per well or as % of cell content. Each result is the mean of quadruplicate determinations from a single cell preparation which has been repeated in similar or identical form three or more times on different cell preparations.

To estimate stimulation of total inositol phosphate formation, cells were incubated with 1 μCi *myo*-[2-^{3}H]-inositol (15 Ci mmol^{-1}) at 37°C for 32–40 h in 0.5 ml of modified BSS containing (mM): NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.5, GIBCO non-essential and essential amino acids at 1%, glutamine 27 mg 100 ml^{-1}, streptomycin 5000 μg 100 ml^{-1}, penicillin 5000 iu 100 ml^{-1}, cytosine arabinoside 5 μM, in 5% CO_2 95% air. Cells were then washed, and preincubated with 10 mM lithium chloride in BSS in the presence or absence of drugs for 10 min. Drugs were then added for the duration of the incubation period as appropriate in BSS plus lithium. The reaction was stopped with cold methanol. Chloroform extraction was followed by isolation of [^{3}H]-inositol phosphates on Dowex-1 (Cl^{-1}) essentially as described by Rooney & Nahorski (1986).

Dihydropyridine, phorbol esters and diacylglycerol kinase and lipase inhibitors were diluted with BSS from stock solutions in dimethylsulphoxide (DMSO).

Materials

Cell culture supplies were from GIBCO, Paisley, Scotland except for Primaria plates (Falcon) which were from Becton-Dickinson, Oxford. *myo*-[2-^{3}H]-inositol was from New England Nuclear. The diacylglycerol kinase inhibitor R 59 022 (6-[2-[4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl]-ethyl]-7-methyl-5*H*-thiazolo[3,2-a]pyrimidin-5-one) was purchased from Janssen, Olen, Belgium. The diacylglycerol lipase inhibitor RG 80267 (1,6-*bis*(cyclohexyloximinocarbonylamino) hexane) (Revlon) was a kind gift of Rorer Central Research, Washington, Pasadena, USA, while the isomers of 202 791 (4-(benzoxadiazolyl)-1,4-dihydro-2,6-dimethyl-5-nitro-3-pyridin-carbonate-isopropylester) were kindly donated by Sandoz Ltd, Basle, Switzerland. Other chemicals and drugs were from Sigma Chemical Co. plc, Poole, Dorset, U.K. or Fisons plc, Loughborough, U.K.

Results

Effect of TPA, diacylglycerol kinase inhibitor and diacylglycerol lipase inhibitor on noradrenaline release

The time course, dose-response curve and calcium-dependence for the release of noradrenaline in response to TPA is shown in Figure 1a,b and c, respectively. The rate of release was characteristically slow and consequently subsequent experiments used a 30 min incubation. The EC_{50} for TPA was between 8 and 35 nM (3 determinations). Consistent with previous observations, phorbol ester-stimulated release was dependent on added extracellular calcium (Figure 1c), being maximal at 0.3 mM. The dependence on extracellular calcium suggests that calcium entry may play a role in TPA-stimulated release, thus the effects of two dihydropyridine calcium channel drugs were studied (Table 1). The calcium channel blocker (−)-202 791 reduced noradrenaline release in response to TPA while its stereoisomer, (+)-202 791, a calcium channel enhancer, caused a small release alone but had a greater than additive effect when combined with TPA.

The endogenous PKC activator diacylglycerol is metabolised through either phosphorylation to phosphatidic acid, by diacylglycerol kinase (DG kinase), or deacylation at the 2 position to monoacylglycerol, by diacylglycerol lipase (DG lipase). It was therefore of interest to see whether comparable effects to TPA could be produced by inhibition of one or both of these pathways. Hence, we investigated the effect of a DG kinase inhibitor (R 59 022) and a DG lipase inhibitor (RG 80267) on noradrenaline release. Neither the vehicle alone (dimethylsulphoxide 0.3%) nor the DG lipase inhibitor RG 80267 (up to 50 μM) had any effect on release over a period of 30 min. The DG kinase inhibitor R59 022 did induce enhanced efflux at 30 μM and above (Figure 2a), after incubation periods of over 8 min (Figure 2b). The DG lipase inhibitor failed to stimulate release at any time up to 60 min (Figure 2b).

Since bradykinin and histamine stimulate inositol phosphate production in chromaffin cells as well as release (Plevin & Boarder, 1988), then they must also stimulate formation of diacylglycerol. It was therefore possible that inhibition of dia-

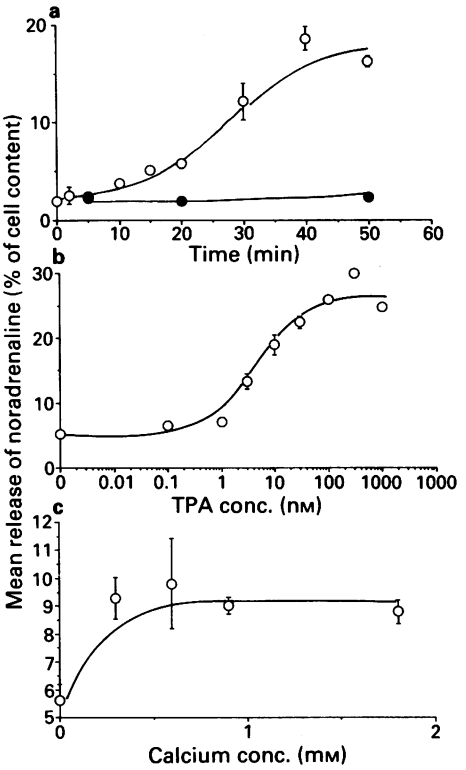

Figure 1 Characteristics of tetradecanoyl phorbol acetate (TPA)-stimulated release of noradrenaline. (a) Release in response to incubation for increasing time with 1 μM TPA (○) or dimethylsulphoxide control (●). (b) Release after 30 min incubation with increasing concentrations of TPA. (c) Release in response to incubation for 30 min with 100 nM TPA in increasing concentrations of extracellular calcium. Basal release in the absence of added calcium was 3.15 ± 0.25%. Release is expressed as % of total noradrenaline cell content. Values are means and vertical bars show s.e.mean of quadruplicates from a single experiment.

Table 1 Effect of dihydropyridine on tetradecanoyl phorbol acetate (TPA)-stimulated release

	Control	*TPA* (100 nM)
Control	27.8 ± 1.3	125.0 ± 7.9
(−)-202 791	31.3 ± 0.5	77.2 ± 7.4
(+)-202 791	54.8 ± 3.2	219.9 ± 9.8

Data shown are pmol noradrenaline released per well over a 30 min incubation period, with dihydropyridine present during a 10 min preincubation as well as during the incubation period. Dimethyl sulphoxide was present at 0.01% throughout. Values are means ± s.e.mean of quadruplicates from a single experiment.

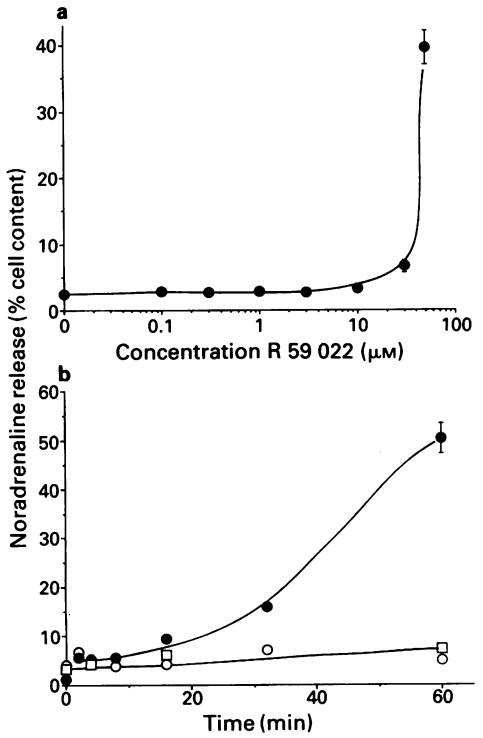

Figure 2 Release of noradrenaline in the presence of inhibitors for diacylglycerol (DG) kinase and diacylglycerol lipase. (a) Noradrenaline release (% of cell content) in response to increasing concentrations of the DG kinase inhibitor R59 022 (●). The dimethylsulphoxide vehicle up to 0.3% had no effect on release. (b) Release (% of cell content) after various times of incubation with 30 μM RG 80267 (○), 30 μM R 59 022 (●) or 0.3% dimethylsulphoxide (□). Values are means and vertical lines show s.e.mean of quadruplicates from a single experiment.

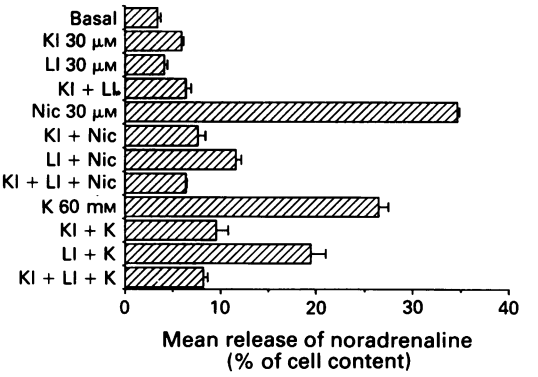

Figure 3 Noradrenaline release in response to nicotine (Nic) and high K^+ (K): effect of diacylglycerol (DG) kinase (KI) and DG lipase (LI) inhibitors. The inhibitors were present where appropriate during a 12 min preincubation period as well as during the 6 min incubation period, in the presence or absence of 30 μM nicotine or 60 mM K^+, during which release was measured. Both inhibitors were present at 30 μM, and dimethylsulphoxide at 0.3% was included where inhibitors were not added. Values are means and horizontal bars show s.e.mean of quadruplicates from a single experiment.

cylglycerol breakdown would modify release by these agents. It was found that bradykinin-stimulated release was unaffected by either the kinase inhibitor or the lipase inhibitor, added separately or together at 30 μM (data not shown). The histamine-stimulated release was reduced by 30 μM kinase inhibitor (data not shown); this was, however, the expected consequence of the antihistamine nature of the drug and was unrelated to its metabolic effects.

Noradrenaline release stimulated by either nicotine or high K^+ (depolarization) was inhibited by preincubation with either inhibitor (30 μM). This is illustrated by results from one such experiment shown in Figure 3; with a stimulation period of 6 min it was found that the nicotine-evoked stimulation could be almost eliminated by either R 59 022 or RG 80267. The high K^+-stimulated release was similarly affected by the kinase inhibitor R 59 022, but was reduced to a lesser extent by the lipase inhibitor RG 80267.

Thus, it appears that neither inhibitor of diacylglycerol metabolism inhibited bradykinin-stimulated release, but they both substantially attenuated release stimulated by nicotine and high K^+ depolarization.

Effect of TPA, diacylglycerol kinase inhibitor and diacylglycerol lipase inhibitor on agonist-stimulated inositol phospholipid breakdown

In the experiments to investigate the effects of TPA on agonist-stimulated formation of total inositol phosphates (in the presence of lithium), the pretreatment time with the phorbol ester was either 10 min (to stimulate PKC) or 24 h (to downregulate PKC). In each case the phorbol ester was also present during the 30 min incubation period in the presence of the agonist. We have previously shown that histamine and bradykinin stimulation of total inositol phosphate formation is linear for 30 min (Plevin & Boarder, 1988). The influence of 1 μM TPA pretreatment for 10 min and 24 h is illustrated by the results in Tables 2 and 3 respectively. Surprisingly, both pretreatment periods produced a substantial loss in agonist-stimulated formation of inositol phosphates. The effect of 10 min TPA pretreatment was characteristically greater with histamine stimulation than with bradykinin stimulation, while 24 h TPA pretreatment almost eliminated (histamine) or substantially reduced (bradykinin) the inositol phosphate response to agonists (Table 3).

We investigated the effects of different TPA concentrations, of the relatively inactive 4-methoxy TPA and of the structurally disparate PKC activator mezerein on bradykinin- and

Table 2 Effect of tetradecanoyl phorbol acetate (TPA) pretreatment for 10 min on bradykinin and histamine stimulation of total inositol phosphate formation

	DMSO control	*TPA (1 μM)*
Control	4625 ± 319	3979 ± 426
Bradykinin	9854 ± 664	4934 ± 401
Histamine	26546 ± 734	5494 ± 637

Data shown are d.p.m. of [^{3}H]-inositol phosphate accumulated in the presence of 10 mM lithium during a 30 min incubation period. TPA was present where appropriate, with dimethyl sulphoxide (DMSO) vehicle at 0.1%, during a 10 min preincubation as well as the incubation period. Values are means ± s.e.mean of quadruplicates from a single experiment.

Table 3 Effect of 24 h pretreatment with tetradecanoyl phorbol acetate (TPA) on bradykinin- and histamine-stimulated total inositol phosphate formation

	DMSO Control	*TPA (1 μM)*
Control	2391 ± 135	2001 ± 244
Bradykinin	9049 ± 1794	6875 ± 657
Histamine	22239 ± 7943	4118 ± 945

Data shown are d.p.m. of [^{3}H]-inositol phosphate accumulated in the presence of 10 mM lithium during a 30 min incubation period. TPA was present, where appropriate, with dimethylsulphoxide (DMSO) vehicle at 0.1% during a 24 h preincubation as well as the incubation period. Values are means ± s.e.mean of quadruplicates from a single experiment.

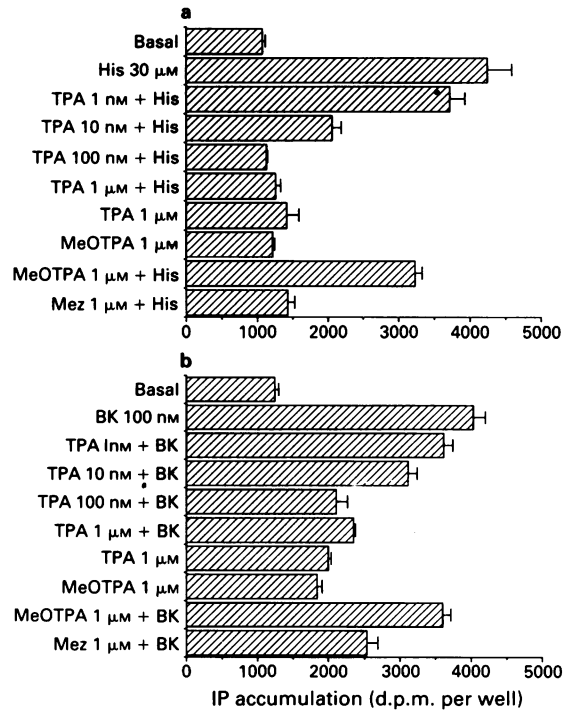

Figure 4 Total [^{3}H]-inositol phosphate (IP) formation in response to stimulation for 30 min with (a) 30 μM histamine (His) or (b) 10 nM bradykinin (BK). Tetradecanoyl phorbol acetate (TPA) was present at the concentrations shown, 4-methoxy TPA (MeOTPA) 1 μM or mezerein (Mez) 1 μM were present during a 10 min preincubation as well as the 30 min incubation period. Values are means and horizontal bars show s.e.mean of quadruplicates from a single experiment.

histamine-stimulated formation of inositol phosphates. These results are illustrated in Figure 4. When stimulation was with histamine substantial effects of TPA could be seen at 10 nM, with essentially maximal effects at 100 nM. 4-MethoxyTPA at 1 μM was no more effective than TPA at 1 nM. Mezerein (1 μM), while having no effect of its own, effectively inhibited histamine-stimulated formation of inositol phosphates. A very similar pattern of results was obtained when stimulation was with bradykinin (Figure 4b). These results are consistent with the regulation by PKC of histamine- and bradykinin-stimulation of inositol phosphates.

The indication that phorbol ester stimulation of PKC might regulate the chromaffin cell response to histamine and bradykinin led us to investigate the role of endogenously activated PKC, using the inhibitors of diacylglycerol kinase (R 59 022) and lipase (RG 80267). When included before and during incubations with bradykinin, R 59 022 was without effect (Table 4). Results of experiments in which the effect of R 59 022 on histamine stimulation was investigated are not presented since it is known that R 59 022 is an H_1-receptor antagonist (de Chaffoy de Courcelles *et al.*, 1985). RG 80267 had no effect on either bradykinin- or histamine-stimulated inositol phosphate formation. A combination of the two inhibitors also failed to influence the accumulation of inositol phosphate in response to bradykinin.

Discussion

The initial observation of Knight & Baker (1983), who used permeabilized cells to show that TPA induced an increased sensitivity of the exocytotic process for calcium, presumably by activating PKC, was influential in directing attention to the role of stimulation of this enzyme in exocytosis. However, here we are concerned with the role of PKC in the initiation of stimulus secretion coupling at the cell membrane. The time course of TPA-stimulated release described was found to be very slow and sustained and dependent on extracellular calcium, in confirmation of previous observations (Brocklehurst *et al.*, 1985). There are perhaps two explanations for this calcium-dependency. Firstly, incubation in nominally calcium-free medium results in a reduction in free intracellular calcium levels, to which TPA-stimulated release is sensitive (Brocklehurst *et al.*, 1985; Pocotte *et al.*, 1985). Secondly, TPA leads to an enhanced calcium influx, without which TPA-stimulated release cannot occur. We found that in chromaffin cells the release in response to TPA was partly blocked by the dihydropyridine antagonist (−)-202 791. Chromaffin cells have clearly been shown to possess L-type dihydropyridine sensitive voltage gated calcium channels which may mediate stimulus-secretion coupling (e.g. Garcia *et al.*, 1984; Boarder *et al.*, 1988; Owen *et al.*, 1989b) and so one explanation for this observation is that TPA-stimulated release is, in part dependent upon an enhanced opening of these channels caused, directly or indirectly, by stimulated PKC activity. The data we present here contain one additional indication that TPA is affecting calcium entry through L-type channels: the calcium channel agonist (+)-202 791 enhanced the release stimulated by TPA. This is consistent with the demonstration of dihydropyridine-sensitive phorbol ester-stimulated calcium influx in cloned rat pituitary cells (Albert *et al.*, 1987). Effects of TPA on calcium fluxes in other cell types include an enhancement of calcium channel opening in *Aplysia* (De Riemer *et al.*, 1985), facilitation of depolarization enhanced calcium influx in intact adrenal medulla of the rat (Wakade *et al.*, 1986), inhibition of depolarization-enhanced calcium flux in PC12 cells (Harris *et al.*, 1986; Di Virgilio *et al.*, 1986) and attenuation of calcium influx in neutrophils (McCarthy *et al.*, 1989).

An interesting alternative to TPA as an activator of PKC is the inhibition of DG kinase and/or DG lipase, in an attempt to elevate the levels of diacylglycerol, an endogenous activator (alongside calcium) of PKC. The DG kinase inhibitor R 59 022 has been shown to potentiate thrombin-induced diacylglycerol production in platelets and inhibit phosphatidic acid production in neutrophils (de Chaffoy de Courcelles *et al.*, 1985; Mege *et al.*, 1988), while RG 80267 has been shown to increase basal diacylglycerol levels and potentiate hormonal stimulated diacylglycerol production (Sutherland & Amin,

Table 4 Effect of inhibitors of diacylglycerol lipase and kinase on bradykinin- and histamine-stimulated inositol phosphate production

	Control	*RG 80267*	*R 59 022*	*RG 80267 and R 59 022*
Control	2373 ± 343	2816 ± 84	3079 ± 511	2396 ± 469
Bradykinin (100 nM)	6540 ± 376	6261 ± 498	5899 ± 619	5559 ± 304
Histamine (30 μM)	12270 ± 1133	12918 ± 414	*	*

The diacylglycerol lipase inhibitor RG 80267 was 10 μM and the diacylglycerol kinase inhibitor R 59 022 was 50 μM. Data shown are d.p.m. of [^{3}H]-inositol phosphates produced during a 30 min incubation period with the inhibitors present during both this incubation period and a preceding 10 min pre-incubation. Values are means ± s.e.mean of quadruplicates from a single experiment. * Effect of R 59 022 on histamine stimulated [^{3}H]-inositol phosphate production was not valid due to antihistamine nature of the inhibitor.

1982; Chang *et al.*, 1988). In experiments run in parallel with those described here, we have shown that both these metabolic inhibitors increase the accumulation of diacylglycerol in chromaffin cells at 30 μM and 50 μM, with the effect of R 59 022 being greater than that of RG 80627 (Owen & Boarder, unpublished observations). Here we showed that the DG kinase inhibitor was able to enhance release at these concentrations. By contrast, the lipase inhibitor was not able to elicit increased noradrenaline efflux. Combined with the effect of these compounds on diacylglycerol accumulation in chromaffin cells (Owen & Boarder, unpublished observations), these results may indicate a relationship between enhancement of diacylglycerol accumulation and the stimulation of release.

Bradykinin-stimulated noradrenaline release was unaffected by the DG kinase and lipase inhibitors suggesting that accumulation of diacylglycerol plays little role in bradykinin-stimulated release. This is consistent with the differing sensitivity of bradykinin- and TPA-stimulated release to dihydropyridines: the TPA-stimulated release is partially sensitive (this paper) while bradykinin-stimulated release is insensitive (Owen *et al.*, 1989) to dihydropyridine calcium channel blockers. The contrasting observation that nicotine- and high K^+-stimulated release is inhibited by both kinase and lipase inhibitors is most likely due to an effect at the level of calcium entry, since the lack of effect on responses to bradkykinin shows that the exocytotic process is not impaired. The inhibition of nicotine- and high K^+-stimulated release may be through PKC activation and subsequent phosphorylation of a component of calcium entry, or a 'non-specific' membrane effect perturbing channels and/or nicotinic receptors.

A possible role for bradykinin-stimulated PKC in chromaffin cells is modulation of receptors and associated effector mechanisms. For example, we have previously provided evidence that angiotensin II enhances prostaglandin stimulation of adenylate cyclase in cultured adrenal medulla cells, by a mechanism involving diacylglycerol production and the activation of PKC (Boarder *et al.*, 1988). In addition, a number of examples exist in a variety of cell types of inositol phospholipid-linked receptor responses which are down-regulated by phorbol ester-stimulated PKC (e.g. Rittenhouse & Sasson, 1985; Drummond, 1985; Aiyar *et al.*, 1986), with indications that endogenously produced PKC activation may be involved (Helper *et al.*, 1988; King & Rittenhouse, 1989; Crouch & Lapetina, 1989; Pfeilschifter *et al.*, 1989). We showed that the inositol phosphate response to bradykinin and histamine in chromaffin cells was attenuated by 10 min prior activation of PKC. These results indicate that stimulation of PKC can result in a reduced response to these agonists, and raise the possibility that enhanced production of diacylglycerol may play a feedback role in the response. Pretreatment for 24 h with phorbol esters, a protocol which causes loss of PKC activity in a variety of cell types, including PC12 cells and adrenal chromaffin cells (Matthies *et al.*, 1987; Bittner & Holz, 1990), might therefore be expected to enhance the inositol response, as shown recently in different cell types (Helper *et al.*, 1988; Pfeilschifter *et al.*, 1989). However, our observation of reduced responses for both bradykinin and histamine stimulation is difficult to interpret: it may be due to a reduced response to the agonist following a long period of prior PKC stimulation by TPA, or a possible dependency of the response on intact PKC activity in the cells. A further investigation is needed to monitor the changes in PKC occurring in these cells on lengthy treatment with phorbol esters.

As previously discussed, diacylglycerol is metabolised by kinase or lipase pathways, so a further strategy is to potentiate the consequences of increased synthesis of diacylglycerol by inhibiting one or both of these pathways. Agonist-enhanced diacylglycerol formation may only be able to activate a feedback loop when diacylglycerol breakdown is impaired. This would result in a reduced inositol phosphate formation in response to agonists in the presence of the diacylglycerol kinase and/or lipase inhibitors. However, we found no effect of either or both of these inhibitors on bradykinin-stimulated accumulation of inositol phosphates. Furthermore, when histamine was used to stimulate these cells, there was no effect on the DG lipase inhibitor.

These results demonstrate that protein kinase C activation by phorbol esters can inhibit the receptor-stimulated synthesis of inositol phosphates in chromaffin cells. However, the results provide no support for the suggestion that diacylglycerol produced as a result of receptor stimulation in chromaffin cells acts to down-regulate the inositol phospholipid receptor effector system. This may help to explain why in chromaffin cells the agonist stimulation of inositol phosphate production is linear for 45 min (Plevin & Boarder, 1988), while other systems which have this feedback loop desensitize rapidly (e.g. Helper *et al.*, 1988).

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