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Differential potentiation of mitogen-stimulated phosphoinositide hydrolysis in protein kinase C-depleted Swiss 3T3 cells

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In Swiss 3T3 cells, depletion of protein kinase C (PKC) by prolonged incubation with phorbol esters potentiates the formation of total inositol phosphates in response to bombesin or vasopressin [Blakeley, Corps & Brown (1989) Biochem. J. **258**, 177–185]. The characteristics of the accumulation of inositol phosphates in control and PKC-depleted cells stimulated by bombesin, vasopressin or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have now been compared. The potentiation of the $PGF_{2\alpha}$ response was greater than that of the vasopressin response which was, in turn, greater than that of the bombesin response. The time courses of the responses to all three agonists were biphasic, and both phases of the response were amplified in the PKC-depleted cells. These results provide further evidence for the involvement of a PKC-mediated negative-feedback loop regulating phosphoinositide hydrolysis in response to several 3T3 cell mitogens. The differential potentiation of the response to these agonists suggests that PKC might act at multiple sites within the signal transduction pathway.

INTRODUCTION

An increase in PtdIns(4,5)P_2 hydrolysis is one of the earliest responses of cells to many hormones, neurotransmitters and mitogens (see Berridge, 1987). In several cell types responding to various phosphoinositide-hydrolysing agonists, the accumulation of inositol phosphates and the mobilization of Ca^{2+} is inhibited by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and related activators of protein kinase C (PKC). An inhibitory effect of this type has been demonstrated in, for example, thrombin-stimulated platelets (MacIntyre *et al.*, 1985; Watson & Lapetina, 1985), thyroliberin-stimulated GH3 cells (Drummond, 1985), vasopressin-stimulated WRK-1 mammary tumour cells (Monaco & Mufson, 1986), thrombin-stimulated hamster fibroblasts (L'Allemain *et al.*, 1986), substance P-stimulated rat parotid cells (Sugiya *et al.*, 1988) and bombesin-stimulated Swiss 3T3 cells (Brown *et al.*, 1987; Blakeley *et al.*, 1989).

These results raise the interesting possibility that a negative-feedback loop involving activation of PKC by diacylglycerol may normally serve to regulate phosphoinositide hydrolysis. In support of this possibility, it has been shown that Swiss 3T3 cells in which PKC has been down-modulated by prolonged exposure to phorbol esters exhibit both an increased production of total inositol phosphates and a prolonged Ca^{2+} transient in response to bombesin (Brown *et al.*, 1987; Corps *et al.*, 1989). An enhanced production of inositol phosphates after depletion of PKC was also observed in WB rat liver cells or vascular smooth muscle cells stimulated by angiotensin II, and in NG108-15 neuroblastoma cells stimulated by bradykinin (Hepler *et al.*, 1988; Fu *et al.*, 1988; Pfeilschifter *et al.*, 1989). In this study we have compared the time courses and dose–response relationships for the formation of inositol phosphates in control versus PKC-depleted cells stimulated by bombesin, vasopressin or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

MATERIALS AND METHODS

Cell-culture reagents were obtained from Flow Laboratories. *myo*-[2-^{3}H]Inositol, ^{125}I-streptavidin, biotinylated anti-mouse Ig and a monoclonal antibody against PKC were from Amersham International. Swiss 3T3 cells express only PKC-α (Rose-John *et al.*, 1988), and the antibody recognizes a conserved region in PKC-α and -β. TPA and $PGF_{2\alpha}$ were purchased from Sigma. Bombesin and [Arg^8]vasopressin were obtained from Bachem.

Stock cultures of Swiss 3T3 cells were maintained as described previously (Brown & Blakeley, 1983). For experimental use, cells were seeded into 3.5 cm plastic culture dishes in 2 ml of Dulbecco's modified Eagle's medium supplemented with 10% calf serum. The extraction and measurement of inositol phosphate fractions from mitogen-stimulated cells was performed as described previously (Heslop *et al.*, 1986; Brown *et al.*, 1987). Cells were labelled for the final 48–72 h of culture by adding *myo*-[^{3}H]inositol directly to the growth medium: 0.8 μCi/ml for measurement of total inositol phosphates and 2.5 μCi/ml when InsP_1, InsP_2 and InsP_3 fractions were measured separately. After careful washing to remove unincorporated label, the cells were incubated in phosphate-buffered saline (138 mM-NaCl, 2.8 mM-KCl, 8 mM-Na_2HPO_4, 1.45 mM-KH_2PO_4, 0.91 mM-$CaCl_2$ and 0.49 mM-$MgCl_2$, pH 7.4) containing 10 mM-LiCl and mitogens as indicated in the Figure legends. At the end of the incubation, ^{3}H-labelled inositol phosphates were extracted into 0.5 ml of 15% (w/v) trichloroacetic acid (4 °C) and analysed by anion-exchange chromatography on small columns of Dowex 1-X8 (Berridge *et al.*, 1983). ^{3}H-labelled InsP_1, InsP_2 and InsP_3 fractions were eluted either separately, or together as a single (Ins$P_{tot.}$) fraction (Blakeley *et al.*, 1989).

RESULTS AND DISCUSSION

Prolonged incubation of various cell types with phorbol esters causes a down-modulation of PKC. This effect is due to a phorbol ester-stimulated increase in the rate of degradation of the kinase with no change in its rate of synthesis (Woodgett & Hunter, 1987; Young *et al.*, 1987). The levels of immunoreactive PKC in extracts of Swiss 3T3 cells treated for 48 h in the absence or presence of 300 ng of TPA/ml are shown in Fig. 1. In the TPA-pretreated cells, the level of the kinase was considerably decreased and fell below the detection limit of the immunoblot.

Compared with control cultures, these PKC-depleted cells

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PKC, protein kinase C; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; G-protein, guanine-nucleotide-binding protein; PDGF, platelet-derived growth factor.

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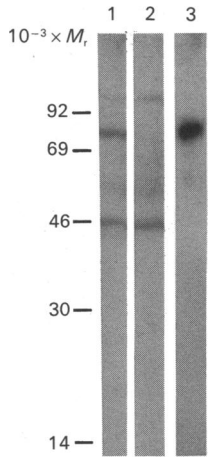

Fig. 1. Depletion of cellular PKC by prolonged treatment with TPA

Five-day-old cultures, grown on 9 cm dishes, were treated with TPA (300 ng/ml) added directly to the growth medium. After 48 h, the cells from each dish were scraped into 200 μl of 20 mM-Tris (pH 7.5) containing 2 mM-EDTA, 0.5 mM-EGTA, 1% Nonidet P-40, 5 mM-dithiothreitol, 0.25 mM-leupeptin, 1 mM-phenylmethanesulphonyl fluoride and aprotinin (100 μg/ml). The appropriate volume of 5× concentrated sample buffer was added and the sample was boiled for 3 min. Samples were applied to a 10% polyacrylamide resolving gel with a 4% stacking gel and electrophoresed at 30 mA for approx. 3 h. Proteins were transferred from the gel to nitrocellulose and the blots were blocked using MTA buffer (Glenney, 1986). Blots were incubated overnight at 4 °C in MTA buffer containing a 1:100 dilution of a monoclonal antibody against PKC. Immunoreactive proteins were revealed by successive incubations with biotinylated anti-mouse Ig and ^{125}I-streptavidin. The migration positions on the blot of Rainbow M_r marker proteins (Amersham) are indicated. Lane 1, control cells; lane 2, TPA-pretreated cells; lane 3, PKC purified from bovine brain.

exhibited a markedly potentiated formation of total inositol phosphates when stimulated by vasopressin, $PGF_{2\alpha}$ or bombesin at all doses tested (Fig. 2). At the highest doses tested, the potentiation was 2.3-fold for bombesin, 5.2-fold for vasopressin and 12.4-fold for $PGF_{2\alpha}$ (Fig. 2). Although the number of cells per dish was slightly increased by TPA pretreatment, this increase (37 ± 4% in 14 experiments) is much too small to account for the potentiated responses observed. In several experiments we have observed that the potentiation of the $PGF_{2\alpha}$ response is always greater than that of the vasopressin response which, in turn, is more effectively potentiated than the bombesin response. This differential potentiation of the various agonists is not simply related to the smaller effects of $PGF_{2\alpha}$ and vasopressin in control cells, since the response to low doses of bombesin was not similarly potentiated (Fig. 2).

The marked potentiation of the response to the different agonists was also observed when the time course of the formation of inositol phosphates was investigated (Fig. 3). We have previously reported a biphasic accumulation of inositol phosphates in bombesin-stimulated cells, most evident in the InsP_3 and InsP_2 fractions, which reach a peak value within 1–2 min after bombesin addition, and then decrease somewhat (to 5 min) before rising again (Blakeley *et al.*, 1989; Brown *et al.*, 1989). Despite the much smaller responses induced, a similarly biphasic trend was also discernible in control cultures stimulated by vasopressin or $PGF_{2\alpha}$ (Fig. 3). Moreover, the biphasic nature of the response to these latter agonists was clearly evident in the potentiated response of PKC-depleted cells (Fig. 3). These results indicate a two-phase response to all three agonists and, in addition, suggest that both phases of the response are amplified after depletion of PKC.

The basal levels of the inositol phosphate fractions in unstimulated cells were increased only slightly in the PKC-depleted cells (Fig. 3). Furthermore, no sizeable accumulation of inositol phosphates was observed when LiCl was added to either control or PKC-depleted cells in the absence of an agonist (Fig. 3; results not shown). In previous experiments we have observed a very small increase in the InsP_1 fraction of PKC-depleted cells after addition of LiCl (Brown *et al.*, 1987).

The mechanism by which PKC inhibits phosphoinositide hydrolysis in response to receptors which are (putatively) coupled to phospholipase C activation via guanine-nucleotide-binding proteins (G-proteins) remains to be established. PKC-mediated phosphorylation of various receptors is well-documented (see Sibley *et al.*, 1988), and phosphorylation of G-proteins and phospholipase C has been reported (Katada *et al.*, 1985; O'Brien *et al.*, 1987; Rhee *et al.*, 1989). It seems possible that the differential inhibitory effect of PKC on phosphoinositide hydrolysis stimulated by different mitogens could be due to multiple sites of phosphorylation (receptor, G-protein, phospholipase C)

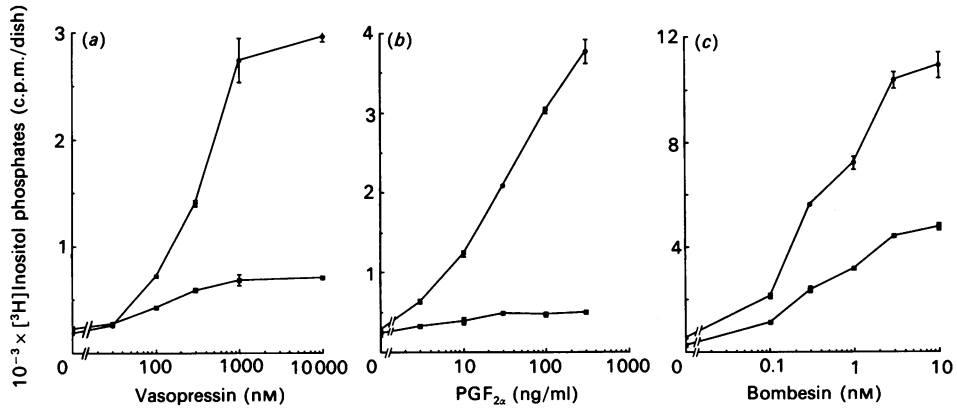

Fig. 2. Dose–responses for mitogen-stimulated formation of inositol phosphates in control or PKC-depleted cells

Total ^{3}H-labelled inositol phosphates were measured after a 30 min incubation of control (■) or PKC-depleted (●) cells with the indicated concentrations of mitogens in the presence of 10 mM-LiCl. The PKC-depleted cells were prepared by adding TPA (200 ng/ml) to the growth medium for 48 h before the experiments. The values shown are the means (±S.E.M.) of triplicate determinations on separate dishes of cells. Error bars not visible fall within the symbols.

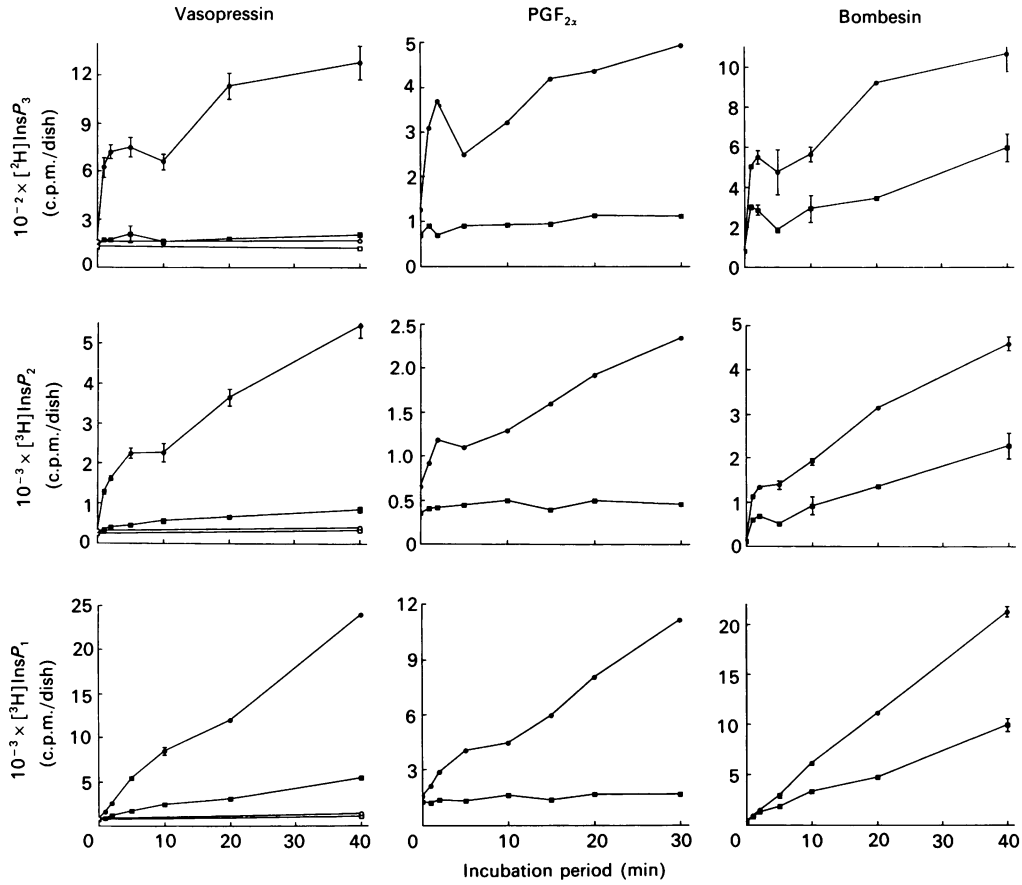

Fig. 3. Time courses of mitogen-stimulated production of inositol phosphates in control and PKC-depleted cells

^{3}H-labelled InsP_3, InsP_2 and InsP_1 fractions were measured at the indicated times after addition of vasopressin (1 μM), $PGF_{2\alpha}$ (300 ng/ml) or bombesin (10 nM) to control (■) or PKC-depleted (●) cells in the presence of 10 mM-LiCl (added 10 min before the mitogens). The effects of LiCl addition in the absence of mitogens are shown by the open symbols in the vasopressin experiment. The values shown are the means of triplicate (vasopressin and bombesin experiments) or duplicate ($PGF_{2\alpha}$ experiment) determinations on separate dishes of cells.

by the kinase in the signal transduction pathway. The availability of antibodies against phospholipases C will permit this question to be addressed, but antibodies to the receptors and the G-protein(s) involved will be required for a full investigation.

In marked contrast to the results presented here, the formation of inositol phosphates in response to platelet-derived growth factor (PDGF) and fibroblast growth factor is not inhibited by acute exposure to phorbol esters, nor potentiated in PKC-depleted cells (Sturani *et al.*, 1986; Blakeley *et al.*, 1989; Brown *et al.*, 1989). These results suggest that the mechanisms by which these polypeptides stimulate PtdIns(4,5)P_2 hydrolysis in Swiss 3T3 cells are quite different from the action of peptides such as bombesin and vasopressin. Indeed, it has recently been shown that the tyrosine kinase domain of the PDGF receptor is able to phosphorylate phospholipase C-γ to high stoichiometry (Meisenhelder *et al.*, 1989; Wahl *et al.*, 1989). In addition, purified preparations of PDGF receptors have been reported to stimulate phospholipase C-γ phosphorylation *in vitro* (Meisenhelder *et al.*, 1989). Although it has not yet been demonstrated that this phosphorylation regulates enzyme activity, this is an attractive hypothesis to explain the actions of this receptor on PtdIns(4,5)P_2 hydrolysis. Whatever the mechanism, it is clear that it is not subject to negative feedback in the same way as the receptors for bombesin, vasopressin and $PGF_{2\alpha}$.

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