Protein kinase C-mediated negative-feedback inhibition of unstimulated and bombesin-stimulated polyphosphoinositide hydrolysis in Swiss-mouse 3T3 cells

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Bombesin-related peptides stimulate a rapid increase in polyphosphoinositide hydrolysis in Swiss-mouse 3T3 cells. These peptides generate an increase in the efflux of $^{45}\text{Ca}^{2+}$ from pre-labelled cells, a response consistent with an inositol trisphosphate-mediated mobilization of intracellular Ca^{2+} . The bombesin-stimulated release of cellular $^{45}\text{Ca}^{2+}$ is inhibited by tumour-promoting phorbol esters (e.g. 12-*O*-tetradecanoylphorbol 13-acetate, TPA). Although there are several possible sites of action at which this effect might occur, our results indicate that TPA induces an uncoupling of bombesin-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) without decreasing cellular binding of bombesin. In cultured cells, protein kinase C can be down-modulated by a prolonged incubation of the cells with phorbol esters. Such pretreatment greatly decreased the inhibitory effect of TPA on bombesin-stimulated PIP_2 hydrolysis, suggesting that this action of the phorbol ester is mediated via protein kinase C. Since diacylglycerol is an endogenous activator of protein kinase C and a direct product of PIP_2 hydrolysis, these results suggest that protein kinase C inhibition of polyphosphoinositide hydrolysis may function as a negative-feedback pathway. Cells in which protein kinase C has been down-modulated show elevated basal and bombesin-stimulated production of inositol phosphates, providing evidence that such a feedback loop limits polyphosphoinositide turnover in both unstimulated and mitogen-stimulated cells.

INTRODUCTION

Bombesin, a 14-amino-acid peptide first isolated from the skin of the frog *Bombina orientalis* (Anastasi *et al.*, 1971), is one of a family of closely related amphibian, avian and mammalian peptides which have a highly homologous C-terminal octapeptide (Walsh *et al.*, 1979). The best characterized of the mammalian peptides are gastrin-releasing peptide, originally purified from pig gastric tissue (McDonald *et al.*, 1979), and the neuro-medins B and C, from pig spinal cord (Minamino *et al.*, 1983, 1984). Peptides of the bombesin family elicit a wide range of physiological responses, including smooth-muscle contraction (Minamino *et al.*, 1983, 1984) and the release of several peptide hormones, including gastrin, cholecystokinin, prolactin and growth hormone (Rivier *et al.*, 1978; Walsh *et al.*, 1979; McDonald *et al.*, 1979; Ghatei *et al.*, 1982; Westendorf & Schonbrunn, 1982). In addition to these acute responses, repeated administration of bombesin to rats has been shown to cause pancreatic (Lezoché *et al.*, 1981) and gastric cell (Lehy *et al.*, 1983) hyperplasia. However, it was not possible to determine whether this effect on cell proliferation was a direct effect of bombesin, or was an indirect response, owing to the stimulated release of other mitogenic factors.

Bombesin and gastrin-releasing peptide have been shown to be potent mitogens for some cell types in culture, including Swiss-mouse 3T3 cells (Rozengurt &

Sinnett-Smith, 1983), normal bronchial epithelial cells (Willey *et al.*, 1984) and cell lines derived from small-cell cancer of the lung (SCCL) (Cuttitta *et al.*, 1985; Weber *et al.*, 1985). Since human SCCL and SCCL-derived cell lines are known to synthesize and secrete bombesin-like peptides (Erisman *et al.*, 1982; Moody *et al.*, 1983; Sorenson *et al.*, 1982), it is possible that these peptides may function as autocrine growth factors involved in the abnormal growth of the tumours. Evidence in favour of this proposition has been reported by Cuttitta *et al.* (1985), who showed that a highly specific monoclonal antibody against bombesin markedly suppressed the growth of SCCL cells both *in vitro* and in nude mice.

These findings emphasize the importance of understanding the molecular mechanisms by which bombesin and related peptides exert their effects on cell proliferation. Swiss 3T3 cells, which possess specific high-affinity receptors for radiolabelled gastrin-releasing peptide (Zachary & Rozengurt, 1985) and [^{125}I]bombesin (Brown & Laurie, 1986), should prove useful for this purpose. The binding of bombesin to its receptors on Swiss 3T3 cells results in a rapid stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (Brown *et al.*, 1984; Heslop *et al.*, 1986), generating inositol 1,4,5-trisphosphate (1,4,5- IP_3) and diacylglycerol, an endogenous activator of protein kinase C (see Berridge & Irvine, 1984; Nishizuka, 1984). Hydrolysis of PIP_2 in 3T3 cells is also stimulated by vasopressin (Brown *et al.*, 1984), prostaglandin $\text{F}_{2\alpha}$ (MacPhee *et al.*, 1984), and

Abbreviations used: PIP_2 , phosphatidylinositol 4,5-bisphosphate; IP_1 , IP_2 , IP_3 , IP_4 , IP_5 , IP_6 , inositol mono-, bis-, tris-, tetrakis-, pentakis- and hexakis-phosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline.

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platelet-derived growth factor (Berridge *et al.*, 1984; Chu *et al.*, 1985), but not by insulin (Berridge *et al.*, 1985; Heslop *et al.*, 1986), insulin-like growth factor 1 (K. D. Brown, unpublished work) or epidermal growth factor (Besterman *et al.*, 1986). The PIP_2 -hydrolysing agonists induce an IP_3 -mediated mobilization of intracellular Ca^{2+} from bound stores, which can be demonstrated either as an increased efflux of $^{45}\text{Ca}^{2+}$ from pre-labelled cells (Berridge *et al.*, 1984; Lopez-Rivas & Rozengurt, 1984) or as an elevation of intracellular free Ca^{2+} concentration measured with quin2 (Hesketh *et al.*, 1985; Mendoza *et al.*, 1986).

Agonist-induced mobilization of intracellular Ca^{2+} has been shown to be decreased or abolished by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and related activators of protein kinase C. The effect has been demonstrated in, for example, angiotensin- or phenylephrine-stimulated hepatocytes (Brock *et al.*, 1985; Cooper *et al.*, 1985), thyroliberin-stimulated GH3 pituitary tumour cells (Drummond, 1985), carbachol-stimulated astrocytoma cells (Orellana *et al.*, 1985), vasopressin-stimulated WRK-1 rat mammary cells and Swiss mouse 3T3 cells (Monaco & Mufson, 1986; Mendoza *et al.*, 1986), and thrombin-stimulated platelets (MacIntyre *et al.*, 1985; Rittenhouse & Sasson, 1985). The molecular mechanisms underlying the inhibitory effect of TPA on Ca^{2+} mobilization are not fully understood. The effect could, in principle, be due either to the inhibition of one or more of the events leading to the release of Ca^{2+} from intracellular stores, or to an alteration in the rate at which mobilized Ca^{2+} is extruded from the cells. We show here that phorbol esters inhibit the bombesin-stimulated release of Ca^{2+} from Swiss 3T3 cells. In addition, we have demonstrated that a major site of inhibitory action is a TPA-mediated decrease in the hydrolysis of PIP_2 . The inhibitory action of TPA on PIP_2 hydrolysis is mediated via protein kinase C, since it is not observed in cells in which the kinase has been down-modulated by prolonged pretreatment with the phorbol ester. Finally, we have observed elevated basal and stimulated rates of PIP_2 hydrolysis in the cells in which protein kinase C has been down-modulated. This result suggests that stimulation of protein kinase C by diacylglycerol, formed during PIP_2 hydrolysis, may activate a negative-feedback loop, limiting production of inositol phosphates in Swiss 3T3 cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin and newborn-calf serum were obtained from Flow Laboratories. [*methyl*- ^3H]Thymidine, ^{125}I , $^{45}\text{Ca}^{2+}$, *myo*-[2- ^3H]inositol, *myo*-[2- ^3H]inositol 1-phosphate, *myo*-[2- ^3H]inositol 1,4-bisphosphate and *myo*-[2- ^3H]inositol 1,4,5-trisphosphate were from Amersham International. Phorbol dibutyrate and TPA, both from Sigma, were dissolved in dimethyl sulphoxide at 1 mg/ml and diluted immediately before use in DMEM containing 1 mg of bovine serum albumin/ml. Bombesin and [*Tyr* 4]bombesin were from Bachem U.K. [*Tyr* 4]Bombesin was iodinated by the soluble-lactoperoxidase method, and separated from ^{125}I that had not reacted by using a Sep-Pak C_{18} cartridge (Waters) as described previously (Brown & Laurie, 1986).

Cell cultures

Stock cultures of Swiss-mouse 3T3 cells were maintained and passaged as described previously (Brown & Blakeley, 1983). For experimental use, cells were seeded into 35 mm-diam. culture dishes in DMEM containing 10% (v/v) newborn-calf serum, and used after 7 days' growth, by which time the cells were confluent and quiescent.

Efflux of $^{45}\text{Ca}^{2+}$ from pre-labelled cells

These experiments were performed by a modification of the procedure described by Berridge *et al.* (1984). Cells were pre-labelled by an overnight incubation in DMEM containing $^{45}\text{Ca}^{2+}$ (4.4 $\mu\text{Ci/ml}$). The radioactive medium was removed and the cells were washed four times with phosphate-buffered saline (PBS; pH 7.4) containing 2 mM EDTA and a further four times with PBS without EDTA. [The composition of PBS is (mM): NaCl, 138; KCl, 2.8; Na_2HPO_4 , 8; KH_2PO_4 , 1.45; CaCl_2 , 0.91; MgCl_2 , 0.49.] The efflux of $^{45}\text{Ca}^{2+}$ was measured by incubating the cells at 37 °C in 1 ml of PBS, which was replaced at 2 min intervals with fresh solution. At the times indicated in the Figures, control PBS was replaced with PBS containing test reagents as described in the Figure legends. Each 1 ml efflux sample was added to 9 ml of scintillation fluid and counted for $^{45}\text{Ca}^{2+}$ radioactivity. The radioactivity remaining in the cells at the end of the experiment was extracted (room temperature for 30 min) into 500 μl of 0.1 M-HCl and counted. The efflux data are presented as a fractional efflux, i.e. the amount of radioactivity leaving the cells during each 2 min interval is expressed as a percentage of that present in the cells at the start of the interval.

Extraction, measurement, and identification of inositol phosphates

Cells were pre-labelled for 72 h by adding *myo*-[^3H]inositol (5 or 10 $\mu\text{Ci/ml}$) directly to the growth medium 3–4 days after seeding. The labelling medium was removed and the cells were incubated for 45 min in PBS (pH 7.4) to remove as much as possible of the unincorporated [^3H]inositol. The cells were incubated for the required stimulation period at 37 °C in PBS (pH 7.4) containing 10 mM-LiCl and the test reagents at the concentrations indicated in the individual Figure and Table legends. The solution was removed by suction and replaced by 0.5 ml of 15% (w/v) trichloroacetic acid (4 °C), and the dishes were kept at 4 °C to extract cellular inositol phosphates. The extracts were transferred to test tubes and the cells were washed with 0.5 ml of water. The water washes were combined with the original extracts, and the acid was removed by four extractions with water-saturated diethyl ether. Residual ether was removed from the samples under a stream of nitrogen. The extracted samples were stored at -20 °C.

^3H -labelled inositol phosphates in the cell extracts were separated by anion-exchange chromatography over Dowex 1-X8 (100–200 mesh; formate form) by the method of Berridge *et al.* (1983), with minor modifications as described below. The thawed samples were adjusted to pH approx. 7.5 by the addition of 10 μl of 1.5 M-NaOH and then applied to 0.7 cm $\times$ 1.5 cm beds of resin packed into small Econocolumns (Bio-Rad 737-1242). The columns were packed, immediately before use, with fresh resin for each experiment. The

loaded columns were washed with 10 ml of water to remove [^3H]inositol, and then with 5 ml of 60 mM-sodium formate to elute glycerophosphoinositol. [^3H]inositol mono-, bis- and tris-phosphates (IP_1 , IP_2 and IP_3 respectively) were eluted with sequential 5 ml portions of 0.1 M-formic acid/0.2 M-ammonium formate, 0.1 M-formic acid/0.5 M-ammonium formate and 0.1 M-formic acid/1.0 M-ammonium formate. The elution positions were confirmed with ^3H -labelled standards. Other studies have shown that the fraction eluted from the column with 0.1 M-formic acid/1.0 M-ammonium formate includes both 1,4,5- IP_3 and 1,3,4- IP_3 (Irvine *et al.*, 1985), together with inositol 1,3,4,5-tetrakisphosphate (IP_4) (Batty *et al.*, 1985; Heslop *et al.*, 1985). However, as pointed out by Taylor *et al.* (1986), this ' IP_3 fraction' is a valid measure of phospholipase C activity, because 1,4,5- IP_3 formed directly by PIP_2 hydrolysis appears to be the only precursor for the formation of 1,3,4,5- IP_4 and 1,3,4- IP_3 (Hawkins *et al.*, 1986; Irvine *et al.*, 1986). Residual radioactivity on the column, representing, by analogy with the data of Batty *et al.* (1985) and Heslop *et al.* (1985), [^3H]inositol pentakisphosphate (IP_5) and [^3H]inositol hexakisphosphate (IP_6), was eluted with 0.1 M-formic acid/2.5 M-ammonium formate. The column eluates were collected directly into scintillation vials, to which was added 10 ml of scintillation fluid (ACS; Amersham). After cooling to 4 °C, the gelled samples were counted for ^3H radioactivity.

Binding of ^{125}I -labelled [Tyr^4]bombesin

Cells were rinsed with 1–2 ml of binding medium, which consisted of DMEM containing 0.1% (w/v) bovine serum albumin, 0.1 μM -KI and 50 mM-Bes. The cells were then incubated for 30 min at 37 °C with 1 ml of binding medium containing ^{125}I -[Tyr^4]bombesin (270 Ci/mmol). After incubation, unbound radioactivity was removed by washing the cells four times with cold (4 °C) PBS containing 0.1% albumin and 0.1 μM -KI. The washed cells were solubilized (20 min at 37 °C) into 1 ml of 0.5 M-NaOH, and cell-associated radioactivity was determined with a gamma counter. Non-specific binding (defined as that which was not displaced by a 200-fold or greater excess of unlabelled [Tyr^4]bombesin) was subtracted from total cell-associated radioactivity to give values for specific binding.

RESULTS

Bombesin-stimulated release of $^{45}\text{Ca}^{2+}$ from Swiss 3T3 cells

Bombesin stimulates DNA synthesis in Swiss 3T3 cells with a half-maximal response at peptide concentrations of 0.1–0.3 nM and a maximal response at 1–3 nM (Rozengurt & Sinnott-Smith, 1983; Corps *et al.*, 1985). Over a similar concentration range, bombesin and related mammalian peptides stimulated a rapid but transient increase in the release of $^{45}\text{Ca}^{2+}$ from pre-labelled cells (Fig. 1). The results for bombesin are typical of those obtained in several experiments, which, taken together, show a close correspondence between the dose-response for the stimulation of Ca^{2+} release and that for the stimulation of DNA synthesis. The greatest effect on $^{45}\text{Ca}^{2+}$ efflux was always evident during the first 2 min interval after bombesin addition and, despite the continued presence of the peptide, $^{45}\text{Ca}^{2+}$ release had

Fig. 1. Effect of bombesin on the efflux of $^{45}\text{Ca}^{2+}$ from confluent quiescent monolayers of Swiss 3T3 cells

Cells were grown for 7 days and prelabelled with $^{45}\text{Ca}^{2+}$ by an overnight incubation. The cells were washed and $^{45}\text{Ca}^{2+}$ efflux was measured during successive 2 min intervals as described in the Materials and methods section. Beginning at 12 min (longer arrow), and continuing for the remainder of the experiment, samples of the efflux solution contained no addition (○), 1 nM-bombesin (●, ■), 10 nM-GRP (▲), or 10 nM-neuromedin C (▼). One of the bombesin-treated dishes (■) also received EGTA (1 mM) beginning at 6 min (shorter arrow). The amount of $^{45}\text{Ca}^{2+}$ leaving the cells during each 2 min interval is expressed as a fraction of that present at the beginning of the interval.

returned close to unstimulated control values after 6 min. We have found (results not shown) that the stimulation of $^{45}\text{Ca}^{2+}$ release by bombesin could be almost completely blocked by a short preincubation with [$\text{D-Arg}^1, \text{D-Pro}^2, \text{D-Trp}^{7,9}, \text{Leu}^{11}$]substance P, which has been found to be a competitive antagonist of bombesin action in these (Corps *et al.*, 1985; Zachary & Rozengurt, 1985) and other cells (Jensen *et al.*, 1984). The bombesin-stimulated release of $^{45}\text{Ca}^{2+}$ was not affected by the addition of EGTA to the medium (Fig. 1), suggesting that increased $^{45}\text{Ca}^{2+}$ release does not result from a displacement of intracellular bound $^{45}\text{Ca}^{2+}$ by a stimulated influx of extracellular Ca^{2+} .

In contrast with the effect of bombesin, insulin produced no alteration of cellular $^{45}\text{Ca}^{2+}$ release from Swiss 3T3 cells (results not shown). This observation is consistent with the lack of effect of insulin on PIP_2 hydrolysis (Berridge *et al.*, 1984; Heslop *et al.*, 1986). In addition, although insulin synergizes with bombesin in stimulating DNA synthesis in Swiss 3T3 cells, insulin failed to potentiate the bombesin-mediated release of $^{45}\text{Ca}^{2+}$ from the cells (results not shown).

Phorbol esters inhibit bombesin-stimulated $^{45}\text{Ca}^{2+}$ release from Swiss 3T3 cells

The tumour promotor, TPA, when added alone produced no significant alteration in cellular $^{45}\text{Ca}^{2+}$ release (Fig. 2). In four separate experiments similar to that shown in Fig. 2, the percentage of the initial total cell

Fig. 2. Inhibition of bombesin-stimulated $^{45}\text{Ca}^{2+}$ efflux from Swiss 3T3 cells by TPA

Cells were grown for 7 days and prelabelled with $^{45}\text{Ca}^{2+}$ by an overnight incubation. The cells were washed and $^{45}\text{Ca}^{2+}$ efflux was measured during successive 2 min intervals as described in the Materials and methods section. The amount of $^{45}\text{Ca}^{2+}$ leaving the cells during each 2 min interval is expressed as a fraction of that present at the beginning of the interval. Additions to the efflux solutions were as follows. (a): $\circ$, no addition; $\bullet$, 0.1 nM-bombesin from 12 min; $\blacksquare$, 20 nM-TPA from 6 min and 0.1 nM-bombesin from 12 min. (b): $\square$, 20 nM-TPA from 6 min; $\bullet$, 3 nM-bombesin from 12 min; $\blacksquare$, 20 nM-TPA from 6 min and 3 nM-bombesin from 12 min.

$^{45}\text{Ca}^{2+}$ remaining in control cells after 6 min was $79 \pm 1\%$, decreasing to $67 \pm 1\%$ after a further 6 min. In cells treated with 20 nM-TPA for the second 6 min interval, the corresponding values were $80 \pm 1\%$ and $68 \pm 2\%$. Because effects of phorbol esters on agonist-induced Ca^{2+} mobilization have been observed in several cell types (see the Introduction), we have investigated the action of TPA on the bombesin-mediated $^{45}\text{Ca}^{2+}$ efflux from Swiss 3T3 cells. A brief (6 min) pre-exposure of cells to TPA (20 nM) completely blocked the stimulatory effect of 0.1 nM-bombesin on $^{45}\text{Ca}^{2+}$ efflux (Fig. 2a) and substantially decreased the effect of 3 nM bombesin (Fig. 2b). Similar inhibitory effects on the stimulated $^{45}\text{Ca}^{2+}$ efflux were obtained by using a related tumour promoter, phorbol dibutyrate, in place of TPA (results not shown).

There are several sites within the putative pathway of

Fig. 3. Possible sites of action for the TPA/protein kinase C-mediated inhibition of bombesin-stimulated $^{45}\text{Ca}^{2+}$ release

There are several possible sites at which protein kinase C could exert an inhibitory influence on bombesin-stimulated $^{45}\text{Ca}^{2+}$ release. (1) The kinase could act on the bombesin receptor, resulting in a decrease in receptor affinity or number. (2) The transduction pathway linking the activated receptor to the hydrolysis of PIP_2 could be uncoupled by an action of the kinase. (3) The kinase could act at the endoplasmic reticulum to inhibit either the binding of 1,4,5- IP_3 to its putative receptor or the release of Ca^{2+} from bound stores. (4) Activated protein kinase C could inhibit Ca^{2+} extrusion through the plasma membrane. Abbreviations: PI, phosphatidylinositol and its 4-phosphate; DG, diacylglycerol.

bombesin-mediated cellular activation at which TPA, presumably acting via protein kinase C (see below), could exert an inhibitory influence on $^{45}\text{Ca}^{2+}$ efflux. Four such possibilities are illustrated in Fig. 3. The simplest explanation would be that TPA blocks or inhibits the binding of bombesin to its cellular receptors. However, we have failed to find any effect of TPA on the binding of ^{125}I -labelled [Tyr⁴]bombesin to the cells. There was no effect of concentrations of TPA as high as 100 nM on the cellular binding of ^{125}I -[Tyr⁴]bombesin (1 nM) (Fig. 4a). Furthermore, TPA (20 nM) did not decrease ^{125}I -[Tyr⁴]bombesin binding measured over a wide range of ligand concentrations (Fig. 4b). Scatchard analysis of these data indicates that TPA does not alter either the affinity or the number of bombesin receptors on Swiss 3T3 cells.

Another possible site of action of TPA is at the endoplasmic reticulum, either by inhibiting the binding of 1,4,5- IP_3 to its putative receptors (Spät *et al.*, 1986a,b) or by decreasing the subsequent release of Ca^{2+} (see Fig. 3). Preliminary results (not shown) of investigations of this possibility suggest that TPA does not decrease the 1,4,5- IP_3 -mediated release of $^{45}\text{Ca}^{2+}$ from saponin-permeabilized cells. A further possibility is that TPA could inhibit the extrusion of mobilized $^{45}\text{Ca}^{2+}$ from the cells by an action on plasma-membrane Ca^{2+} transport. However, this seems unlikely, since TPA did not alter basal $^{45}\text{Ca}^{2+}$ efflux from the cells (Fig. 2), and because phorbol esters do not increase the mitogen-induced

Fig. 4. TPA does not affect the binding of ^{125}I -[Tyr⁴]bombesin to Swiss 3T3 cells

(a) Confluent monolayers of cells were rinsed with binding medium and incubated with binding medium containing 1 nM- ^{125}I -[Tyr⁴]bombesin and the indicated concentration of TPA for 30 min at 37 °C. Cell-associated ^{125}I -[Tyr⁴]bombesin was measured as described in the Materials and methods section. The results have been corrected for non-specific binding (not displaced by a minimum 200-fold excess of unlabelled bombesin). Each point represents the mean value of three determinations of ^{125}I -[Tyr⁴]bombesin binding to separate dishes of cells, expressed as a percentage of binding to control cells. (b) Cells were incubated with binding medium containing the indicated concentrations of ^{125}I -[Tyr⁴]bombesin, in the absence (○) or presence (●) of 20 nM-TPA, for 30 min at 37 °C. Each point represents a single value for specific ^{125}I -[Tyr⁴]bombesin binding.

elevation of intracellular free Ca^{2+} concentration measured with quin 2 (Hesketh *et al.*, 1985; Mendoza *et al.*, 1986; Monaco & Mufson, 1986).

Finally, it is possible that TPA uncouples the signalling pathway from the occupied bombesin receptor to the phosphodiesterase responsible for PIP_2 hydrolysis. To investigate this possibility, we have incubated cells with ^3H inositol to label PIP_2 and then measured the bombesin-stimulated production of ^3H -labelled inositol phosphates in control cells and in cells preincubated for 15 min with TPA. The results (Fig. 5) show a pronounced dose-dependent inhibition of the bombesin-stimulated ^3H -labelled IP_1 , IP_2 and IP_3 fractions

Fig. 5. TPA inhibits bombesin-stimulated inositol phosphate generation in Swiss 3T3 cells

Cells were grown for 7 days and labelled with ^3H inositol for the final 72 h by adding it directly to the growth medium. The medium was removed, and the cells were washed with PBS (pH 7.4) and incubated for 45 min at 37 °C in PBS to remove as much as possible of the unincorporated radioactivity. The indicated concentration of TPA was added for the final 15 min of this incubation. The solutions were removed and replaced by 1 ml of PBS (pH 7.4) containing 10 mM-LiCl, 3 nM-bombesin and the indicated concentration of TPA, and the dishes were incubated for 30 min at 37 °C. The cells were extracted into cold trichloroacetic acid, and ^3H -labelled inositol phosphates were separated and measured as described in the Materials and methods section. Each point represents the mean value ($\pm$ S.E.M., except where these lie within the symbol) of four determinations of the inositol phosphates from separate dishes of cells: ○, mean values for production of inositol phosphates by unstimulated cells. In contrast with its inhibitory effects on bombesin-stimulated inositol phosphate generation, TPA does not decrease basal accumulation.

Addition	^3H inositol phosphate (c.p.m./dish)		
	IP_1 fraction	IP_2 fraction	IP_3 fraction
None	830 $\pm$ 12	214 $\pm$ 10	91 $\pm$ 1
TPA (30 nM)	911 $\pm$ 34	247 $\pm$ 11	100 $\pm$ 2

generated in the presence of the phorbol ester. A similar dose-response was obtained for inhibition of the production of each of the three inositol phosphate fractions, with a maximal inhibitory effect at a TPA concentration of ~ 10 nM and half-maximal inhibition at ~ 2 nM (Fig. 5). In contrast with this inhibitory effect on bombesin-stimulated generation of inositol phosphates, TPA failed to inhibit basal production of inositol phosphates (see legend to Fig. 5).

Down-regulation of protein kinase C decreases the inhibitory effects of phorbol esters on bombesin-stimulated PIP_2 hydrolysis

In Swiss 3T3 cells, as in other cell types, protein kinase C activity can be greatly diminished by pretreatment of the cells for 1–2 days with high concentrations of TPA. The loss of the kinase can be shown both as a decrease in enzymic activity (Rodriguez-Pena & Rozengurt, 1984; Blackshear *et al.*, 1985) and by a dramatic fall in the binding of phorbol esters such as [^3H]phorbol dibutyrate (Collins & Rozengurt, 1984; Blackshear *et al.*, 1985). Other results have indicated that these changes are accompanied by a decrease in immunoreactive protein kinase C, owing to a greatly increased rate of kinase degradation in the treated cells (Ballester & Rosen, 1985). In the context of PIP_2 -hydrolysing agonists, this procedure should prove very valuable in determining which cellular responses are attributable to protein kinase C activation, since such responses should be greatly attenuated in TPA-pretreated cells compared with control cells. In control Swiss 3T3 cells, bombesin stimulated a marked increase in the [^3H]-labelled IP_1 , IP_2 and IP_3 fractions, and this increase was diminished by the presence of 30 nM-TPA (Table 1). In contrast, in cells pretreated for 48 h with 300 nM-TPA, bombesin retained the ability to stimulate inositol phosphate generation, but this stimulation was no longer inhibited by the presence of TPA (Table 1). Furthermore, the TPA-pretreated cells showed an enhanced response to bombesin stimulation, and the basal unstimulated production of inositol phosphates was increased some

2-fold in the treated cells compared with control cells (Table 1). These effects are not due to a substantial alteration in polyphosphoinositide pool size, since this changed little (29% increase) in the TPA-pretreated cells (results not shown). These results suggest, firstly, that phorbol ester-induced inhibition of polyphosphoinositide hydrolysis is mediated via protein kinase C (since inhibition is not seen in cells in which kinase C has been down-modulated) and, secondly, that there is a decrease (by protein kinase C down-modulation) of a constitutive, kinase C-mediated, inhibitory action on PIP_2 hydrolysis, which normally limits production of inositol phosphates in both stimulated and unstimulated cells.

Increased basal generation of inositol phosphates in cells in which protein kinase C has been down-regulated

We have further investigated the activity of this putative feedback relationship in an experiment in which the rates of accumulation of [^3H]-labelled inositol phosphates were monitored in control and in TPA-pretreated cells. The results (Fig. 6) show that the radioactivities in the IP_1 , IP_2 and IP_3 fractions were all elevated in unstimulated cells in which protein kinase C had been down-modulated as compared with control untreated cells. The radioactivity in the column fraction presumed to contain IP_5 and IP_6 was also increased in the treated cells (Fig. 6). The radioactivity in this fraction and the IP_2 fraction does not change over the 60 min incubation. In contrast, the [^3H]-labelled IP_1 fraction accumulates during this period (Fig. 6), owing to the presence of 10 mM-LiCl and the consequent inhibition of the dephosphorylation of IP_1 to inositol (Hallcher & Sherman, 1980; Berridge *et al.*, 1982). The rate of accumulation of the IP_1 fraction is clearly more rapid in the cells in which protein kinase C has been down-regulated, as would be predicted if a tonic inhibitory feedback effect had been decreased. Although much less pronounced than that seen for IP_1 , the IP_3 fraction also showed a tendency to increase during the course of the experiment, and this effect was most pronounced in the TPA-pretreated cells.

Table 1. Effect of prolonged pretreatment with phorbol ester on bombesin-stimulated PIP_2 hydrolysis and its inhibition by TPA

Cells were grown for 7 days and labelled with [^3H]inositol for the final 72 h by adding it directly to the growth medium. Some of the dishes of cells received TPA (300 nM) for the final 48 h of the growth period. The labelling medium was removed and the cells were washed with PBS (pH 7.4) and incubated for 45 min at 37 °C in PBS to remove as much as possible of the unincorporated radioactivity. TPA (30 nM) was added to some dishes of cells for the final 15 min of this incubation. The solutions were removed and replaced by 1 ml of PBS containing 10 mM-LiCl and the indicated concentrations of the test reagents. The cells were incubated at 37 °C for 30 min. The cells were extracted into cold trichloroacetic acid, and the extracted [^3H]-labelled inositol phosphates were separated and measured as described in the Materials and methods section. Each point represents the mean value ($\pm$ S.E.M.) of four determinations of the inositol phosphates from separate dishes of cells.

Pretreatment	Addition	[^3H]Inositol phosphate (c.p.m./dish)		
		IP_1 fraction	IP_2 fraction	IP_3 fraction
None	None	889 $\pm$ 50	312 $\pm$ 14	156 $\pm$ 8
	Bombesin (3 nM)	13396 $\pm$ 494	1525 $\pm$ 219	317 $\pm$ 19
	Bombesin (3 nM) + TPA (30 nM)	7338 $\pm$ 136	1089 $\pm$ 158	242 $\pm$ 9
TPA (300 nM for 48 h)	None	2157 $\pm$ 133	640 $\pm$ 88	300 $\pm$ 11
	Bombesin	21555 $\pm$ 267	2916 $\pm$ 409	631 $\pm$ 55
	Bombesin + TPA	20911 $\pm$ 812	3100 $\pm$ 393	636 $\pm$ 35

Fig. 6. Increases in ³H-labelled inositol phosphates in Swiss 3T3 cells pretreated with TPA to down-regulate protein kinase C

Cells were grown for 7 days and labelled with [³H]inositol for the final 72 h by adding it directly to the growth medium. Some of the dishes of cells received TPA (300 nM) for the final 48 h of the growth period. The labelling medium was removed, and the cells were washed with PBS (pH 7.4) and incubated for 40 min at 37 °C in PBS to remove as much as possible of the unincorporated radioactivity. The buffer contained 10 mM-LiCl for the final 10 min of this incubation. At the indicated times, cells were extracted into cold trichloroacetic acid, and ³H-labelled inositol phosphates were separated and measured as described in the Materials and methods section. Each point represents the mean value ($\pm$ S.E.M., except where these lie within the symbol) of four determinations of the inositol phosphates from separate dishes of cells. White symbols, control cells; black symbols, TPA-pretreated cells. (a) IP₁ fraction; (b) IP₂ fraction (squares) and IP₃ fraction (circles); (c) IP₅/IP₆ fraction.

DISCUSSION

Several studies have demonstrated that elevation of the intracellular free Ca²⁺ concentration, in response to various Ca²⁺-mobilizing agonists in a number of cell types, is inhibited by the presence of phorbol esters (see the Introduction). The effects of TPA appear to be due to an inhibitory action of phorbol esters on agonist-stimulated polyphosphoinositide hydrolysis (Brock *et al.*, 1985; MacIntyre *et al.*, 1985; Cotechia *et al.*, 1985; Corvera *et al.*, 1986). The results presented here extend these findings to the stimulation of Ca²⁺ release and PIP₂ hydrolysis in Swiss 3T3 cells by the mitogenic peptide bombesin.

TPA caused a marked decrease in the production of inositol phosphates in rabbit aortic smooth-muscle cells (Cotechia *et al.*, 1985), in smooth-muscle-derived DDT₁MF-2 cells (Leeb-Lundberg *et al.*, 1985) and in cultured rat hepatocytes (Lynch *et al.*, 1985; Corvera *et al.*, 1986) in response to α_1 -adrenergic stimulation by noradrenaline. In those studies the effects of TPA on PIP₂ hydrolysis were accompanied by a decrease in the affinity or number of α_1 -adrenoceptors, and the authors raised the possibility that the effects on phosphoinositide hydrolysis were due to decreased agonist binding resulting from a protein kinase C-mediated phosphorylation of the receptor. In support of a receptor-active receptor-specific effect of phorbol esters is the report that angiotensin-II-stimulated effects on phosphatidylinositol turnover in cultured rat hepatocytes were not affected by TPA (Corvera *et al.*, 1986).

In contrast, there are several examples of a phorbol ester-mediated inhibition of phosphoinositide hydrolysis in the absence of an effect on agonist binding. Angiotensin-II-stimulated PIP₂ hydrolysis in rat aortic smooth-muscle cells has been found to be inhibited by TPA, and this inhibitory effect is not accompanied by alteration in either the affinity or the number of cellular receptors for ¹²⁵I-angiotensin II (Brock *et al.*, 1985; Griendling *et al.*, 1986). TPA has been reported to inhibit vasopressin-stimulated IP₃ production in rat hepatocytes without altering the characteristics of vasopressin binding (Lynch *et al.*, 1985). Similar results have been obtained for the stimulation of rat mammary WRK-1 cells by vasopressin (Monaco & Mufson, 1986). Activation of muscarinic acetylcholine receptors by carbachol is linked to a stimulation of PIP₂ hydrolysis, and, in 1321N1 astrocytoma cells (Orellana *et al.*, 1985) and PC12 cells (Vicentini *et al.*, 1985), the stimulation is inhibited by TPA, with little or no effect on the binding properties of the muscarinic receptor. However, Liles *et al.* (1986) have observed that, in NIE-115 neuroblastoma cells, TPA induces rapid internalization and subsequent degradation of muscarinic acetylcholine receptors. In the present work we have been unable to observe an effect of phorbol esters on the binding of ¹²⁵I-[Tyr⁴]bombesin to Swiss 3T3 cells, even at high concentrations of TPA. We have repeatedly looked at binding from low concentrations of ligand (0.25–1.0 nM) and have found no evidence for a conversion of high-affinity receptors into a lower-affinity state. We conclude that, if such an affinity change occurs, it must involve a small proportion of very-high-affinity sites that are not detectable at the specific radioactivity of our ¹²⁵I-[Tyr⁴]bombesin. These results indicate that the TPA-mediated inhibition of bombesin-stimulated PIP₂ hydrolysis in Swiss 3T3 cells

occurs at a post-receptor site. The coupling of occupied receptors to phosphodiesterase activation is thought to involve G proteins (see Taylor & Merritt, 1986), and one can speculate that phosphorylation of these proteins might be involved in post-receptor inhibition of PIP_2 hydrolysis by TPA. We have no simple explanation for the apparent paradox that TPA inhibits stimulated, but not unstimulated, production of inositol phosphates. It may be that there is a low degree of basal PIP_2 hydrolysis which is not susceptible to inhibition by phorbol esters, perhaps because it is catalysed by a different phosphodiesterase or is from a separate PIP_2 pool. We suppose that, in the basal state, a small TPA/protein kinase C-sensitive component is fully inhibited and becomes apparent only when the kinase is down-regulated.

It is generally assumed that the inhibitory actions of phorbol esters on phosphoinositide hydrolysis and Ca^{2+} mobilization are mediated via protein kinase C. However, little direct evidence is available on this question. In some cases an inhibitory action of 1-oleoyl-2-acetyl-glycerol has been demonstrated (Brock *et al.*, 1985; Cooper *et al.*, 1985; Kojima *et al.*, 1986), although the effect is generally much weaker than that of the phorbol esters. Indeed, Monaco & Mufson (1986) found no effect of oleoyl-acetyl-glycerol on vasopressin-stimulated phosphoinositide metabolism in WRK-1 cells. However, Mendoza *et al.* (1986) have reported that oleoyl-acetyl-glycerol, like phorbol dibutyrate, inhibited vasopressin-stimulated Ca^{2+} mobilization in Swiss 3T3 cells. In addition, it was found that the inhibitory action of phorbol dibutyrate on vasopressin-stimulated $^{45}\text{Ca}^{2+}$ mobilization was no longer evident when cells had been pretreated with phorbol dibutyrate to down-regulate protein kinase C. We have extended this work in showing that phorbol esters inhibit bombesin-stimulated $^{45}\text{Ca}^{2+}$ mobilization in Swiss 3T3 cells, without changing the cellular binding of agonist, and that the mechanism probably involves inhibition of PIP_2 hydrolysis. Pretreatment of these cells for 48 h with high concentrations of phorbol esters causes a pronounced down-modulation of cellular protein kinase C, measured either as enzyme activity in cell extracts or as phorbol ester binding to intact cells (Rodriguez-Pena & Rozengurt, 1984; Collins & Rozengurt, 1984; Blackshear *et al.*, 1985, 1986). Furthermore, the phosphorylation of a protein of M_r 80000, which is a well-characterized protein kinase C substrate (Blackshear *et al.*, 1986; Isacke *et al.*, 1986), is stimulated by TPA in control cultures, but not in the down-modulated cells (Blackshear *et al.*, 1985). We have found (results not shown) that the down-modulated cells fail to exhibit TPA-mediated inhibition of ^{125}I -EGF binding, an effect strongly correlated with activation of protein kinase C (Brown *et al.*, 1979, 1984; Friedman *et al.*, 1984; Hunter *et al.*, 1984; Davis & Czech, 1985, 1986; Downward *et al.*, 1985; Lin *et al.*, 1986). Although the molecular mechanism causing the down-modulation of protein kinase C is not fully understood, it appears to involve the proteolytic processing of the activated, translocated, enzyme (Ballester & Rosen, 1985). Our finding that a prolonged pretreatment of the cells with TPA blocks the inhibitory action of TPA on PIP_2 hydrolysis indicates that the inhibition is mediated via protein kinase C.

These results raise the possibility that a negative-feedback loop, involving protein kinase C, may be intimately involved in the regulation of phosphoinositide

hydrolysis, since diacylglycerol, an endogenous activator of protein kinase C, is a product of the hydrolysis. We have provided evidence in favour of this possibility by showing that basal production of inositol phosphates is elevated in cells down-modulated by protein kinase C compared with control cells. Furthermore, such cells show a greater production of inositol phosphates in response to bombesin than do control cells. An important consequence of this putative feedback loop is that it may be one factor limiting the production of 1,4,5- IP_3 and the subsequent mobilization of intracellular Ca^{2+} . This possibility could be tested by measuring 1,4,5- IP_3 production and intracellular Ca^{2+} concentrations in unstimulated and stimulated control cells compared with those down-modulated by protein kinase C.

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