Analysis of protein kinase C requirement for exocytosis in permeabilized rat basophilic leukaemia RBL-2H3 cells: a GTP-binding protein(s) as a potential target for protein kinase C

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The role of protein kinase C in calcium-dependent exocytosis was investigated in permeabilized rat basophilic leukaemia cells. When protein kinase C was down-regulated by phorbol myristate acetate (1 μ M for 3–6 h) or inhibited by pharmacological agents such as calphostin C (1 μ M) or a protein kinase C-specific pseudo-substrate peptide inhibitor (100–200 μ M), cells lost the ability to secrete in response to 10 μ M free Ca^{2+} . In contrast, a short treatment (15 min) with phorbol myristate acetate, which maximally activates protein kinase C, potentiated the effects of calcium. Biochemical analysis of protein kinase C-deprived cells indicated that loss of the Ca^{2+} -induced secretory response correlated with disappearance of protein kinase C- α . In addition, at the concentrations effective for exocytosis, calcium caused trans-

location of protein kinase C- α to the membrane fraction and stimulated phospholipase C, suggesting that, in permeabilized cells, protein kinase C can be activated by calcium through generation of the phospholipase C metabolite diacylglycerol. The δ , ϵ and ζ Ca^{2+} -independent protein kinase C isoenzymes were insensitive to phorbol myristate acetate-induced down-regulation and did not, as expected, translocate to the particulate fraction in response to calcium. Interestingly, secretory competence was restored in cells depleted of protein kinase C or in which protein kinase C itself was inhibited by non-hydrolysable GTP analogues, but not by GTP, suggesting that protein kinase C might regulate the ability of a G protein(s) directly controlling the exocytotic machinery to be activated by endogenous GTP.

INTRODUCTION

Protein kinase C (PKC) has been shown to play different roles in exocytosis in a variety of cell types. In both intact (Pocotte et al., 1985) and permeabilized (Pocotte et al., 1985; TerBush et al., 1988; TerBush and Holz, 1990; Isosaki et al., 1991) chromaffin cells, one of the most thoroughly investigated secretory systems, as well as in PC12 cells, a pheochromocytoma-derived cell line (Pozzan et al., 1984), PKC has an important modulatory, but not essential, role in Ca^{2+} -dependent secretion. Stimulatory effects of PKC activation on exocytosis have been reported in various other cell lines, including receptor-activated intact platelets (Hannun et al., 1987), permeabilized platelets (Coorsen et al., 1990), neutrophils (Di Virgilio et al., 1984; Smolen et al., 1989), pituitary cells (Ronning and Martin, 1986; van der Merwe et al., 1990; Stojilkovic et al., 1991) and insulin-secreting cells (Wollheim and Regazzi, 1990). In permeabilized mast cells, PKC has been proposed to be essential in mediating the response of the secretory machinery to calcium (Howell et al., 1989; Churcher and Gomperts, 1990; Churcher et al., 1990). Indeed, a cell system in which PKC plays an essential role in Ca^{2+} -triggered secretion would be an interesting tool to study the molecular mechanisms controlling exocytosis. Antigen-stimulated secretion from rat basophilic leukaemic (RBL) cells has been proposed to absolutely

require PKC activity (Gat-Yablonski and Sagi-Eisenberg, 1990) and strong evidence has recently been presented showing an obligatory role for both the Ca^{2+} -dependent and Ca^{2+} -independent PKC isoenzymes in antigen-stimulated permeabilized RBL cells (Ozawa et al., 1993). Conclusive evidence for an essential role for PKC in the exocytotic machinery itself, however, is difficult to obtain in receptor-stimulated cell systems such as the one mentioned above, since PKC affects not only the exocytotic response but also membrane receptors and transduction pathways which in turn regulate secretion (White and Metzger, 1988; Stojilkovic et al., 1991; Benhamou et al., 1992), thereby potentially obscuring direct effects on the secretory machinery.

We attempted to characterize the role of PKC in Ca^{2+} -induced exocytosis in RBL cells by using an experimental approach allowing direct manipulation of the secretory machinery, independently of receptor stimulation, and consisting of permeabilization by streptolysin O (SLO) of RBL cells. RBL cells are a well characterized secretory system which can be permeabilized by SLO and yet maintain fully the ability to secrete (Ali et al., 1989; De Matteis et al., 1991). These cells express two of the Ca^{2+} -dependent PKC isoenzymes (α and β), the down-regulation of which has been thoroughly described and shown to be complete within a few hours of PMA treatment (Huang et al., 1989). Ca^{2+} -

Abbreviations used: RBL, rat basophilic leukaemia cells; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; 5HT, 5-hydroxytryptamine (serotonin); SLO, streptolysin O; α -PDD, phorbol 12,13 α -didecanoate; β -PDD, phorbol 12,13 β -didecanoate; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; G_{ex} , hypothetical G protein coupled to exocytotic machinery; G_{p} , G protein coupled to phospholipase C; G_{i} , inhibitory G protein; PKC, protein kinase C; PKI, PKC inhibitor peptide derived from PKC (residues 19–36; RFARKGALRQKNVHEVK); PLC, phospholipase C; PMSF, phenylmethanesulphonyl fluoride.

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triggered, receptor-independent, exocytosis in permeabilized RBL cells has also been well characterized and proposed to occur through G protein activation (De Matteis et al., 1991), in a manner similar to other vesicular transport systems (Mayorga et al., 1989; Balch, 1990; Chavrier et al., 1990; Gorvel et al., 1991; Bomsel and Mostov, 1992; Leyte et al., 1992).

We show here that a conventional Ca^{2+} -dependent PKC isoenzyme is required to support the secretory response to calcium in permeabilized RBL cells, and it is suggested that PKC- α might be essential for the control of calcium-triggered exocytosis in RBL cells. Working hypotheses are also provided concerning (a) the mechanism through which the kinase is recruited and activated during calcium stimulation, and (b) the identity of the molecular target(s) (possibly G proteins) of the kinase that are involved in secretion.

EXPERIMENTAL

Sources

Minimum essential medium with Eagle's salts, medium 199 and foetal calf serum were purchased from GIBCO (New York, U.S.A.). 5-[1,2- ^3H]Hydroxytryptamine binoxalate (^3H]5HT), *myo*-[2- ^3H]inositol and [γ - ^{32}P]ATP were from NEN/Dupont. ATP disodium salt, leupeptin, phenylmethanesulphonyl fluoride (PMSF), phosphatidylserine, phorbol 12-myristate 13-acetate (PMA) and histone type III-S were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calphostin C was from Calbiochem (San Diego, CA, U.S.A.) and all the other nucleotides were from Boehringer (Mannheim, Germany). SLO was from Wellcome Reagents Limited (Beckenham, Kent, U.K.). PKC inhibitor peptide [PKI; PKC-(19–36)] was purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). Monoclonal anti-PKC- α antibodies were from UBI (New York, NY, U.S.A.). Polyclonal anti-PKC- α , anti-PKC- δ , anti-PKC- ϵ and anti-PKC- ζ antibodies, purchased from Gibco-BRL, were a gift from Dr. M. Molino, Laboratory of Platelet and Leukocyte Pharmacology, Consorzio Mario Negri Sud, S. Maria Imbaro (Chieti), Italy; anti-PKC- β I and anti-PKC- β II antisera were a gift from Dr. S. Jaken, W. Alton Jones Cell Science Center, Lake Placid, NY, U.S.A. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were from Zymed (San Francisco, CA, U.S.A.). Biotinylated anti-mouse and anti-rabbit IgGs and streptavidin-alkaline phosphatase conjugate were from Gibco-BRL. All other reagents were obtained from various manufacturers at the highest available purity.

Cell culture

RBL-2H3 cells were grown in 75 cm^2 tissue culture flasks in Eagle's minimum essential medium supplemented with 16% foetal calf serum. Adherent cells were subcultured weekly after detachment in a buffer containing 125 mM NaCl, 1.5 mM EDTA, 5.6 mM glucose and 10 mM Hepes at pH 7.2.

5HT release from permeabilized cells

5HT release from permeabilized RBL cells was performed exactly as previously described (De Matteis et al., 1991). In some experiments the following extensive permeabilization protocol of secretion was followed. Cells loaded with [^3H]5HT as described (De Matteis et al., 1991) were washed three times with Tyrode's solution (125 mM NaCl, 5 mM KCl, 5.6 mM glucose, 10 mM Hepes, pH 7.2) to remove unincorporated radioactivity and treated at 4 °C with Tyrode's solution containing 1 mM EGTA and 3 units/ml SLO for 10 min to achieve membrane binding of the toxin. The permeabilization buffer was substituted for

Tyrode's solution containing 1.8 mM MgCl_2 , 10 mM ATP and 1 mM EGTA for 3 min at 37 °C. Finally, a release buffer consisting of the appropriate concentrations of free Ca^{2+} according to Cockcroft and Gomperts (1979) using the affinity constants reported by Tsien and Pozzan (1989) [corrected for pH and ATP concentrations in a preparation of rat brain cytosol diluted to 3 mg/ml (see below) or BSA in the same buffer (as a control)] was added to the cells. Release was terminated after 4 min, at 37 °C as described (De Matteis et al., 1991). With both protocols, release was expressed as a percentage of the total incorporated radioactivity.

For the preparation of rat brain cytosol the following procedure was used. Ten whole brains dissected from CD/COBS rats (Charles River Italia) were washed and homogenized on ice in 40 ml of homogenization buffer (20 mM Hepes, pH 7.2, 1.8 mM MgCl_2 , 1 mM EGTA, 10 mM ATP, 5 mM dithiothreitol, 1 mM PMSF, 0.5 mM *o*-phenanthroline, 10 $\mu\text{g/ml}$ leupeptin, 1 μM pepstatin) with a Janke and Kunkel Ultra-Turrax tissue grinder (Germany) at maximum setting, with three or four 30 s bursts. The homogenate was first clarified at 15000 g for 30 min at 4 °C and then ultracentrifuged at 100000 g for 90 min at 4 °C. Finally, the cytosol was assayed for total protein concentration (usually around 10–12 mg/ml), rapidly frozen in liquid N_2 and stored in aliquots at –80 °C up to a maximum of 3 months, after which activity progressively declined. Prior to use, the cytosol preparation was adjusted to 115 mM NaCl, 5 mM KCl and 5.6 mM glucose and diluted to 4 mg of protein/ml.

PKC assay

Total PKC activity was measured by incorporation of ^{32}P into histone III-S using the procedure of Kikkawa et al. (1983) with slight modifications. Cells were detached and plated on 9 cm Petri dishes at a density of 10^7 cells/10 ml per dish. The following day, cells were washed three times with Tyrode's solution, permeabilized for 5 min as described above, resuspended in 5 ml of homogenization buffer (20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol, 0.1 mg/ml leupeptin, 0.1 mg/ml PMSF and 250 mM sucrose) and disrupted at 4 °C by sonication for 10 s (Sonics and Materials sonicator model Vibra-Cell VC50 with microprobe at setting 60). The homogenate was then centrifuged at 100000 g for 60 min at 4 °C. The pellet was resuspended in homogenization buffer and 0.5% Triton X-100 was added to both supernatants and resuspended pellets. After 30 min at 4 °C, membrane and cytosolic fractions were passed through a DEAE-Sephacel (Pharmacia, Sweden) chromatography column and eluted in a final volume of 1 ml. To measure total PKC activity, the cell homogenate was treated with 0.5% Triton X-100 for 30 min and then passed through the same chromatographic column. Histone phosphorylation was carried out for 10 min at 30 °C by adding 30 μl of lysate to 170 μl of reaction buffer (50 mM Pipes, pH 7.0, 10 mM MgCl_2 , 2 mM EGTA, 0.1 mM dithiothreitol, 500 $\mu\text{g/ml}$ histone type III-S, 12.5 μM ATP) containing 1 μCi of [γ - ^{32}P]ATP, supplemented with 3 mM CaCl_2 alone or with the further addition of 0.5 μM PMA and 40 $\mu\text{g/ml}$ phosphatidylserine to discriminate calcium/phospholipid-dependent kinase activity from calcium-dependent activity. The reaction was stopped by the addition of ice-cold 25% trichloroacetic acid solution and pellets were washed twice by precipitation. Finally, protein pellets were dissolved and radioactivity was counted by liquid scintillation spectroscopy. PKC activity is expressed as pmol of incorporated P_i phosphorus/min per mg of total cellular protein. Protein content was assessed using the Bio-Rad protein assay kit.

PKC Immunoblotting

Cells destined for PKC down-regulation experiments were treated for various times with PMA before further processing. For Ca^{2+} -dependent PKC translocation experiments, cells were first permeabilized and stimulated for secretion as described above. In both cases cells were washed three times with Tyrode's solution after the various treatments, scraped and collected by centrifugation. Pellets were suspended in lysis buffer (120 mM NaCl, 5 mM EGTA, 20 mM Pipes, pH 6.6, 1 mM dithiothreitol, 250 mM sucrose, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ PMSF, 0.5 mM aprotinin, 10 $\mu\text{g}/\text{ml}$ trypsin inhibitor, 1 mM benzamide) and disrupted at 4 °C by sonication for 10 s as specified above. The homogenate was then centrifuged at 1500 g for 10 min. The resulting post-nuclear lysate (total homogenate) was, in some experiments, fractionated further into membrane and cytosolic fractions by centrifuging it at 130000 g for 1.5 h and resuspending the membrane fraction in lysis buffer containing 0.1% Triton X-100. For Ca^{2+} -dependent translocation experiments, CaCl_2 was added to the lysis buffer to achieve 0.3 μM free Ca^{2+} throughout the preparation. After determination of protein content by the assay of Lowry et al. (1951), Laemmli (1970) sample buffer was added to the homogenates and proteins were separated by SDS/PAGE on 10% polyacrylamide gels, transferred to nitrocellulose filters and probed with one of the anti-PKC antibodies. Antigens were detected using horseradish peroxidase-conjugated second antibodies and the chromogenic substrate 4-chloro-1-naphthol, or with the biotin-streptavidin-alkaline phosphatase method.

Phospholipase C (PLC) assay

InsP_3 release was taken as marker of PLC activity. Cells were detached, resuspended in complete growth medium and plated at a density of 10^6 cells/ml in 12-well plates 2 days before the release experiment. On the day before release was measured, complete growth medium was replaced with medium 199 containing either [^3H]inositol (5 $\mu\text{Ci}/\text{ml}$) or [^3H]5HT (1 $\mu\text{Ci}/\text{ml}$). The release experiment using permeabilized cells was conducted in parallel for InsP_3 and 5HT, under identical conditions, using the solutions described above, with the addition of 500 μM 2,3-diphosphoglyceric acid and 5 mM LiCl. In plates labelled with [^3H]inositol the reaction was stopped by the addition of 1 ml of ice-cold methanol. Cells were then scraped and wells were rinsed with a solution of methanol/water (5:12, v/v) (0.85 ml); 1.25 ml of chloroform was then added. Samples (1 ml) of the aqueous phase were loaded on Dowex formate columns and the separation of the products of inositol lipid hydrolysis was carried out as described by Berridge et al. (1983).

RESULTS

Analysis of PKC activity

The presence and activity of PKC were studied by both semi-quantitative immunoblot analysis and an enzymic assay in SLO-permeabilized cells in which the kinase had been activated by acute exposure to PMA, inhibited by specific blockers, or down-regulated by chronic PMA treatment. The Ca^{2+} -dependent conventional α , βI and βII isoenzymes of PKC were rapidly and completely down-regulated by PMA in RBL cells with typical time courses (Figure 1a; Huang et al., 1989); the β form decayed more rapidly (it became undetectable by immunoblot analysis after 10–30 min of exposure to PMA) than the α isoenzyme, which has disappeared completely after 3 h of treatment. As recently shown (Ozawa et al., 1993), the non-conventional Ca^{2+} -independent isoenzymes of PKC, namely δ , ϵ and ζ , also appeared

to be expressed in RBL cells, but seemed to be quite resistant to PMA-induced down-regulation (results not shown). Figure 1(b) shows the time-dependent changes of PKC activity in membrane and cytosolic fractions from cells exposed to 0.1 or 1 μM PMA for 15 min to 6 h and then permeabilized with SLO for 5 min. In control cells, PKC was almost exclusively cytosolic; SLO-induced permeabilization itself caused a 50–70% loss of activity (results not shown). A 15 min treatment with PMA induced the disappearance of PKC from the cytosol and its simultaneous appearance in the membrane fraction, indicating that translocation has occurred (Farago and Nishizuka, 1990). If PMA treatment was continued, membrane PKC activity progressively declined with a rate that was dependent on the PMA concentration, and reached undetectable levels by around 1 h (in cells treated with 1 μM PMA). Surprisingly, cells that had received a short (15 min) PMA treatment exhibited markedly higher levels of total PKC activity than control cells (Figure 1b). This was most probably due to the existence of an endogenous PKC inhibitor, protein phosphatases or proteases in the cytosol, which interfered with the kinase assay in homogenates from control cells (where the enzyme is cytosolic) but not in PMA-treated cells (where the enzyme is mostly membrane-bound). Mixing experiments showed, in fact, that PKC activity in washed membranes was substantially reduced by the addition of cytosol and that

Figure 1 PMA-induced down-regulation and translocation of PKC in RBL cells

(a) Cells were treated with 100 nM (2 and 15 min) or 1 μM (1, 3 and 6 h) PMA and then processed for immunoblotting to detect the α , βI and βII isoenzymes of PKC. Under the experimental conditions used in this study, we always obtained fairly reliable correlations between signal and relative amount of detected protein when known amounts of standards were used. Similar results were obtained in three independent experiments. (b) Cells were exposed to 100 nM PMA (■, ●) or to 1 μM PMA (□, ○) in complete growth medium for the indicated times. They were then permeabilized and processed as described in the Experimental section for the measurement of membrane (■, □) and cytosolic (●, ○) PKC activity. Data are means of three determinations; S.D.s did not exceed 10% of the means. Similar results were obtained in four independent experiments.

Figure 2 Relationship between Ca^{2+} -triggered release and PKC activity

Control or PMA-treated cells (100 nM for 15 min; 1 μM for 60, 180 and 360 min) were permeabilized. PKC activity (●) and 5HT release (○) were then measured as described in the Experimental section. Stimulated release is defined as the difference between Ca^{2+} -triggered (10 μM Ca^{2+}) and basal (0.01 μM Ca^{2+}) 5HT release. Data are means of two to three determinations; S.D.s never exceeded 5% (5HT release) and 10% (PKC activity) of the means. Similar results were obtained in four independent experiments.

protease inhibitors did not modify this inhibition (results not shown).

Correlation between Ca^{2+} -triggered secretion and PKC isoenzyme down-regulation

Ca^{2+} -dependent secretion and membrane-bound PKC activity were measured in cells that had been treated with PMA (100 nM PMA for 15 min to stimulate maximal PKC activity; 1 μM PMA for longer times to obtain a rapid and complete down-regulation of PKC) (Figure 2). Secretion was maximal after exposure to 100 nM PMA for 15 min, at which time membrane PKC activity reached its peak (Figures 1b and 2). It then progressively declined upon longer PMA treatments, in parallel with PKC activity, and became undetectable in coincidence with the disappearance of the α isoenzyme (Figure 1a). Permeabilized PKC-depleted cells were no longer able to secrete when exposed to 10 μM Ca^{2+} . The loss of the secretory response was not due to a non-specific effect of PMA since the inactive analogue phorbol 12,13 α -didecanoate (α -PDD) failed to modify secretion, whereas the active isomer phorbol 12,13 β -didecanoate (β -PDD) had effects similar to those of PMA, albeit at higher concentrations (results not shown). We also examined whether the lack of secretory response in PKC-depleted cells might result from a lower Ca^{2+} sensitivity and/or to a somewhat slower response of the secretory machinery, by using higher Ca^{2+} concentrations (Figure 3a) or longer stimulation times (Figure 3b). PKC-depleted cells, however, were unable to secrete either in response to 100 μM Ca^{2+} or during 12 min of stimulation (Figures 3a and 3b).

The role of PKC in Ca^{2+} -triggered secretion was confirmed by experiments with inhibitors of the enzyme (Table 1). The pseudo-substrate peptide inhibitor of PKC, PKI (House and Kemp, 1987; Malinow et al., 1989; TerBush and Holz, 1990), reduced the secretory response to Ca^{2+} by 60–70% in permeabilized cells. Pretreatment with the highly specific inhibitor calphostin C (Kobayashi et al., 1989; Bruns et al., 1991) also decreased Ca^{2+} -triggered release by 58% at 1 μM . When cells were extensively permeabilized with high concentrations of SLO as described in the Experimental section, they completely lost the ability to secrete in response to Ca^{2+} . Secretion, however, could be recon-

Figure 3 Effect of increasing Ca^{2+} concentration and time course of secretion in PMA-treated cells

Control cells (●), or cells treated for 15 min with 100 nM PMA (○) or for 6 h with 1 μM PMA (△) were permeabilized and then treated as follows. In (a), cells were exposed to solutions containing the indicated Ca^{2+} concentration. In (b), permeabilized cells were exposed to solutions containing 10 μM Ca^{2+} for 3 min. The solutions were renewed every 3 min and the radioactive content was determined. The release rate is the ratio (%) between the radioactivity released during each 3 min period and the total radioactivity present in the cells at the beginning of that period. Data are means of three determinations; S.D.s never exceeded 5% of the means. Similar results were obtained in four independent experiments.

Table 1 Effects of PKC inhibitors on exocytosis stimulated by Ca^{2+} or Ca^{2+} + GTP[S] in permeabilized RBL cells

Cells were permeabilized and stimulated with 10 μM Ca^{2+} or 10 μM Ca^{2+} plus 10 μM GTP[S] in the presence or absence of 100 or 200 μM PKI (applied during both the permeabilization and release periods) or 1 μM calphostin C (applied under fluorescent light for 15 min prior to permeabilization). Stimulated release is defined as in the legend of Figure 2. Data are means $\pm$ S.D. of three determinations, except for those with PKI (two independent experiments). Values in parentheses indicate release as a percentage of control release (no inhibitor present).

Condition	5HT release (%)	
	Ca^{2+}	Ca^{2+} + GTP[S]
Control	11.8 $\pm$ 3.3 (100)	16.9 $\pm$ 3.0 (100)
PKI (100 μM)	5.1 $\pm$ 0.2 (43)	14.1 $\pm$ 0.9 (83)
PKI (200 μM)	4.4 $\pm$ 0.7 (37)	14.2 $\pm$ 1.8 (84)
Calphostin C (1 μM)	5.0 $\pm$ 0.4 (42)	11.2 $\pm$ 0.9 (66)

stituted to almost normal levels by adding a dilute preparation of rat brain cytosol containing 10 μM free Ca^{2+} . When this protocol was applied to PKC-depleted cells, the preparation of rat brain cytosol was still able to restore the Ca^{2+} -dependent secretory response (Table 2).

Table 2 Reconstitution of secretion in exhaustively permeabilized PKC-depleted RBL cells

Control cells or cells treated for 6 h with $1 \mu\text{M}$ PMA were extensively permeabilized as described in the Experimental section and release was stimulated by adding $10 \mu\text{M}$ free Ca^{2+} in a preparation of rat brain cytosol or BSA in the same buffer (as a negative control). Results are means $\pm$ S.D.

Condition	5HT release (%)	
	Control	PKC-depleted
BSA	4.5 ± 1.1	0.9 ± 0.8
Brain cytosol	14.4 ± 4.8	7.9 ± 2.7

Figure 4 Ca^{2+} -dependence of 5HT release, InsP_3 production and PKC- α translocation in permeabilized RBL cells

Cells were permeabilized and exposed to solutions containing the specified Ca^{2+} concentration. (a) InsP_3 production (○) and 5-HT release (●) were determined as described in the Experimental section. Similar results were obtained in four independent experiments, and S.D.s never exceeded 5% (5HT release) or 10% (InsP_3 production) of the means. (b) Translocation of PKC- α to the particulate fraction when the Ca^{2+} concentration was increased from non-stimulatory (1, 10 nM) to stimulatory (2, $10 \mu\text{M}$) levels is shown. Similar results were obtained in two independent experiments.

Calcium activates PLC and induces translocation of PKC in SLO-permeabilized cells

The requirement for PKC in Ca^{2+} -triggered secretion suggests that one of the mechanisms by which Ca^{2+} induces secretion is the activation of PKC. Since the main endogenous activator of PKC is the PLC metabolite diacylglycerol (Farago and Nishizuka, 1990), and since PLC is a Ca^{2+} -dependent enzyme (Rhee et al., 1989), we examined whether Ca^{2+} might activate PLC under conditions where it stimulates release, using InsP_3 generation as a marker of PLC activity (Berridge et al., 1983). The production of InsP_3 was indeed stimulated by Ca^{2+} . The Ca^{2+} concentration-response curves for InsP_3 generation and 5HT release are shown in Figure 4(a). Maximal responses were achieved at $10 \mu\text{M}$ Ca^{2+} for 5HT and $> 30 \mu\text{M}$ Ca^{2+} for InsP_3 . Neomycin, an inhibitor of PLC, reduced both InsP_3 production and the secretory response, by 60% and 40% respectively (results not

Figure 5 Effect of guanine nucleotides on Ca^{2+} -triggered 5HT release in cells with different levels of PKC activity

(a) Cells were treated for 15 min with 100 nM PMA or for 60, 180 and 360 min with $1 \mu\text{M}$ PMA. Treated and control cells were then permeabilized and exposed to $10 \mu\text{M}$ Ca^{2+} either alone (A) or with $10 \mu\text{M}$ GTP[S] (B), 1 mM GTP (C) or 3 mM GDP[S] (D). The shaded areas indicate the net effects of the nucleotides as calculated by subtracting the Ca^{2+} -alone-evoked release from that induced by Ca^{2+} plus the nucleotide. (b) Control cells (○) and cells treated for 6 h with $1 \mu\text{M}$ PMA (●) were permeabilized and exposed to $10 \mu\text{M}$ Ca^{2+} -containing solutions with the indicated concentrations of GTP[S]. In both cases data are means of two to three determinations; S.D.s never exceeded 5% of the means. Similar results were obtained in four independent experiments.

shown). We also investigated whether Ca^{2+} could directly cause translocation of PKC to the particulate fraction under the same experimental conditions in which exocytosis was studied. Indeed, in permeabilized cells, stimulation of secretion with $10 \mu\text{M}$ Ca^{2+} induced the translocation of PKC- α to the particulate fraction (Figure 4b) but not, as expected, the δ , ϵ and ζ isoenzymes (results not shown). These latter observations were obtained by including $10 \mu\text{g/ml}$ leupeptin in the release buffer; this did not impair or modify the secretory response (results not shown).

Guanosine 5'-[γ -thio]triphosphate (GTP[S]) restores the secretory response to Ca^{2+} in PKC-depleted cells

As both PKC and a G_e protein(s) (De Matteis et al., 1991) appear to be required for Ca^{2+} triggered secretion in RBL cells, we sought to study their possible interactions by examining the effects of guanine nucleotides in cells with different levels of PKC activity. When the secretagogue effects of Ca^{2+} alone, Ca^{2+} plus GTP, or Ca^{2+} plus GTP[S] were compared in cells treated with PMA for different times, three distinct patterns were obtained (Figure 5a). The effect of Ca^{2+} alone peaked after 15 min of PMA

treatment, then declined rapidly upon continuing the treatment. The stimulation by Ca^{2+} plus GTP began declining only after 60 min. Both stimuli, however, completely lost their effectiveness in PKC-depleted cells (after 6 h of PMA treatment). Ca^{2+} plus GTP[S], however, retained substantial activity even after a 6 h PMA treatment. The restoring activity of GTP[S] on Ca^{2+} -triggered secretion was totally abolished by guanosine 5'-[β -thio]diphosphate (GDP[S]). Figure 5(b) shows the dose-response curves for GTP[S] in control and PKC-depleted cells. The EC_{50} values and the maximal effective concentrations were similar, but the maximal effect of the nucleotide was larger in PKC-depleted cells. Other non-hydrolysable analogues of GTP had effects similar to those of GTP[S]. The rank order of potency was $\text{GTP[S]} > \text{guanosine 5'-}[\beta\gamma\text{-imido}]\text{triphosphate} > \text{guanosine 5'-}[\beta\gamma\text{-methylene}]\text{triphosphate}$ (results not shown). Thus, GTP[S] but not GTP was able to support the secretory response in cells deprived of PKC. This conclusion was confirmed by experiments in which PKC activity was suppressed by synthetic inhibitors. Ca^{2+} alone was a poor secretagogue in cells treated with the PKI peptide or calphostin C (Table 1), but the addition of GTP[S] restored its secretagogue activity. Indeed, as in the case of PKC-depleted cells, the relative effect of GTP[S] was higher in PKC-inhibited cells, so that absolute values of secretory response were higher than (in PKI-treated cells) or equal to (in calphostin C-treated cells) control release (Table 1). Complete inhibition of the secretory response was observed at higher doses of calphostin C in RBL cells (Ozawa et al., 1993; R. Buccione and M. A. De Matteis, unpublished work), but this might be due to unspecific cytotoxicity (Bruns et al., 1991) and was in fact not reversed by addition of GTP[S] (results not shown).

DISCUSSION

It is reported here that, in RBL cells, PKC activity is essential for Ca^{2+} -triggered release. A preliminary characterization seems to indicate the Ca^{2+} -dependent α isoenzyme as the candidate for the control of Ca^{2+} -triggered receptor-independent exocytosis. Evidence is also presented that in these cells PKC may be activated directly both by Ca^{2+} -promoted translocation and by diacylglycerol generated by PLC during Ca^{2+} -triggered secretion. PKC may act in the secretory process by regulating, among other things, a G protein directly controlling the exocytotic machinery (G_e; Gomperts, 1990; De Matteis et al., 1991).

Requirement for PKC in the secretory process

There are 10 reported molecular isoenzymes of PKC, but which of them is involved in exocytosis is still controversial. Of the Ca^{2+} -dependent isoenzymes the α and β isoforms, but not the γ isoform, have been identified in RBL cells. A recombinant β isoenzyme is able to reconstitute receptor-mediated secretion in permeabilized PKC-deleted RBL cells (Ozawa et al., 1993), and both the α and β types, but not the γ form, are able to reconstitute PMA-induced release of luteinizing hormone in PKC-depleted pituitary cells (Naor et al., 1989) or PC12 cells (Ben Shlomo et al., 1991). The α and β isoenzymes can be discriminated by their different rates of down-regulation in RBL cells (Figure 1a; Huang et al., 1989), the β form decaying more rapidly (it becomes undetectable by immunoblot after 10–30 min of exposure to PMA) than the α isoenzyme; the latter disappears completely only after 3 h of treatment (Figure 1a; Huang et al., 1989). A comparison of these rates with the decay kinetics of Ca^{2+} -triggered release (Figure 2) suggests that the α form is more likely to be the one supporting release. A similar correlation was found between the intensity of the secretory response and the levels of membrane-bound PKC activity, both after acute PMA

treatment, which potentiated the secretory response, and after prolonged PMA treatment, which led to gradual loss of both PKC activity and secretory competence. It appeared, however, that the decay of membrane-bound PKC activity was more rapid (undetectable by 1–2 h) than the decrease in PKC- α immunoreactivity, which was still detectable at 3 h. This apparent discrepancy is unresolved at this time, although one could suggest various explanations; for example the fact that measurable PKC activity could be mostly due to the β isoenzymes or that the enzymic assay is less sensitive than immunoblot detection. Ozawa et al. (1993), based on reconstitution studies with recombinant isoenzymes of PKC in PKC-depleted permeabilized washed RBL cells, were recently able to show that receptor-mediated secretion relied heavily on the Ca^{2+} -dependent β -isoenzyme and partially on the Ca^{2+} -independent δ isoenzyme. Our system, however, is based on the use of permeabilized RBL cells that retain about 30% of the residual Ca^{2+} -dependent PKC activity and are fully responsive to stimulation. Moreover, the purpose of this study was to examine the secretory response to Ca^{2+} rather than to antigen which, through the activation of the IgE receptor, exerts pleiotropic effects. We confirm that the Ca^{2+} -independent (δ , ϵ and ζ) isoenzymes of PKC (Ono et al., 1988, 1989; Koide et al., 1992; Ogita et al., 1992) appear to be expressed in RBL cells, but we suggest that they are unlikely to be involved in the late stages of Ca^{2+} -triggered exocytosis in RBL cells because of their insensitivity to Ca^{2+} and their resistance to PMA-induced down-regulation (our results not shown; Strulovici et al., 1991; Ways et al., 1992; Ozawa et al., 1993; for reviews, see Asoaka et al., 1992; Azzi et al., 1992), which instead leads to inhibition of secretion.

To summarize, we propose that while PKC- β is certainly required for IgE receptor-stimulated release (Ozawa et al., 1993) and might contribute to Ca^{2+} -triggered release, PKC- α appears sufficient to support the latter. Reconstitution experiments with recombinant isoenzymes are required to ascertain whether PKC- α , PKC- β or both are sufficient to support Ca^{2+} -triggered secretion.

The loss of secretory competence in PKC-depleted cells was probably due to the loss of an essential function of PKC in the exocytotic reaction, rather than to non-specific or toxic effects of PMA or PKC depletion. This was indicated by the following evidence: (a) reconstitution of the secretory response with rat brain cytosol in PMA-down-regulated cells demonstrates that the secretory apparatus remains intact and capable of normal function once appropriate soluble factors are added back to the cell ghosts; (b) the decay of secretion was induced by the active PMA analogue β -PDD but not by the inactive isomer α -PDD; (c) the response was not due to secretory granule depletion (a problem encountered with other cell types; see Stojilkovic et al., 1991) because in RBL cells PMA alone does not induce release, but rather potentiates the response to Ca^{2+} ; (d) it was not likely to be related to the prolonged period of PKC stimulation which precedes and accompanies down-regulation (Wilson, 1990), since Ca^{2+} -induced release closely parallels the levels of membrane-bound, and thus activated, PKC. In addition, both PKI and calphostin C are inhibitory on Ca^{2+} -induced release although, not surprisingly, they are 'leaky' in their effect (Table 1).

As to how and when PKC might function during the release process, our results seem to indicate that, at least in permeabilized cells, PKC may be rapidly recruited by Ca^{2+} during the secretory event, since stimulatory concentrations (micromolar) of Ca^{2+} stimulate InsP_3 formation. The inhibitory effect of neomycin on secretion at concentrations that inhibit PLC also points to the involvement of this enzyme in Ca^{2+} -triggered secretion. Micromolar Ca^{2+} also induces translocation of PKC- α to the particulate

fraction in permeabilized cells, where 30% of the total PKC is retained. It cannot be excluded, however, that a fraction of the secretory response relies on a phosphoprotein generated by PKC prior to permeabilization. An elevation in cytosolic Ca^{2+} might also stimulate phospholipase D, thereby activating PKC through the diacylglycerol yielded by dephosphorylation of phosphatidic acid, the product of phospholipase D activity (Lin and Gilfillan, 1992).

Role of G proteins

A central observation in this study is that the non-hydrolysable guanine nucleotide analogue GTP[S], but not GTP, overcomes the effect of PKC depletion and restores secretory competence in PKC-depleted cells. In fact, when Ca^{2+} alone or Ca^{2+} plus GTP are used as stimuli, the secretory response is lost in PKC-depleted cells. By contrast, under the same conditions, the combination of Ca^{2+} and GTP[S] is still capable of eliciting secretion. Earlier work by Gomperts and collaborators (Howell et al., 1989; Churcher and Gomperts, 1990; Churcher et al., 1990) showed that, in permeabilized rat mast cells, protein phosphorylation catalysed by PKC was essential in maintaining responsiveness to Ca^{2+} . GTP[S] allowed a dephosphorylation event permissive for secretion, but still required the presence of a PKC-phosphorylated substrate(s) or the activity of PKC itself. The results we obtained in RBL cells are somewhat different in this respect, as the secretory machinery appears to be still functional in PKC-depleted and PKI peptide- or calphostin C-treated RBL cells, and can be activated by shifting a G protein(s) to the active conformation with GTP[S]. What seems to be lost in PKC-depleted RBL cells is thus the ability of a G protein(s) to be activated by GTP. This could be due to a reduced rate of activation (GDP-GTP exchange) or to an increased rate of inactivation (GTP hydrolysis) of the G protein. The efficacy of non-hydrolysable analogues such as GTP[S] could therefore be explained by their ability to force the equilibrium towards the GTP-bound form. These considerations, together with the fact that full activation of PKC by PMA does not compensate for the GDP[S] block of G proteins and subsequently of exocytosis (Figure 5a), suggest that PKC might be necessary to activate a G protein (Sagi-Eisenberg, 1989), or maybe, in the case of the monomeric ras-like GTP-binding proteins, the accessory proteins GTPase activating protein (GAP) or GDP/GTP exchange factors (see Macara, 1991, for a review). Some reports do indicate that PKC-catalysed serine phosphorylation and pp60^{src} -catalysed tyrosine phosphorylation (enhanced by PKC activation) of G_{α} subunits (Lounsbury et al., 1991; Hausdorff et al., 1992) and PKA-catalysed phosphorylation of a ras-like monomeric GTP-binding protein (Hata et al., 1991) all seem to regulate G proteins by increasing their ability to exchange GDP for GTP.

Concluding remarks

A G protein(s) is unlikely to be the only target of PKC in the regulated secretory machinery, since activation of PKC by acute PMA treatment strongly potentiates the stimulatory effect of GTP[S]. In RBL and chromaffin cells other such targets could include the myosin light chain and calpactin (Ludowyke et al., 1989; Burgoyne, 1991). PKC activity appears also to be required in some instances for the activity of tyrosine kinases in RBL cell exocytosis (Benhamou et al., 1992). Our current view is that, in RBL cells, Ca^{2+} has complex effects in regulated exocytosis: it stimulates diacylglycerol generation via PLC, thereby activating a Ca^{2+} -dependent PKC ($\alpha?$); the kinase, in turn, activates a G protein(s) as well as probably other substrates, which then converge with Ca^{2+} on to a common step. We also suggest that

the fraction of the exocytotic response regulated by Ca^{2+} -independent isoenzymes (Ozawa et al., 1993) might be related to receptor activation and not part of the purely Ca^{2+} -dependent response in RBL cells.

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