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Differential down-regulation of protein kinase C selectively affects IgE-dependent exocytosis and inositol trisphosphate formation

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Short-term treatment of rat basophilic leukaemia (RBL-2H3) cells with the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) activates protein kinase C (PKC) and results in the inhibition of the IgE-dependent formation of inositol phosphates, but in the potentiation of serotonin secretion. Long-term treatment with TPA, which depletes the cells of their endogenous PKC, eliminates both Ca^{2+}-ionophore- and TPA- as well as IgE-dependent secretion, but it potentiates by 1.7-fold IgE-induced inositol phosphate formation. Taken together, these observations strongly suggest that the dual actions of TPA on IgE-dependent responses are both mediated by PKC. The opposing effects of TPA are differentially down-regulated. Following TPA treatment, the rate by which the cells lose their ability to undergo exocytosis is faster than the rate at which inhibition of inositol phosphates formation is relieved and their production potentiated. In addition, both processes show different sensitivities to inhibitors of PKC action. Whereas IgE-dependent secretion is completely blocked by the PKC inhibitors K252a, H-7 and sphingosine [concns. causing 50% inhibition (IC_{50} values) = 25 ng/ml, 80 μM and 30 μM respectively], these inhibitors do not relieve inhibition of inositol phosphate formation by TPA, nor do they potentiate this response. These results may imply that the bidirectional control exerted by PKC on IgE-dependent responses is mediated by its different isoenzymes.

INTRODUCTION

A variety of cellular responses, including IgE-dependent exocytosis in mast and rat basophilic leukaemia (RBL-2H3) cells [1], involve stimulation of PtdIns(4,5)P_2 breakdown, leading to the generation of diacylglycerol (DAG), which activates protein kinase C (PKC), and of InsP_3, which mobilizes Ca^{2+} from intracellular stores (reviewed in [2,3]). We [4] and others [5] have previously shown that the phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) acts in a synergistic manner with Ca^{2+} ionophores to stimulate secretion. Nevertheless, we have also demonstrated that TPA inhibits the rise in the intracellular Ca^{2+} concentration that follows aggregation of the receptors for IgE [6]. These findings were confirmed and extended to numerous cellular systems (reviewed in [2]). Moreover, inhibition of the Ca^{2+} signal was found to be closely correlated with inhibition of PtdIns(4,5)P_2 breakdown (reviewed in [3]). Hence the dual action of TPA on cell function is not confined to mast cells only but represents a universal mechanism. In an attempt to resolve the mechanisms underlying the opposing effects of TPA, we proposed [7] that two forms of PKC may exist, one responsible for regulation of Ca^{2+} in a negative-feedback control, and the other involved in activation of exocytosis. Although this hypothesis was rather speculative when proposed, it is now clear that indeed more than one species of PKC enzyme exist [8–10]. At least seven subspecies of PKC have been identified that share a common, closely related, structure and yet are clearly distinct. Moreover, based on the findings that these subspecies differ in their cellular and intracellular distributions (reviewed in [10]) and that they show different activation patterns in response to cofactors such as Ca^{2+}, DAG, phosphatidylserine (PtdSer) and fatty acids [10], the possibility arises that the different isoenzymes of PKC may play different physiological roles. RBL cells express both the Type II and Type III isoenzymes of PKC [11]. We have therefore undertaken this study to examine whether the dual actions of TPA are both mediated by PKC, and, if so, whether different isoenzymes of PKC may be involved. For this purpose we have subjected RBL cells to short- and long-term treatments with TPA, conditions that respectively activate and down-regulate PKC. The effects of these treatments on IgE-dependent exocytosis and PtdIns(4,5)P_2 breakdown have been evaluated.

MATERIALS AND METHODS

Chemicals

myo-[^{3}H]Inositol (13.8 Ci/mmol), [^{3}H]serotonin (10.8 Ci/mmol) and monoclonal antibodies against PKC (MC5) were purchased from Amersham. Alkaline phosphatase-conjugated anti-(mouse IgG) antibodies were from Promega. Ca^{2+} ionophore A23187 was from Calbiochem; monoclonal, dinitrophenyl (DNP)-specific, IgE was a generous gift from Dr. Z. Eshhar (Weizmann Institute) and DNP_8-BSA was synthesized as described in [12]. All other reagents used were from Sigma.

Cells

RBL-2H3 cells were maintained as previously described [4] in 250 ml tissue culture flasks (Nunc) in Eagle's minimal essential medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum (Biolab, Jerusalem, Israel), 4 mM-glutamine and antibiotics, in 5% CO_2 at 36 °C.

Secretion

Secretion was determined as previously described [4]. Briefly, cells grown in 24-well Costar plates were preloaded for 16–20 h with [^{3}H]serotonin (5 μCi/10^6 cells), and incubated for 90 min with a monoclonal DNP-specific IgE-class antibody. They were then washed three times and further incubated in 0.4 ml of Tyrode buffer (in mM: 137 NaCl, 2.7 KCl, 0.4 NaH_2PO_4 and 20 Hepes, pH 7.5) supplemented with 5.6 mM-glucose, 1.8 mM-$CaCl_2$ and 0.1% BSA. After triggering with the indicated reagents, 0.1 ml samples from the supernatants were taken for counting of radioactivity. Release is presented as a percentage of

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PLC, phospholipase C; PtdSer, phosphatidylserine; PKC, protein kinase C; DAG, diacylglycerol; DNP, dinitrophenyl; G-protein, GTP-binding protein; IC_{50}, concn. causing 50% inhibition.

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the total [^{3}H]serotonin taken up by the cells. The latter value was determined by lysing the cells with 1 M-NaOH.

Analysis of inositol phosphates

Cells were preloaded for 16–20 h with *myo*-[^{3}H]inositol (4 μCi/10^6 cells). The cells were washed three times in Tyrode buffer, and portions (1×10^6 cells) were subsequently incubated with the desired reagents at a final volume of 0.4 ml at 37 °C. Reaction was terminated by adding 1.5 ml of chloroform/methanol (1:2, v/v), followed by 0.5 ml of chloroform and 0.5 ml of water. The upper phases were then applied on to Dowex-1 columns (formate form) [13]. The inositol phosphates were eluted sequentially using 0.1 M-formic acid/0.2 M-ammonium formate (for InsP_1), 0.1 M-formic acid/0.4 M-ammonium formate (for InsP_2) and 0.1 M-formic acid/1.0 M-ammonium formate (for InsP_3).

Phosphorylation of cytosolic proteins

Control cells or cells pretreated for the indicated time periods with the desired TPA concentrations were subsequently washed three times with buffered saline and suspended in a buffer containing β-glycerophosphate (80 mM), EGTA (20 mM), $MgCl_2$ (15 mM), aprotinin (2.5 μg/ml), trypsin inhibitor (10 μg/ml), vanadate (2 mM) and phenylmethanesulphonyl fluoride (1 mM), pH 7.4. The cells were lysed by repeated freezing in liquid N_2 and thawing. Supernatants were collected by centrifugation at 12000 *g* for 15 min at 4 °C. For phosphorylation, 20 μg portions were incubated in the presence or absence of PtdSer (200 μg/ml), and the reaction was initiated by the addition of a reaction mixture yielding the following concentrations in a final volume of 100 μl: Na-Hepes, pH 7.6 (20 mM), magnesium acetate (10 mM), Ca^{2+} (2 mM), [γ-^{32}P]ATP (50 μM, 5 μCi) and dithiothreitol (2 mM). Reactions were carried out for 15 min at 30 °C and terminated by adding 50 μl of Laemmli's [14] sample buffer containing 5 mM-ATP. Samples were boiled for 5 min and proteins were separated on SDS/PAGE (10% gels) [14]. Gels were dried and radiographed.

Immunodetection of PKC

PKC levels and localization were determined by our modification to the method described in [15]. Cells grown in 24-well Costar plates were pretreated for the indicated time periods with TPA and washed three times with ice-cold buffered saline. Cytosolic proteins were extracted by adding 200 μl of a buffer containing Mops, pH 7.2 (20 mM), EGTA (10 mM), EDTA (5 mM) and digitonin (0.5 mg/ml) to each well. The supernatants containing the cytosolic proteins were collected. Subsequently, 200 μl of the same buffer containing 0.5% Triton X-100 was added and the supernatants containing the membrane Triton-soluble proteins were collected. Portions of both fractions were added to Laemmli's [14] sample buffer, boiled for 5 min and separated on SDS/PAGE (10% gels). The gels were transferred to nitrocellulose papers, blocked with BSA (1%) in buffered saline and incubated for 18 h with a monoclonal anti-PKC antibody. Following four 5 min washes with 0.1% Tween 20 in buffered saline, incubation with alkaline phosphatase-conjugated anti-(mouse IgG) antibodies continued for 2 h. The blots were then washed and the colour reaction was developed according to the manufacturer's instructions.

Presentation of data

The results presented are mean values of duplicate determinations of a representative experiment. Similar results were obtained on at least four separate experiments in each case.

RESULTS

Activation of PKC by TPA involves translocation of the enzyme from the cytosol to the membrane [16]. However, in its active membrane-bound conformation the enzyme becomes susceptible to proteolysis and it is degraded and lost from the cell [17]. To determine levels and subcellular distribution of PKC, control or TPA-treated cells were immediately solubilized in 0.5% Triton X-100 to obtain a total cell extract (Fig. 1*a*) or, alternatively, cells were first permeabilized in a lysis buffer containing 0.05% digitonin (Fig. 1*b*), which has previously been shown to release cytosolic proteins while leaving the membrane-bound proteins intact [15]. The membrane proteins were subsequently extracted in the same buffer supplemented with 0.5% Triton X-100 (Fig. 1*c*). PKC levels in total cell extracts as well as in the cytosolic and membrane fractions were determined by Western blot analysis using monoclonal anti-PKC antibodies

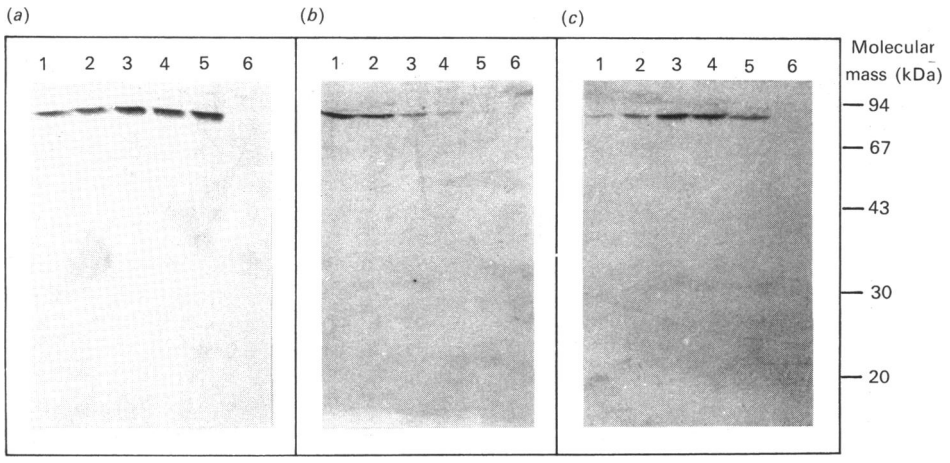

Fig. 1. Effects of short- and long-term treatment with TPA on immunodetectable PKC levels

Cells grown in 24-well Costar plates were incubated with TPA (100 ng/ml) for the following time periods: 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 30 min (lane 4), 60 min (lane 5) or 18 h (lane 6). They were subsequently permeabilized in 0.5% Triton X-100 (*a*) or fractionated to give cytosolic (*b*) and membrane (*c*) fractions by subsequent permeabilization in 0.05% digitonin and 0.5% Triton X-100-containing buffers, as described in the Materials and methods section. PKC levels in these fractions were determined by Western blotting analysis as described in the Materials and methods section.

that recognize an epitope located at the hinge that connects the regulatory and catalytic domains of both Type II and Type III isoenzymes.

As illustrated in Fig. 1, in resting RBL cells most (> 90%) of the PKC resided in the cytosolic fraction (lane 1). Following TPA treatments for 5 min (lane 2), 10 min (lane 3) or 30 min (lane 4), there was no significant change in PKC levels detected in the total cell extract (Fig. 1*a*). However, TPA treatment had a pronounced effect on the subcellular distribution of PKC. Already after 10 and 30 min of cell treatment with TPA there was hardly any immunoreactivity left in the cytosolic fraction (Fig. 1*b*, lanes 3 and 4), whereas the immunoreactive band present in the membrane fraction became much stronger (Fig. 1*c*, lanes 3 and 4). This is most probably due to the translocation of cytosolic PKC to the membrane as a result of TPA treatment. Following a longer incubation period with TPA (60 min), the cellular level of PKC did not seem to be altered if the cells were immediately lysed in the Triton-containing buffer (Fig. 1, lanes 5). However, if cells were first subfractionated the level of PKC recovered in the membrane fraction (Fig. 1*c*, lane 5) did not account for all of the cytosolic PKC present in control cells (Fig. 1*b*, lane 1), implying that proteolysis of the translocated PKC has occurred. Indeed, in agreement with previous studies [11,17], longer incubation periods with TPA (18 h; lanes 6) caused the complete disappearance of PKC from the total cell extract as well as from the membrane fraction.

To ascertain that TPA specifically affected PKC and not other cellular proteins, the effects of TPA treatments on the profile of protein phosphorylation that is either dependent or independent of PKC were examined. As shown in Fig. 2, in the presence of Mg^{2+}, [γ-^{32}P]ATP and Ca^{2+} (2 mM), the pattern of phosphorylation of cytosolic fractions derived from cells treated with TPA for 30 min (lane c) or 18 h (lane e) was similar to that of control cells (lane a). The major protein phosphorylated under these conditions (p95) could be elongation factor EF-2 that was shown previously to serve as a major substrate for a Ca^{2+}/calmodulin-dependent protein kinase III [18]. These findings reveal that short- or long-term treatments with TPA have no effect on the location or catalytic activity of Ca^{2+}-dependent kinases or their substrates that are present in the cytosolic fraction of RBL cells. In contrast, marked differences in the phosphorylation profile, depending on TPA treatment, were observed in the presence of PtdSer. Although addition of PtdSer to cytosol derived from control cells induced the phosphorylation of several new proteins of 18, 30, 38, 46, 64, 80 and 150 kDa (lane b), these phosphoproteins failed to appear following addition of PtdSer to cytosols derived from cells treated with TPA for 30 min or 18 h (lanes d and f). The Ca^{2+} and PtdSer dependency of the phosphorylation of these proteins suggests that they represent substrates of PKC that undergo phosphorylation *in vitro* when the cytosolic PKC is activated. Their failure to undergo phosphorylation in the TPA-treated cytosols is compatible with the observation that, following such treatments, no PKC is left in the cytosol of these cells. Indeed, addition of exogenous purified PKC to the depleted cytosol restored the phosphorylation of all of the substrates *in vitro* (lane g). Thus TPA treatment affected PKC but not any of its potential cytosolic substrates.

Following short-term treatment with TPA (100 ng/ml, 30 min), which results in translocation of PKC to the membrane fraction, secretion in response to Ca^{2+} ionophore (A23187, 100 ng/ml) was markedly potentiated (Fig. 3, lane b compared with lane a). Similarly, secretion in response to an antigen against which IgE is directed was significantly (by 1.7-fold) potentiated (Fig. 3, lane e compared with lane d). Longer incubation periods with TPA (18 h), which result in down-regulation and complete loss of PKC from the cells, caused complete inhibition of both ionophore/TPA- and antigen-induced secretion (Fig. 3, lanes c and f).

To further evaluate the involvement of PKC in exocytosis, the effects of several reagents, previously reported to inhibit PKC activity, on IgE-dependent exocytosis were studied. The three inhibitors tested, K252a [19], H-7 [20] and sphingosine [21], were all found to inhibit antigen-induced serotonin secretion (Fig. 4). Inhibition was dose-dependent with IC_{50} values (concns. causing 50% inhibition) of 25 ng/ml, 80 μM and 30 μM respectively.

In agreement with previous studies [1], addition of antigen markedly stimulated InsP_3 formation (Fig. 5, lane b compared

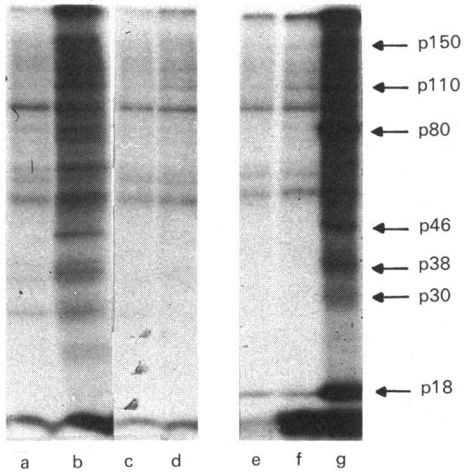

Fig. 2. Effects of TPA treatment on PKC-mediated phosphorylation

Cytosol fractions, prepared as described in the Materials and methods section from control (lanes a and b) or TPA-treated (for 30 min, c and d; for 18 h, e, f and g) cells, were phosphorylated in the presence of Ca^{2+} (2 mM) with (b, d, f and g) or without (a, c and e) PtdSer (200 μg/ml). Purified PKC (0.25 μg) was added in lane g. Protein phosphorylation was analysed as described in the Materials and methods section.

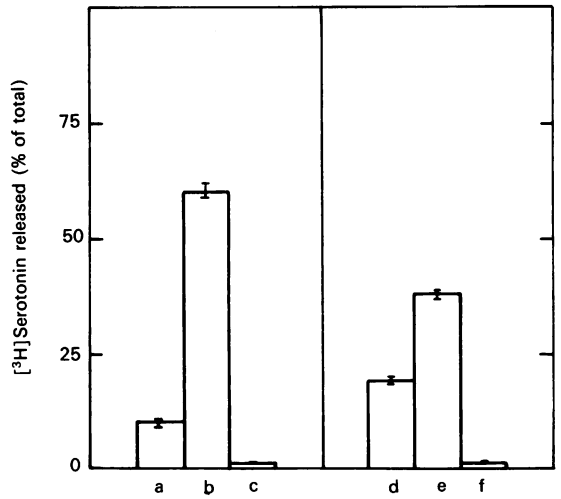

Fig. 3. Effects of short- and long-term treatment with TPA on serotonin secretion from RBL cells

IgE-bearing cells preloaded with [^{3}H]serotonin were incubated with TPA (100 ng/ml) for 30 min (b, e) or 18 h (c, f), or without TPA (a, d). The cells were subsequently triggered either with a Ca^{2+} ionophore (A23187, 100 ng/ml) (a–c) or with antigen (DNP_8-BSA, 150 ng/ml) (d–f) for 20 min. Serotonin secretion was determined as described in the Materials and methods section.

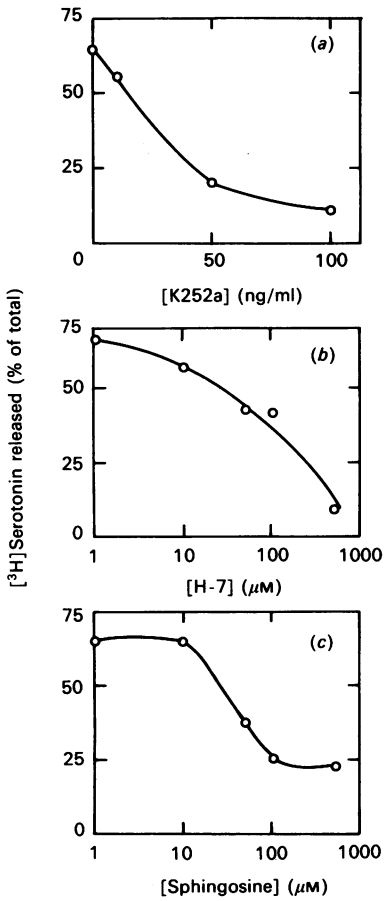

Fig. 4. Effect of PKC inhibitors on antigen-induced serotonin secretion

IgE-bearing cells preloaded with [^{3}H]serotonin were preincubated for 30 min with the indicated concentrations of the PKC inhibitors K252a (*a*), H-7 (*b*) or sphingosine (*c*). Antigen (DNP_8-BSA, 150 ng/ml) was subsequently added and the incubation was continued for a further 20 min. Serotonin secretion was determined as described in the Materials and methods section.

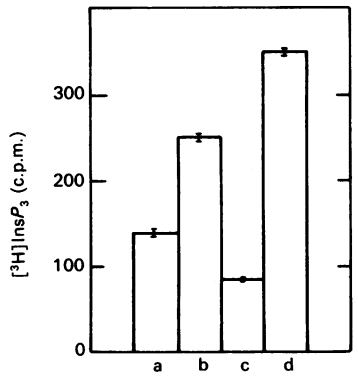

Fig. 5. Effects of short- and long-term treatment with TPA on antigen-induced $InsP_3$ formation

IgE-bearing cells preloaded with [^{3}H]inositol were incubated with TPA (100 ng/ml) for 30 min (c) or 18 h (d) or without TPA (a, b). The cells were subsequently incubated for 10 min with (b–d) or without (a) antigen (DNP_8-BSA, 150 ng/ml). Inositol phosphates formed were analysed as described in the Materials and methods section.

with lane a). However, whereas short incubation periods (30 min) completely inhibited antigen-induced $InsP_3$ formation (Fig. 5, lane c compared with lane b), TPA had no inhibitory effect on $InsP_3$ formation after longer treatments (18 h). Moreover, under these conditions the response to antigen was potentiated by 1.73 ± 0.20-fold ($n = 4$) (Fig. 5, land d).

The effects of TPA were specific. They could not be mimicked by the vehicle (dimethyl sulphoxide) or by the inactive analogue of TPA (methyl-TPA) (results not shown). They could however be mimicked by phorbol dibutyrate, another active phorbol ester (results not shown).

The subspecies of PKC have recently been shown to differ in their susceptibility to proteolysis [22]. Moreover, they have been shown to undergo differential down-regulation in response to

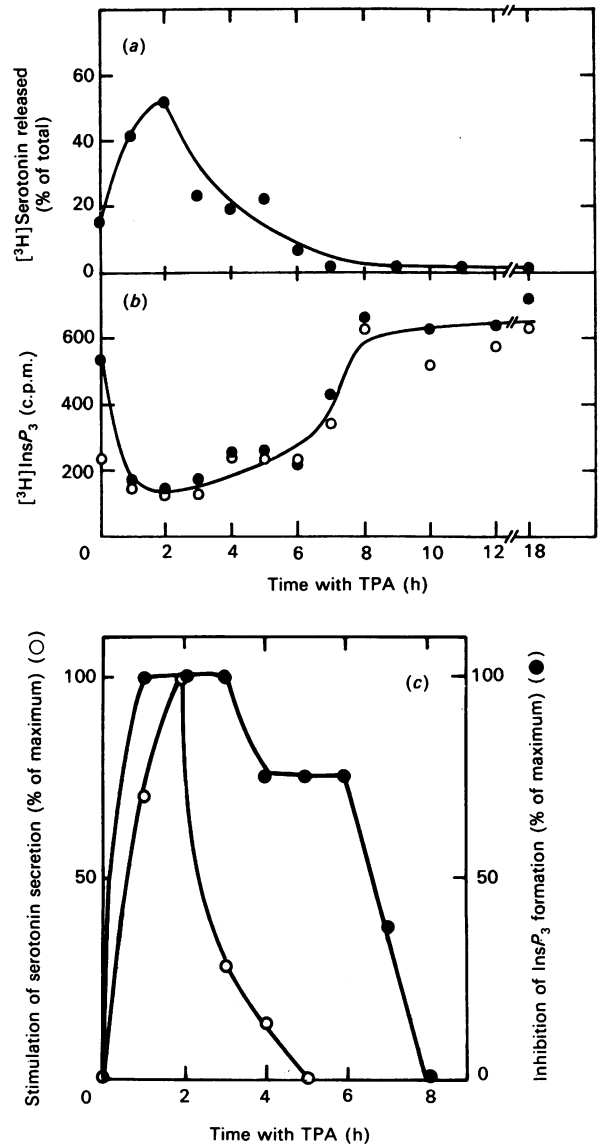

Fig. 6. Differential down-regulation of serotonin secretion and inhibition of $InsP_3$ formation by TPA

IgE-bearing cells preloaded with either [^{3}H]serotonin (*a*) or [^{3}H]-inositol (*b*) were incubated with TPA (100 ng/ml) for the indicated time periods. The cells were subsequently triggered with antigen (DNP_8-BSA, 150 ng/ml) (●) or with TPA (100 ng/ml) followed by antigen (○) and serotonin secretion and $InsP_3$ formation were determined. (*c*) Analysis of panels (*a*) and (*b*), where maximal effects (100%) refer to the values of serotonin secreted and inositol phosphates formed after 2 h of TPA treatment.

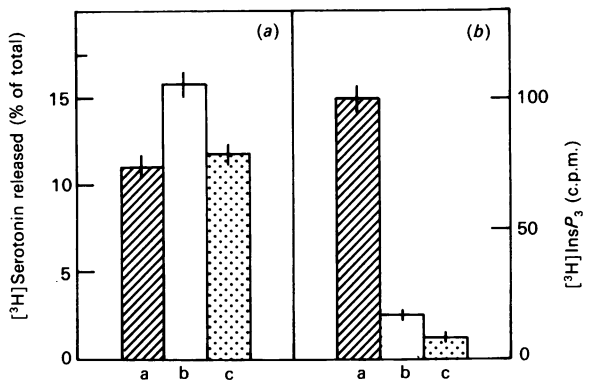

Fig. 7. Effect of sphingosine on TPA actions on antigen-induced InsP_3 formation and serotonin secretion

IgE-bearing cells were preloaded with [^{3}H]serotonin (*a*) or [^{3}H]inositol (*b*). Antigen (DNP_8-BSA, 150 ng/ml) was subsequently added and serotonin secretion and InsP_3 formation were determined as described in the Materials and methods section. a, Control cells; b, cells that were preincubated for 30 min with TPA (100 ng/ml); c, cells that were preincubated for 30 min with sphingosine (100 μM) followed by 30 min incubation with TPA (100 ng/ml).

TPA [11,23]. These observations prompted us to investigate in detail the kinetics of inhibition of IgE-dependent exocytosis following TPA treatment and compare it with the kinetics of stimulation of InsP_3 production. As shown in Fig. 6, these processes clearly differed in their kinetics. At times when both TPA/ionophore (results not shown) and antigen had already failed to evoke secretion, antigen-induced InsP_3 formation was still largely inhibited. For example, following 3 h of TPA treatment, the potentiating effect of TPA on antigen-induced secretion was lost, and secretion was completely blocked after 5 h (Figs. 6*a* and 6*c*). In contrast, after 5 h of TPA treatment antigen-induced inositol phosphate formation was still inhibited by 75% (Figs. 6*b* and 6*c*). It took 7–8 h of TPA treatment before inositol phosphate production recommenced (Figs. 6*b* and 6*c*). Hence stimulation of exocytosis and inhibition of InsP_3 formation are differentially down-regulated following TPA treatment.

Stimulation of exocytosis by TPA and inhibition of InsP_3 formation were also differentially affected by the PKC inhibitor sphingosine. Whereas preincubation with sphingosine (100 μM, 30 min) eliminated the potentiating effect of TPA on antigen-induced serotonin secretion (Fig. 7*a*, lane c compared with lanes b and a), pretreatment with sphingosine failed to abolish the inhibition exerted by TPA on InsP_3 formation (Fig. 7*b*, lane c compared with lanes b and a). In fact, none of the PKC inhibitors tested in this study exerted any effect on InsP_3 formation measured in the absence or presence of TPA (results not shown). It therefore appears that exocytosis is far more sensitive to inhibition of PKC than is InsP_3 formation. These observations are consistent with previous studies showing that staurosporine, another inhibitor of PKC, inhibits thrombin-induced secretion of serotonin from human platelets while having no effect on thrombin-induced formation of inositol phosphates [24].

DISCUSSION

We and others have shown that TPA potentiates Ca^{2+}-ionophore- and IgE-dependent secretion, whereas it inhibits both IgE-induced InsP_3 formation and the rise in the intracellular concentration of Ca^{2+} [4,6,25,26]. To establish that the dual effects of TPA are indeed both mediated by PKC, we have taken advantage of the fact that prolonged exposure of cells to TPA was previously shown to result in down-regulation of the enzyme due to its enhanced degradation [16]. We predicted that if PKC indeed exerts a bidirectional control on IgE-dependent responses, then depletion of the RBL cells of their endogenous PKC should result in inhibition of IgE-mediated exocytosis but in potentiation of IgE-stimulated InsP_3 formation.

Preincubation of RBL cells with TPA (100 ng/ml) for 30 min resulted in translocation of PKC from the cytosolic to the membrane fraction. This is indicated both by the loss of immunoreactive PKC from the cytosolic fraction and its appearance in the membrane fraction (Fig. 1), and by the loss of Ca^{2+}/PtdSer-dependent phosphorylation in cytosol derived from TPA-treated cells (Fig. 2). Under these conditions, IgE-stimulated InsP_3 formation is completely blocked (Fig. 5), whereas secretion in response to both antigen and Ca^{2+} ionophore is stimulated (Fig. 3).

Long-term treatment with TPA (18 h) resulted in complete loss of PKC from the cells (Fig. 1). In these PKC-depleted cells TPA has no inhibitory effect on antigen-induced InsP_3 formation. Moreover, a marked potentiation (1.73 ± 0.20, $n = 4$) of InsP_3 production in response to aggregation of the receptors for IgE occurs (Fig. 5). In contrast, PKC-depleted cells fail to undergo exocytosis in response to either TPA/ionophore or antigen (Fig. 3). Taken together, these results indicate that PKC exerts a bidirectional control on IgE-dependent functions. PKC is essential for exocytosis initiated both by the receptor for IgE as well as by the combination of Ca^{2+} ionophore and TPA, but it also exerts a negative feedback control on IgE-dependent PtdIns(4,5)P_2 breakdown. The fact that InsP_3 formation is potentiated in PKC-depleted cells suggests that, under physiological conditions, in the absence of TPA, generation of InsP_3 is partially inhibited by PKC that is presumably activated by DAG. Removal of PKC relieves this inhibition, resulting in the enhanced breakdown of PtdIns(4,5)P_2.

The involvement of PKC in IgE-mediated exocytosis is further illustrated in the findings that several inhibitors of PKC (K252a [19], H-7 [20] and sphingosine [21]) significantly inhibit antigen-induced secretion (Fig. 4).

The cellular targets through which PKC exerts its dual actions are at present unknown. Its inhibitory action could be mediated by phosphorylation of the IgE receptor, the phospholipase C (PLC) enzyme or the GTP-binding protein (G-protein) that presumably intervenes between the two. Both PLC [27] and the IgE receptor [28] have been suggested to serve as substrates of PKC in RBL cells, and the G-proteins G_i [29] and transducin [30] have been shown to serve as high-affinity substrates of PKC *in vitro*. Hence further studies are required to identify the endogenous substrates of PKC that mediate its multiple actions. Furthermore, as PKC consists of a family of related enzymes encoded by different genes (reviewed in [10]), different isoenzymes of PKC may mediate its opposing functions. Recent reports [11,22,23] demonstrated that the different subspecies of PKC are differentially down-regulated in response to TPA treatment. In RBL cells [11], PKC Type II is depleted faster than Type III following TPA treatment. Our findings demonstrate that the rate by which the cells lose their potency to undergo exocytosis following TPA treatment is faster than the rate at which inhibition of InsP_3 formation is relieved and replaced by potentiation of this response (Fig. 6). We also show that both processes, exocytosis and InsP_3 formation, manifest different sensitivities to the PKC inhibitor sphingosine. Whereas sphingosine abolishes the potentiating effect of TPA on exocytosis (Fig. 7*a*) and in the absence of TPA completely blocks IgE-dependent secretion (Fig. 4), it does not eliminate the inhibitory action of TPA on InsP_3 formation and by itself does not potentiate InsP_3 formation

(Fig. 7*b*). Hence the distinct kinetics of down-regulation together with the different sensitivities to PKC inhibitors may be due to different affinities of PKC for its substrates which are responsible for the stimulatory or inhibitory signals. Alternatively, these observations may point to the possibility that PKC Type II transmits stimulatory signals and plays a crucial role in activating exocytosis, whereas PKC Type III negatively regulates the Ca^{2+} signal.

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