Evaluation of the role of inositol trisphosphate in IgE-dependent exocytosis

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A close correlation exists between inhibition by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) of inositol trisphosphate (InsP₃) formation and the rise in internal Ca²⁺ concentrations in IgE-stimulated rat basophilic leukemia (RBL-2H3) cells. Inhibition of both processes is dose-dependent, with half-maximal and maximal inhibition occurring at 1.5 and 10 ng of TPA/ml respectively. At a similar range of concentrations TPA does not inhibit, but rather enhances, IgE-dependent secretion. When added to antigen-activated cells, EGTA immediately abrogates secretion and stimulates InsP₃ production. In contrast, EGTA has only a small inhibitory effect on IgE-induced secretion from TPA-activated cells. In antigen-activated cells, EGTA partially inhibits InsP₁ formation, suggesting that, unlike InsP₃, InsP₁ may in part be formed directly from phosphatidylinositol in a Ca²⁺-dependent manner. Together, these findings suggest that under physiological conditions the stimulated formation of InsP₃ is insufficient for triggering secretion, and that Ca²⁺ influx is essential. Moreover, InsP₃ formation is not obligatory for IgE-mediated exocytosis, provided that the cells are activated by TPA. Secretion from TPA-activated cells, which is independent of InsP₃ formation and the rise in internal Ca²⁺, does not require the presence of external Ca²⁺, implying that the presence of external Ca²⁺ during IgE-induced secretion is required for producing the Ca²⁺ signal and not for exocytosis *per se*.

INTRODUCTION

IgE-mediated histamine secretion from rat basophilic leukemia cells (RBL-2H3), a tumour analogue of mucosal mast cells, involves stimulation of PtdIns(4,5)P₂ breakdown [1–6], leading to the concomitant generation of diacylglycerol, which activates protein kinase C (PKC), and InsP₃, which mobilizes Ca²⁺ from intracellular stores [7,8]. We and others have previously shown that in their combined presence, Ca²⁺ ionophores and the PKC-activating phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA) trigger secretion from mast [9] and RBL cells [10,11], implying that the induction of both signals (Ca²⁺ rise and PKC activation) is sufficient to elicit secretion. Therefore receptor-induced PtdIns(4,5)P₂ hydrolysis, which generates both signals, may be a crucial step in the sequence of events leading to exocytosis. Indeed, a close correlation exists between aggregation of the IgE receptors, the extent of PtdIns(4,5)P₂ breakdown and histamine secretion [2]. Furthermore, depletion of the RBL cells of their endogenous PKC by subjecting them to long-term treatment with TPA completely abolishes IgE-dependent secretion [12]. Similarly, in the presence of the PKC inhibitors K252a, H-7 or sphingosine, IgE-dependent secretion is blocked [12]. These findings therefore indicate that PKC plays an essential role in the secretory process. However, PtdIns(4,5)P₂ breakdown, the rise in intracellular Ca²⁺ concn. ([Ca²⁺]_i) and consequently histamine secretion from RBL cells are all dependent on the presence of external Ca²⁺ [1]. Moreover, the rise in [Ca²⁺]_i results both from Ca²⁺ influx through receptor-activated Ca²⁺ channels [13] and from mobilization of Ca²⁺ from intracellular stores [14]. The relative contribution of each of these sources to the final rise in [Ca²⁺]_i has not yet been clarified. Hence it is still questionable whether InsP₃ is a physiological regulator of the IgE-dependent response.

In the present study we have attempted to evaluate the importance of InsP₃ in IgE-dependent stimulus–secretion coupling. For this purpose we have compared the effects of depletion of external Ca²⁺ by EGTA and of blocking the rise in [Ca²⁺]_i by TPA [15] on both InsP₃ formation and secretion. We show that the stimulated formation of InsP₃ neither is sufficient nor is it obligatory for IgE-dependent secretion to occur. Under physiological conditions the presence of external Ca²⁺ is absolutely required to trigger secretion. In contrast, IgE-dependent secretion from TPA-activated cells, which occurs in the absence of stimulated InsP₃ formation, is independent of external Ca²⁺, implying that external Ca²⁺ is required solely for maintaining the Ca²⁺ signal.

MATERIALS AND METHODS

Chemicals

myo-[³H]inositol (13.8 Ci/mmol) and [³H]serotonin (10.8 Ci/mmol) were purchased from Amersham. Monoclonal dinitrophenyl (DNP)-specific IgE was generously donated by Dr. Z. Eshhar (Weizmann Institute), and DNP₈-BSA was synthesized according to the procedure described in [16]. All other reagents used were from Sigma.

Cells and secretion

RBL-2H3 cells were maintained and serotonin secretion was determined as described in the preceding paper [12].

Analysis of inositol phosphates

Inositol phosphates were separated and analysed as described [12,17].

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PLC, phospholipase C; DNP, dinitrophenyl; PKC, protein kinase C; [Ca²⁺]_i, intracellular Ca²⁺ concn.; G-protein, GTP-binding protein.

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Monitoring of $[Ca^{2+}]_i$

Intracellular concentrations of Ca^{2+} were monitored using quin-2 as previously described [15].

RESULTS AND DISCUSSION

Effect of TPA on IgE-dependent inositol phosphate formation

We and others [11,12,15,18] have previously shown that pretreatment of RBL cells with TPA blocks both the rise in $[Ca^{2+}]_i$ and $PtdIns(4,5)P_2$ breakdown induced by antigen. In order to determine to what extent inhibition of both processes by TPA is correlated, the dose-response relationships were studied. As shown in Fig. 1, addition of antigen (DNP₈-BSA, 30 ng/ml) to IgE (DNP-specific)-bearing RBL cells preloaded with *myo*- $[^3H]$ inositol resulted in the enhanced formation of $InsP_1$, $InsP_2$ and $InsP_3$. TPA by itself had no effect on inositol phosphate levels. However, when added before (30 min) antigen, TPA inhibited their antigen-induced formation. Inhibition was dose-dependent, and half-maximal and maximal inhibition occurred at 1.5 and 10 ng of TPA/ml respectively (Figs. 2a–2c). This range of concentrations was similar to that found previously to inhibit the antigen-dependent rise in $[Ca^{2+}]_i$ (Fig. 2d). These observations therefore demonstrate that inhibition of the Ca^{2+} signal by TPA is closely correlated with inhibition of polyphosphoinositide breakdown.

Inhibition of IgE-stimulated $InsP_3$ formation could result either from inhibition of the $PtdIns(4,5)P_2$ -hydrolysing phospholipase C (PLC) or from activation of the $InsP_3$ -dephosphorylating phosphatase. The enzymic activity of the latter has been shown to increase following its phosphorylation by PKC [19,20]. In an attempt to distinguish between these possibilities, the effect of TPA on the rate of $InsP_1$ formation was examined both in the presence and in the absence of Li^+ , which blocks $InsP_1$ dephosphorylation. We envisaged that if the mechanism of PKC

Fig. 1. Effects of TPA on inositol phosphate formation

IgE-bearing cells preloaded with $[^3H]$ inositol were preincubated for 30 min at 37 °C with or without TPA (50 ng/ml). Incubation was continued for additional 10 min in the presence or absence of antigen (Ag: DNP₈-BSA, 30 ng/ml). The reaction was thereafter terminated and inositol phosphates were analysed as described in the Materials and methods section: (a) $InsP_1$, (b) $InsP_2$, (c) $InsP_3$. The data, expressed as percentage of basal levels, represent the mean values of five experiments in which all determinations were carried out in duplicate.

Fig. 2. Effects of TPA on antigen-induced inositol phosphate formation and $[Ca^{2+}]_i$ rise

IgE-bearing cells preloaded with $[^3H]$ inositol (a–c) or with quin-2/AM (d) were preincubated for 30 min at 37 °C with the indicated concentrations of TPA. They were subsequently triggered with antigen DNP₈-BSA (150 ng/ml) for an additional 10 min, and inositol phosphates and $[Ca^{2+}]_i$ were analysed as described in the Materials and methods section. Basal $[Ca^{2+}]_i$ was 200 nM. The results of a representative experiment, carried out in duplicate, are presented.

Fig. 3. Effect of TPA on rate of $InsP_1$ formation in the absence or presence of Li^+

IgE-bearing $[^3H]$ inositol-loaded cells were incubated for 30 min at 37 °C with (●) or without (○) TPA (100 ng/ml) in the absence (a) or presence (b) of 10 mM- $LiCl$. Antigen DNP₈-BSA (150 ng/ml) was subsequently added, and following the indicated time periods at 37 °C, the reaction was terminated and the $InsP_1$ formed was determined as described in the Materials and methods section.

Fig. 4. Effects of TPA on inositol phosphate formation in stimulated cells

IgE-bearing cells preloaded with [^3H]inositol were incubated with antigen DNP₈-BSA (150 ng/ml) for 5 min. TPA (100 ng/ml) (□) or vehicle (dimethyl sulphoxide, 0.01 %, ○) was subsequently added and incubations were continued for the indicated periods. Inositol phosphates were analysed as described in the Materials and methods section.

action involves acceleration of the rate of InsP_3 degradation, then in the presence of Li^+ , TPA treatment should result in an increase in the initial rate of InsP_1 formation. However, as shown in Fig. 3, although Li^+ did block the dephosphorylation of InsP_1 leading to its accumulation (Fig. 3b compared with Fig. 3a), TPA did not accelerate the rate of InsP_1 formation but rather completely inhibited it (Fig. 3b). These results therefore suggest that the inhibitory effect exerted by TPA on InsP_3 formation involves, in addition to the known stimulatory action on the InsP_3 -dephosphorylating phosphatase, also inhibition of PLC.

When added after antigen, TPA has previously been found to accelerate the rate of decay of the Ca^{2+} signal [15]. TPA (100 ng/ml) added to already stimulated cells immediately abrogated the formation of inositol phosphates in a consistent manner (Figs. 4a–4c). As illustrated in Fig. 4(c), when TPA was added 5 min after the antigen, InsP_3 levels immediately declined. The levels of InsP_2 and InsP_1 (Figs. 4a and 4b) remained constant, most probably due to the presence of Li^+ , which blocks their dephosphorylation. These results suggest either that $\text{PtdIns}(4,5)\text{P}_2$ breakdown generates the Ca^{2+} signal, or that it is activated sequentially to the rise in $[\text{Ca}^{2+}]_i$. The enhanced formation of InsP_3 is unlikely to be the consequence of the rise in $[\text{Ca}^{2+}]_i$, as Ca^{2+} ionophores, which raise $[\text{Ca}^{2+}]_i$, do not stimulate InsP_3 formation [1]. Thus the Ca^{2+} signal may be the consequence of $\text{PtdIns}(4,5)\text{P}_2$ breakdown, implying that, consistent with other

Fig. 5. Effects of EGTA on inositol phosphate formation and serotonin secretion in stimulated cells

IgE-bearing cells preloaded with [^3H]inositol (a, b) or [^3H]serotonin (c) were incubated with antigen DNP₈-BSA (150 ng/ml) for 5 min. EGTA (3 mM) (●) or buffer (○) was subsequently added (arrow), and at the indicated times inositol phosphates formed and serotonin released were analysed. The results of a representative experiment carried out in duplicate are presented. Similar results were obtained on three occasions.

reports [21–23], InsP_3 or its metabolites play a role not only in the mobilization of Ca^{2+} from intracellular stores but also in regulating Ca^{2+} channels. Alternatively, both processes (the rise in $[\text{Ca}^{2+}]_i$ and InsP_3 formation) may be independently activated by the same element that is subjected to a negative feedback regulation by PKC [12]. A possible candidate could be a GTP-binding protein (G-protein) that may couple the IgE receptor both to PLC and to the Ca^{2+} channel. Indeed, cholera toxin, which is known to modify G-protein actions, has been shown to enhance antigen-dependent InsP_3 formation [24] and the rise in $[\text{Ca}^{2+}]_i$ [25].

Effect of EGTA on antigen-induced inositol phosphate formation

Stimulation of $\text{PtdIns}(4,5)\text{P}_2$ breakdown has been shown to depend on the initial presence of external Ca^{2+} [1]. However, when added to already stimulated cells (5 min after the antigen), EGTA (3 mM) potentiated InsP_3 formation (Fig. 5b). Nevertheless, addition of EGTA immediately inhibited antigen-induced secretion (Fig. 5c). Under these conditions, EGTA chelates the external Ca^{2+} , thereby abolishing Ca^{2+} influx. This short incubation period with EGTA does not, however, deplete the cells of their endogenous Ca^{2+} pools. These results therefore indicate that the enhanced formation of InsP_3 is insufficient to trigger secretion, and the presence of external Ca^{2+} is required. We

Fig. 6. Effects of TPA on serotonin secretion and InsP_3 formation

Cells, preloaded with either $[^3\text{H}]\text{inositol}$ or $[^3\text{H}]\text{serotonin}$, were pretreated for 30 min at 37°C with the indicated TPA concentrations. The cells were subsequently triggered with antigen $\text{DNP}_8\text{-BSA}$ (30 ng/ml) for an additional 10 min, and InsP_3 formation (●) and release of preloaded serotonin (○) were determined as described in the Materials and methods section. The results of a representative experiment are presented. Similar results were obtained on six occasions.

conclude that, under physiological conditions, IgE-receptor-operated Ca^{2+} channels play a major role in mediating the secretory response. The stimulatory effect of EGTA on InsP_3 formation further argues against the possibility that InsP_3 generation results from a rise in $[\text{Ca}^{2+}]_i$.

In contrast with its potentiating effect on InsP_3 formation, EGTA partially inhibited InsP_1 formation (Fig. 5a). These results suggest that InsP_1 formation is at least partially dependent on Ca^{2+} , suggesting that it is formed both by dephosphorylation of InsP_3 and by direct breakdown of PtdIns . The latter process appears to depend on Ca^{2+} , and may involve a mechanism similar to that proposed from studies with platelets [26], where, following activation of a $\text{PtdIns}(4,5)\text{P}_2$ -hydrolysing PLC and Ca^{2+} influx, the resulting rise in $[\text{Ca}^{2+}]_i$ activates a PtdIns -hydrolysing PLC leading to the direct generation of InsP_1 and diacylglycerol. This process may act as an additional mechanism for activation of PKC. Moreover, this process may be essential for secretion, as its inhibition by EGTA is correlated with inhibition of secretion. Indeed, a Ca^{2+} -dependent PtdIns -hydrolysing PLC has been shown to be present in RBL cells [2].

It is at present unknown why $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis is largely dependent on initial priming with Ca^{2+} , whereas the subsequent removal of Ca^{2+} stimulates InsP_3 formation. The presence of Ca^{2+} appears to be important for the initial coupling of the receptor to PLC, but at the same time it also constrains some inhibitory signal of an as yet undefined nature. This dependency on Ca^{2+} appears to be unique for the RBL cells, as in other cellular systems receptor-stimulated InsP_3 formation can be detected in the absence of external Ca^{2+} .

Effect of EGTA on IgE-dependent secretion from TPA-activated cells

TPA, at concentrations that completely blocked InsP_3 formation and the rise in $[\text{Ca}^{2+}]_i$, had no inhibitory effect on antigen-induced secretion. In fact, TPA was synergistic with antigen under conditions whereby secretion induced by the latter was submaximal (Fig. 6). These results indicate that, in the combined presence of antigen and TPA, the stimulated formation of InsP_3 is not obligatory for IgE-dependent secretion. This

Fig. 7. Effects of EGTA on serotonin secretion from TPA-activated cells

IgE-bearing and $[^3\text{H}]\text{serotonin}$ -loaded cells were preincubated for 30 min at 37°C in the absence (a–d) or presence (e, f) of sphingosine ($100\ \mu\text{M}$). Incubation was continued for further 30 min with (c–f) or without (a, b) TPA (100 ng/ml). Antigen $\text{DNP}_8\text{-BSA}$ (150 ng/ml) was subsequently added, and incubation was continued for an additional 20 min, at the end of which serotonin release was determined as described in the Materials and methods section.

secretion is dependent on the aggregation of the IgE receptors, since TPA by itself does not induce secretion [10].

To test whether release from TPA-activated cells, which is independent of InsP_3 formation and a rise in $[\text{Ca}^{2+}]_i$, is dependent on external Ca^{2+} , cells were pretreated for 30 min with TPA (100 ng/ml) in the presence of external Ca^{2+} , and antigen was subsequently added with or without EGTA (3 mM). As shown in Fig. 7, in the absence of TPA, EGTA inhibited antigen-induced secretion by 70% (a and b). In contrast, TPA-activated cells secreted serotonin in response to antigen in an external- Ca^{2+} -independent manner in the presence of EGTA (c and d). This observation is to our knowledge the first demonstration of external- Ca^{2+} -independent secretion elicited by the IgE receptor. This finding suggests that the requirement for external Ca^{2+} during IgE-induced secretion under physiological conditions is to allow Ca^{2+} influx into the cells and not for some distal steps (e.g. fusion) in the sequence of events leading to exocytosis.

To test whether secretion from TPA-treated cells required activation of PKC, the effect of EGTA on secretion was examined in the presence of the PKC inhibitor sphingosine. In the presence of TPA, sphingosine partially inhibited (by 40%) antigen-induced secretion (Fig. 7, c and e). Addition of EGTA (3 mM) under these conditions resulted in 50% inhibition of secretion (Fig. 7, e and f). External- Ca^{2+} -independent secretion, in the combined presence of antigen and TPA, therefore seems to involve an active PKC. These observations further support the proposal put forward by us [27] and by others [11] that the IgE receptor elicits an additional, as yet unknown, signal that in conjunction with activation of PKC leads to secretion in the absence of elevated $[\text{Ca}^{2+}]_i$.

In conclusion, the results described here reveal that InsP_3 formation and presumably mobilization of internal Ca^{2+} are insufficient for activation of IgE-dependent secretion, and that the presence of external Ca^{2+} is essential. Our results also imply that InsP_3 formation is not obligatory for IgE-mediated secretion. The IgE receptor elicits multiple signals, and the amplification of the PKC signalling pathway by TPA allows dissociation of the cellular response from phosphoinositide hydrolysis and Ca^{2+} fluxes. Under these conditions IgE-mediated secretion is independent of external Ca^{2+} .

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