

Synergistic Signals in the Mechanism of Antigen-induced Exocytosis in 2H3 Cells: Evidence for an Unidentified Signal Required for Histamine Release

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Abstract. The aim of this study was to determine whether the increase in cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in response to antigen (aggregated ovalbumin) on IgE-primed 2H3 cells was sufficient to account for exocytosis. When the $[\text{Ca}^{2+}]_i$ responses to antigen and the Ca^{2+} ionophore A23187 were compared, A23187 was much less effective at releasing histamine at equivalent $[\text{Ca}^{2+}]_i$ increases, and little or no stimulated histamine release occurred with A23187 concentrations that matched the $[\text{Ca}^{2+}]_i$ response to antigen concentrations that stimulated maximal histamine release. The $[\text{Ca}^{2+}]_i$ response to antigen is not, therefore, sufficient to account for exocytosis, although extracellular Ca^{2+} is necessary to initiate both the $[\text{Ca}^{2+}]_i$ response and histamine release: the antigen must generate an additional, unidentified, signal that is required for exocytosis. To determine whether this signal

was the activation of protein kinase C, the effects of the phorbol ester 12-0-tetradecanoyl phorbol 13-acetate (TPA) on the responses to antigen were examined. TPA blocked the antigen-induced $[\text{Ca}^{2+}]_i$ response and the release of inositol phosphates but had little effect on histamine release and did not stimulate exocytosis by itself. The unidentified signal from the antigen is therefore distinct from the activation of protein kinase C and is generated independently of the $[\text{Ca}^{2+}]_i$ response or the release of inositol phosphates. Taken together with other data that imply that there is very little activation of protein kinase C by antigen when the rate of histamine release is maximal, it is concluded that the normal exocytotic response to antigen requires the synergistic action of the $[\text{Ca}^{2+}]_i$ signal together with an unidentified signal that is not mediated by protein kinase C.

STUDIES with a variety of cell systems have led to the view that a rise in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (2) is an obligatory step in the mechanism of exocytosis. The main evidence on which this conclusion was based may be summarized as follows. (a) In some cell systems the stimulation of exocytosis by agents that bind to cell surface receptors required extracellular Ca^{2+} (e.g., adrenal medullary cells [2], pancreatic beta cells [19], and, when stimulated by cross-linking antigen, mast cells [17] and 2H3 basophil leukemic cells [13, 14]). In some cells activation was also shown to be accompanied by an increase in $^{45}\text{Ca}^{2+}$ uptake (16, 19). (b) Ca^{2+} ionophores bypassed receptor-mediated pathways and stimulated exocytosis in some types of cells (e.g., mast cells [15], platelets [12], and neutrophil leukocytes [33]), presumably through the induced increase in $[\text{Ca}^{2+}]_i$. (c) The use of fluorescent $[\text{Ca}^{2+}]_i$ indicators has permitted the direct measurement of increases in $[\text{Ca}^{2+}]_i$ after stimulation of exocytosis in many cells, including mast cells and 2H3 basophil leukemic cells (7, 39). Recent studies have refined this technique and have demonstrated, for example, that exocytosis from the three distinct subcellular storage

organelles in human neutrophils exhibits differential sensitivities to $[\text{Ca}^{2+}]_i$ (25). (d) In experiments on permeabilized adrenal medullary cells (24) and partially broken sea urchin eggs (3), Ca^{2+} caused exocytosis at concentrations similar to the estimated $[\text{Ca}^{2+}]_i$ in stimulated intact cells, provided that ATP was present and an appropriate ionic environment was maintained.

Despite these findings, observations have accrued which cast substantive doubt on the simplistic view that a rise in $[\text{Ca}^{2+}]_i$ is invariably either necessary or sufficient to drive the exocytotic mechanism.

(a) In rat parotid glands it was shown as long ago as 1974 that, although adrenaline caused an increase in $^{45}\text{Ca}^{2+}$ uptake into the cells, agonist-stimulated release of amylase did not require extracellular Ca^{2+} . In addition, subcellular fractionation studies on the same system indicated that adrenaline also stimulated intracellular movement of Ca^{2+} (11). More recently, the use of quin2 in adrenal chromaffin cells revealed that nicotinic receptors mediated an increase in $[\text{Ca}^{2+}]_i$ from 0.1 μM to between 1 and 10 μM that was dependent on extracellular Ca^{2+} , whereas stimulation of exo-

cytosis in the same cells by muscarinic agonists generated a smaller $[Ca^{2+}]_i$ increase to 0.2 μM that appeared to be derived from intracellular Ca^{2+} stores (22).

(b) Studies on the stimulation of exocytosis in neutrophils by the chemotactic peptide f-Met-Leu-Phe, in which the normal ligand-induced rise in $[Ca^{2+}]_i$ was blocked, lead to the conclusion that the peptide generated an important excitatory signal, in addition to the rise in $[Ca^{2+}]_i$, and that exocytosis did not depend simply on the $[Ca^{2+}]_i$ response (5, 30). In other systems, such as bovine parathyroid cells (38), an increase in $[Ca^{2+}]_i$ was reported to inhibit, rather than stimulate, exocytosis.

(c) Examination of single mast cells using a patch clamp technique showed that degranulation in response to GTP γ S did not require Ca^{2+} (13), and other studies suggested that IgE-mediated degranulation did not require the opening of ion channels (26). In similar studies using the $[Ca^{2+}]_i$ indicator fura-2 in single mast cells, the $[Ca^{2+}]_i$ transients observed before exocytosis in most cells were independent of extracellular calcium concentration, reaching maxima of $\sim 5 \mu M$. However, degranulation occurred at variable times after the transients, and occasionally when the $[Ca^{2+}]_i$ had declined to low values ($\sim 0.3 \mu M$), close to the level in unstimulated cells (0.16 μM). Thus, although an elevated $[Ca^{2+}]_i$ enhanced degranulation, it was not an obligatory or sufficient signal (29).

(d) Some of the strongest evidence for a pathway for exocytosis not involving a $[Ca^{2+}]_i$ response was obtained from cell systems (e.g., platelets [31, 36]) in which agents that activate protein kinase C (e.g., the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate [TPA]) caused exocytosis without a detectable effect on $[Ca^{2+}]_i$. However, TPA alone is not able to stimulate exocytosis in all cell systems and there is a wide range of phenotypic variations in response to TPA. For example, TPA alone was largely without effect on bovine adrenal medullary cells (23), although it substantially reduced the Ca^{2+} concentration required for exocytosis when the cells were permeabilized. The effects of TPA on neutrophils were complex: by itself TPA stimulated secretion from specific granules (37, 10) but it inhibited secretion of the lysosomal enzyme beta-glucuronidase in response to f-Met-Leu-Phe. The effect of A23187 on secretion was enhanced by TPA if the $[Ca^{2+}]_i$ increase was limited, but was inhibited at higher $[Ca^{2+}]_i$ levels (4, 28).

Despite these phenotypic variations in responses to TPA, it exerts a strong synergistic effect on exocytosis in combination with A23187 in a variety of cells. This has led to the suggestion that ligands that stimulate exocytosis physiologically may act through the same synergistic combination of a rise in $[Ca^{2+}]_i$ and activation of protein kinase C. In more general terms, the dual signal hypothesis (8) proposed that the exocytotic response resulted from the effects of ligand-induced breakdown of phosphatidylinositol(4,5)-bisphosphate to yield inositol(1,4,5)-trisphosphate, which is able to release intracellular Ca^{2+} , and diacylglycerol, which is a putative endogenous activator of protein kinase C. For example, studies on thrombin, which induces both phosphatidylinositol (4,5)-bisphosphate breakdown and exocytosis in platelets (21), showed that the ligand caused increased phosphorylation of two cytosolic polypeptides having molecular masses of 20 and 40 kD. These were assumed to be diagnostic of an increase in $[Ca^{2+}]_i$ and of activation of protein ki-

nase C, respectively, by comparison with the effect of A23187, which caused phosphorylation of the 20-kD polypeptide, and of TPA, which stimulated phosphorylation of the 40-kD polypeptide. The synergistic effect of A23187 and TPA in platelets was therefore attributed to their ability to mimic the combination of physiological signals for exocytosis. However, the diversity of ligands and agents which cause exocytosis and the observations noted earlier that TPA or A23187 alone have the capacity to cause exocytosis in some cell systems, leave open the question of whether the dual signal hypothesis can account for the normal physiological mechanism of exocytosis in all cells or whether there are other, as yet unidentified, signals which are part of the mechanism.

We have previously shown that the $[Ca^{2+}]_i$ response in IgE-primed 2H3 cells stimulated with antigen is closely correlated with histamine release (7) and that the $[Ca^{2+}]_i$ response occurs in most, if not all, of the cells (32). Here we have examined whether the Ca^{2+} signal was sufficient to initiate the exocytosis mechanism, or whether another signal, particularly activation of protein kinase C, was necessary for the response to antigen. The starting point was to compare the $[Ca^{2+}]_i$ response and histamine release stimulated by antigen or A23187, and the effects of TPA in modulating the responses to either agent.

Materials and Methods

Cell Culture

2H3 cells were maintained in monolayer culture and harvested by treatment with trypsin as described previously (7). In the initial experiments cells were transferred to suspension culture immediately before use. It was found, however, that cells treated in this way gave poor and inconsistent histamine release in response to antigen (aggregated ovalbumin). Therefore, in subsequent experiments cells were harvested from monolayer cultures and transferred either to cluster-well plates, or, before loading with quin2, to suspension culture for incubation overnight.

Assay of Inositol Phosphates and Histamine Release

The procedures used were described in detail elsewhere (6). Cells in cluster-well plates (0.2×10^6 cells/1.6 cm² well) or in suspension (0.3×10^6 cells/ml) were sensitized to ovalbumin by incubation for 16 h at 37°C in complete growth medium containing monoclonal IgE specific for ovalbumin. For assays of inositol phosphates, inositol phospholipids were radiolabeled with myo-[2-³H]inositol (4 $\mu Ci/ml$) throughout the sensitization period. For studies using cell cultures in cluster-wells, the plates were prepared in duplicate: one plate was used for determination of histamine release, the other to monitor the accumulation of [³H]inositol phosphates. After sensitization the culture medium was removed, the cells washed twice, and the medium replaced with a phosphate-free salt solution containing 5 mM LiCl to inhibit inositol 1-phosphate phosphatase. The cultures were incubated for 10 min at 37°C, with or without TPA or other agents as indicated, before the addition of antigen or A23187. Unless otherwise indicated, the cultures were incubated for a further 30 min before assay of histamine or [³H]inositol phosphates as described previously (6).

$[Ca^{2+}]_i$ Measurements

Suspensions of cells in phosphate-free salt solution were loaded with [³H]quin2 acetoxymethyl ester as described previously (7). Aliquots of the cell suspension were loaded separately for each experiment and used immediately to minimize leakage of quin2 from the cells (27). The suspensions were stirred continuously at 37°C and fluorescence measurements were made using a fluorescence spectrophotometer (LSS; Perkin-Elmer Corp., Norwalk, CT) coupled to a computer data station (3600; Perkin-Elmer Corp.), which was used to compute and plot $[Ca^{2+}]_i$ directly. Calibration procedures were based on those described by Hesketh et al. (20) and data

were corrected for the fluorescence of extracellular quin2 determined by the addition of 0.5 mM MnCl₂ to control cell samples. The computer printouts were subjected to Savitzky/Golay smoothing procedures (as supplied in the PECLS software kit, Perkin-Elmer Corp.) to eliminate instrument noise. This did not eliminate interference caused by cell aggregates in the light path (27).

It has recently been observed that in single rat peritoneal mast cells loaded with the [Ca²⁺]_i indicator fura-2 (1) and in suspensions of mast cells containing quin2 (9) there was a large irreversible loss of fluorescence from the cells when challenged with agents known to cause degranulation. This loss was attributed to the exocytosis of fura-2 (40–60%) that had accumulated within secretory granules (1) and was measured directly using [³H]quin2 (33% of total [9]), although in the latter study degranulation itself was not measured. In the present studies with quin2 in 2H3 cells, however, there was no significant exocytosis of indicator. Thus, no increase in extracellular [³H]quin2 was detected in the supernatant from cells after antigen stimulation, compared with unstimulated cells.

Stock solutions of TPA and A23187 (Sigma Chemical Co., St. Louis, MO) were prepared in dimethylsulfoxide, which was always <0.1% vol/vol in the cell suspensions. When responses of the cells to different concentrations of reagents were compared, the concentration of dimethylsulfoxide was kept constant, and appropriate controls for solvent effects were included in all experiments. In experiments where responses to TPA, A23187, or combinations of these reagents were studied, histamine release stimulated by an optimal concentration of aggregated ovalbumin (2.5–10 μg/ml) was also determined to confirm that the cells were capable of exocytosis.

Data from individual experiments were from at least three cell cultures. Where data from several experiments have been averaged, these are presented as means ± SEM of the average values for all experiments. The isomers of inositol triphosphate, inositol (1,4,5)-, and inositol (1,3,4)-triphosphate were not separated and the positions of the phosphate groups are undesignated. Values for the release of histamine are corrected for spontaneous release in the absence of stimulant (net release) as indicated in the figures.

Results and Discussion

Is the [Ca²⁺]_i Increase in Response to Antigen Sufficient to Cause Histamine Release?

Previous studies have shown a correlation between exocytosis and the transient increase in [Ca²⁺]_i in response to the aggregated ovalbumin antigen (7). However, experiments with A23187 indicate that this [Ca²⁺]_i response is not sufficient, by itself, to account for the release of histamine stimulated by antigen: no significant histamine release was obtained in response to concentrations of A23187 (50 and 100 nM), which gave [Ca²⁺]_i increases (Fig. 1) similar to those induced by antigen concentrations (0.05 and 10 μg/ml) that stimulated half-maximal and maximal histamine release, respectively (Table I). A23187 at concentrations <200 nM caused little histamine release (<5%), but higher concentrations, which gave [Ca²⁺]_i increases to ~2 μM, stimulated histamine release to an extent comparable with the maximal response to antigen (Table I and subsequent data, e.g., Fig. 6 B). Therefore, the relationship between [Ca²⁺]_i and histamine release for A23187 or antigen differs widely (Fig. 2). The data strongly suggest that the antigen must generate a response in addition to the increase in [Ca²⁺]_i, which is necessary for exocytosis with antigen.

The data shown in Fig. 2 leave open the question of whether the [Ca²⁺]_i response is a necessary part of the normal mechanism of exocytosis caused by antigen, or merely a consequential response. Previous experiments have shown that removal of Ca²⁺ from the medium before the addition of antigen abolishes not only histamine release but also the [Ca²⁺]_i increase and the release of inositol phosphates. As no other responses to antigen could be detected under these

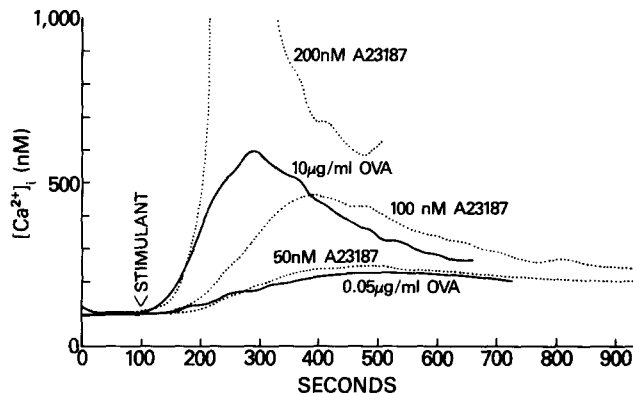


Figure 1. Changes in cytosolic calcium concentration in response to A23187. The [Ca²⁺]_i responses to antigen (aggregated ovalbumin) are compared at antigen concentrations of 0.05 and 10 μg/ml (solid lines), which elicited 50 and 100% of maximal histamine release, respectively. The [Ca²⁺]_i responses to ionophore (broken lines) are shown for 50 and 100 nM A23187, which did not induce histamine release, and for 200 nM A23187, which was the threshold concentration for secretion (see Fig. 5). The magnitude of the [Ca²⁺]_i responses to antigen and A23187 varied between cell preparations, but the experiment shown is typical in that the response to 100 nM A23187 was usually similar to the response to 10 μg/ml ovalbumin. The fluorescence measurements on quin2-loaded cells were compared and transposed to [Ca²⁺]_i as described in Materials and Methods.

conditions, the observations suggested that the primary signal from the antigen–receptor complex was not generated if a low affinity external Ca²⁺-binding site was unoccupied. The data do not, therefore, provide unambiguous evidence that removal of Ca²⁺ from the medium blocks histamine release because the [Ca²⁺]_i response is blocked. To examine this further, experiments were performed in which the [Ca²⁺]_i response was reversed by the addition of 100 μM La³⁺ when the maximum [Ca²⁺]_i response had been generated. This resulted in an immediate reversal of the [Ca²⁺]_i increase and abolished further histamine release, but did not

Table I. Calcium and Histamine Secretion of 2H3 Cells Induced by Antigen, A23187, and TPA

Agent (concentrations)	Maximal increase in [Ca ²⁺] _i	Stimulated release of histamine
	nM	%
A23187		
100 nM	1037 ± 216 (9)	1.7 ± 0.6* (4)
200 nM	1424 ± 107‡ (3)	5.7 ± 2.2 (4)
TPA		
10–100 nM	None (5)	<2.0* (4)
Aggregated ovalbumin		
0.05 μg/ml	123 ± 7 (3)	17.8 ± 1.8 (3)
10 μg/ml	873 ± 243 (8)	44.6 ± 1.7 (3)

Values are means ± SEM. The number of individual experiments are indicated in the parenthesis. [Ca²⁺]_i was measured in suspensions of quin2-loaded cells and release of histamine was measured in monolayer cultures in cluster plates.

* Indicates no significant difference from unstimulated release (4–10%).

‡ At concentrations >200 nM the fluorescence of A23187 interfered with the measurement of Ca²⁺/quin2; when corrections were made for this fluorescence, it was clear that the quin2 was close to saturation with Ca²⁺ and [Ca²⁺]_i was in excess of 2 μM.

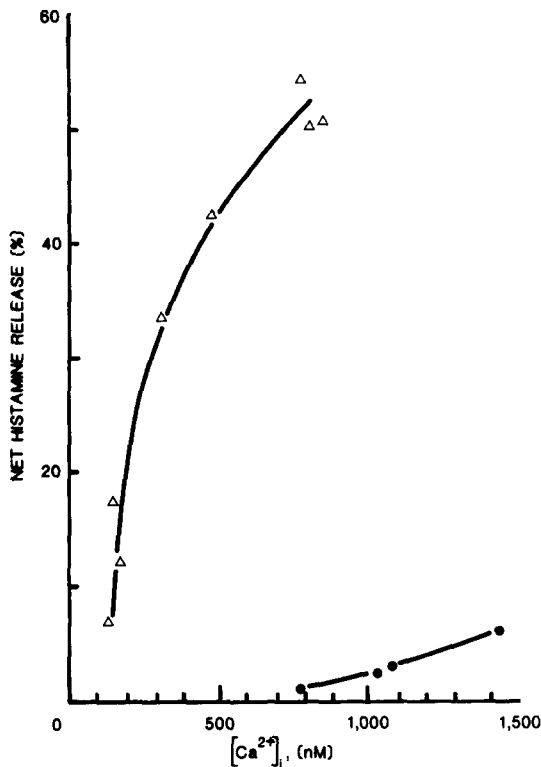


Figure 2. Relationship between $[Ca^{2+}]_i$ and histamine release in response to antigen and A23187. The maximal increases in $[Ca^{2+}]_i$ in response to concentrations of aggregated ovalbumin up to 10 $\mu\text{g/ml}$ (triangles) and of A23187 up to 200 nM (circles) are shown as a function of the stimulated release of histamine. Fluorescence from A23187 at concentrations >200 nM interfered with quin2 fluorescence measurements (see also footnote to Table I). Values are the means of three to nine experiments.

block the continued release of inositol phosphates (Fig. 3). This demonstrates that under these conditions the primary signal from the activated antigen receptor is maintained, but no exocytosis occurs when the $[Ca^{2+}]_i$ increase is blocked by La^{3+} . This is consistent with a requirement for the $[Ca^{2+}]_i$ response for antigen-mediated exocytosis, although an alternative possibility is that La^{3+} blocks the binding of Ca^{2+} to an extracellular site that is required at a stage in the exocytotic pathway subsequent to both the primary signal from the antigen and the $[Ca^{2+}]_i$ response. Other studies, in which external Ca^{2+} was removed by addition of EGTA at various times after the addition of antigen, indicated that breakdown of the inositol phospholipids, although initially dependent on the presence of extracellular Ca^{2+} , became progressively less dependent and finally independent of the presence of Ca^{2+} , while histamine release remained dependent on extracellular Ca^{2+} throughout (Maeyama, K., and M. Beaven, unpublished data). Taken together, the data imply that the $[Ca^{2+}]_i$ increase is normally required for antigen-mediated exocytosis.

Is Protein Kinase C Activation by Antigen the Additional Response Required for Exocytosis?

We note at the outset that in the following analysis it is assumed that appropriate concentrations of TPA and oleoyl acyl glycerol fully activate protein kinase C, as clearly evi-

denced by studies of the isolated enzyme (36). However, the equivalence of the functional effects in intact cells of protein kinase C when activated by these exogenous agents or via endogenously generated diacylglycerol has not been demonstrated experimentally, although it is widely assumed. The receptor-mediated release of diacylglycerol by phospholipase C and the metabolism of the lipid will regulate any activation of protein kinase C temporally. It is also likely that the access of the enzyme to its potential substrates will depend on the location of the diacylglycerol. It should therefore be clearly emphasized that the conclusions drawn concerning any activation of protein kinase C by antigen are based on a comparison of cellular responses to the antigen with the corresponding responses to exogenous activators of protein kinase C.

The above experiments are consistent with the view that exocytosis in response to antigen requires an increase in $[Ca^{2+}]_i$ together with some other synergistic signal. By way of analogy with data from other cell systems, receptor-mediated activation of protein kinase C is a likely candidate for the additional response required for exocytosis, and the effects of TPA on histamine release and the $[Ca^{2+}]_i$ response were therefore examined. TPA alone had no effect on $[Ca^{2+}]_i$ and did not cause any histamine release (Table I).

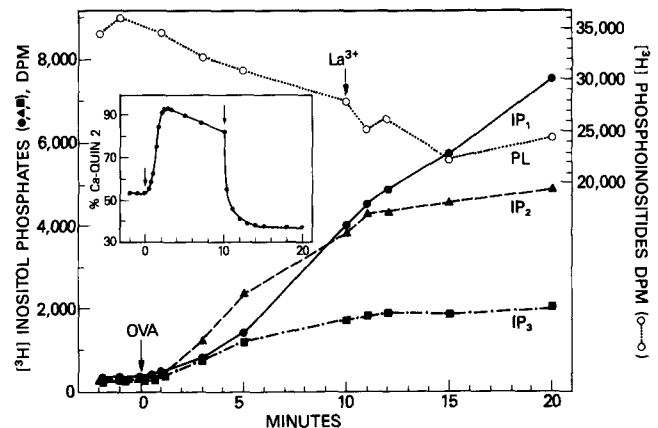


Figure 3. Effect of La^{3+} on the $[Ca^{2+}]_i$ responses and the hydrolysis of phosphoinositides in antigen-stimulated cells. The inositol phospholipids were labeled by overnight incubation of cells with myo- $[^3H]$ inositol before loading of the cells with quin2 as described in Materials and Methods. During the measurement of quin2 fluorescence, samples of the cell suspension were removed for assay of the inositol phosphates (IP_1 , IP_2 , and IP_3). The inositol phosphates were separated on columns of Dowex-1 ($\times 8$, formate form; Sigma Chemical Co.). The IP_3 fraction contained some of the higher inositol polyphosphates in addition to the inositol triphosphate isomers (our unpublished data). Aggregated ovalbumin (OVA) was added at time zero and La^{2+} (100 μM) 10 min later as indicated. This concentration of La^{3+} blocks the $[Ca^{2+}]_i$ response and histamine secretion by $>90\%$ (7). Note that on addition of La^{3+} , quin2 fluorescence declined to below the level in unstimulated cells (inset). This was due to quenching of extracellular quin2 by La^{3+} , as evidenced by control experiments in which the addition of La^{3+} to suspensions of unstimulated cells immediately quenched the fluorescence signal derived from any extracellular quin2 (7, 20), but thereafter had no significant effect on the signal, indicating that the rate of permeation of the plasma membrane by La^{3+} was very slow. No evidence could be obtained for any significant quenching of intracellular quin2 fluorescence by La^{3+} in either stimulated or unstimulated cells.

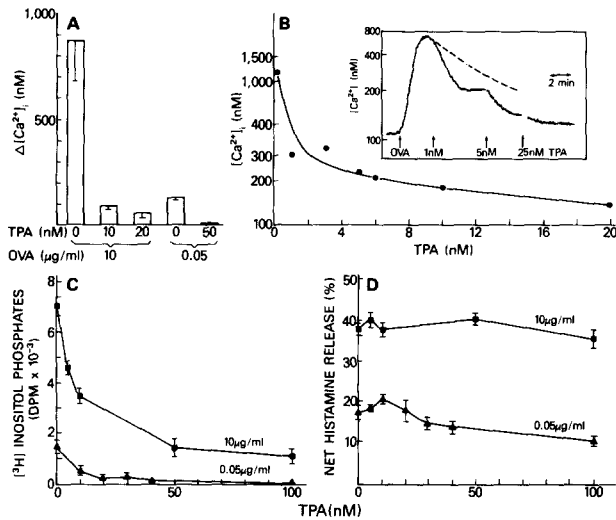


Figure 4. Effects of TPA on the $[Ca^{2+}]_i$ response, the release of $[^3\text{H}]$ inositol phosphates, and secretion of histamine in antigen-stimulated 2H3 cells. (A) The maximal increase in $[Ca^{2+}]_i$ is compared in cells exposed to 0, 10, 20, or 50 nM TPA for 10 min before addition of aggregated ovalbumin at 10 or 0.05 $\mu\text{g/ml}$. (B) The increase in $[Ca^{2+}]_i$ in response to 10 $\mu\text{g/ml}$ of aggregated ovalbumin with the addition of TPA at concentrations from 1 to 20 nM after the maximal increase in $[Ca^{2+}]_i$ was attained. (Inset) A typical $[Ca^{2+}]_i$ response to 10 $\mu\text{g/ml}$ of aggregated ovalbumin with sequential additions of TPA as indicated. The broken line indicates the normal decline in $[Ca^{2+}]_i$ in cells without the addition of TPA (see text). (C) Cells were treated with TPA for 10 min before the addition of 10 or 0.05 $\mu\text{g/ml}$ of aggregated ovalbumin, and the accumulated $[^3\text{H}]$ inositol phosphates were assayed 30 min after addition of the antigen. Values are corrected for the unstimulated release of $[^3\text{H}]$ inositol phosphates (520 to 725 dpm) and are the means \pm SEM from three to eight experiments. (D) Cells were treated with TPA and aggregated ovalbumin as in C and the release of histamine was assayed 30 min later. Values are corrected for unstimulated histamine release (4.0–9.8%) and are the means \pm SEM from three to eight experiments.

However, the effects of TPA on the $[Ca^{2+}]_i$ and histamine responses to antigen differed markedly (Fig. 4).

The $[Ca^{2+}]_i$ response to ovalbumin was strongly antagonized by TPA. The effects of treating the cells with TPA for 10 min before the addition of ovalbumin are shown in Fig.

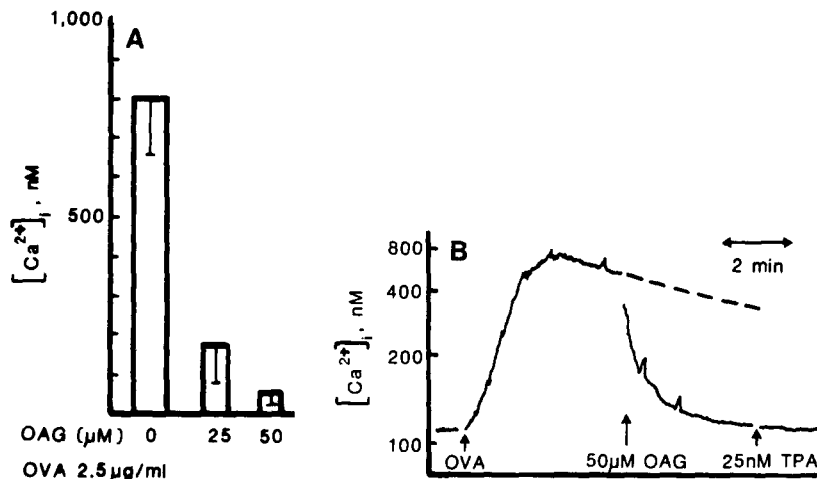


Figure 5. Effect of oleoyl acyl glycerol on the $[Ca^{2+}]_i$ response in antigen-stimulated cells. (A) The maximal increase in $[Ca^{2+}]_i$ is compared in cells exposed to 0, 10, 25, or 50 μM oleoyl acyl glycerol (OAG) for 3 min before the addition of 2.5 $\mu\text{g/ml}$ of aggregated ovalbumin. (B) A typical $[Ca^{2+}]_i$ response to 2.5 $\mu\text{g/ml}$ of aggregated ovalbumin with sequential additions of 50 μM oleoyl acyl glycerol and 20 nM TPA. The dashed line indicates the normal decline in $[Ca^{2+}]_i$ in cells without the addition of TPA.

4 A. 50 nM TPA abolished the $[Ca^{2+}]_i$ response to 0.05 $\mu\text{g/ml}$ ovalbumin (see Fig. 5 A for fluorescence data), and reduced the increase in $[Ca^{2+}]_i$ in response to a saturating dose of ovalbumin (10 $\mu\text{g/ml}$) by $>90\%$. The addition of TPA after the maximal $[Ca^{2+}]_i$ response to 10 $\mu\text{g/ml}$ ovalbumin had been generated caused a rapid decline in $[Ca^{2+}]_i$. The slow spontaneous decline in $[Ca^{2+}]_i$ after the maximal response was greatly accelerated by concentrations of TPA of 1 nM (Fig. 4 B, inset), indicating that any functional activation of protein kinase C by antigen at the time of the maximal $[Ca^{2+}]_i$ response cannot be large compared with the activation caused by 1 nM TPA. The corresponding dose-response curve for the inhibition of the maximal $[Ca^{2+}]_i$ response by subsequent addition of TPA is shown in Fig. 4 B. Experiments were also performed to test whether oleoyl acyl glycerol had the same effect as TPA on the $[Ca^{2+}]_i$ response to antigen, since oleoyl acyl glycerol is a homologue of the putative endogenous activator of protein kinase C. The data in Fig. 5, A and B, show the $[Ca^{2+}]_i$ response to antigen after pretreatment of the cells with oleoyl acyl glycerol, or following addition of the agent after the maximal response to $[Ca^{2+}]_i$ was generated. Similar inhibition of the $[Ca^{2+}]_i$ response was obtained with either agent, and no additional effect of TPA was observed after the addition of 50 μM oleoyl acyl glycerol (Fig. 5 B). The data are therefore consistent with inhibition of the $[Ca^{2+}]_i$ response to antigen by activation of protein kinase C by either agent.

TPA inhibited the release of inositol phosphates in antigen-stimulated cells, as shown in Fig. 4 C. The inhibition of release by TPA closely reflects the effect of TPA on the $[Ca^{2+}]_i$ response. It has been shown above (see Fig. 3) that the Ca^{2+} response can be blocked by La^{3+} without suppression of polyphosphoinositide breakdown. Furthermore, there is little stimulation of inositol phosphate release by A23187 at concentrations up to 200 nM (6). If the release of inositol phosphates and the $[Ca^{2+}]_i$ responses are causally linked, it is the inositol phosphates which cause the $[Ca^{2+}]_i$ increase. This would be of interest in that there is no evidence for substantial antigen-stimulated release of Ca^{2+} from intracellular stores in 2H3 cells. The data would therefore imply that poly-phosphoinositide breakdown induced by antigen is involved, directly or otherwise, in opening the Ca^{2+} signal influx pathway in the plasma membrane of these cells. It may be

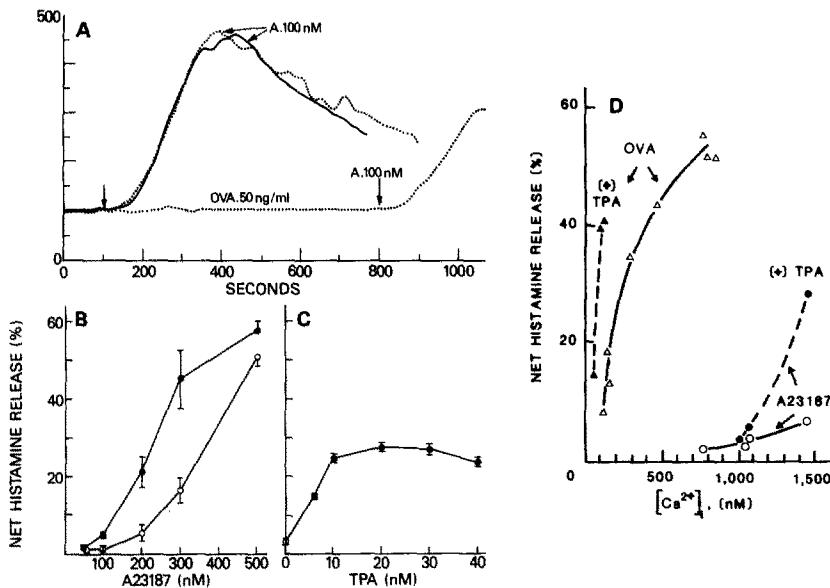


Figure 6. Effect of TPA on the $[Ca^{2+}]_i$ response and histamine release in cells stimulated with A23187. (A) Cells were treated with 50 nM TPA for 10 min before the addition of 100 nM A23187 (solid and broken lines show the $[Ca^{2+}]_i$ responses in the absence and presence of TPA). (In some experiments the decline in $[Ca^{2+}]_i$ after the maximal response to A23187 was retarded by pretreatment with TPA). Also shown is the $[Ca^{2+}]_i$ response to 100 nM A23187 added after 50 μ g/ml of aggregated ovalbumin to cells pretreated with TPA as above. (B) The stimulated release of histamine 30 min after the addition of various A23187 concentrations to cells with (solid circles) or without (open circles) pretreatment with 10 nM TPA for 10 min (means \pm SEM; $n = 3$). (C) The stimulated release of histamine 30 min after the addition of 200 nM A23187 to cells pretreated for 10 min with various concentrations of TPA. Data are from triplicate determinations in a single experiment. (D) The effect of pretreatment with 20

nM TPA for 10 min on stimulated histamine release as a function of the $[Ca^{2+}]_i$ response in cells treated with various concentrations of A23187 (solid circles) or aggregated ovalbumin (solid triangles). The responses from cells in the absence of TPA (open symbols) are replotted from Fig. 2.

noted that if TPA itself blocked the primary signal from the antigen receptor, this would account for the suppression of both the $[Ca^{2+}]_i$ response and the release of inositol phosphates (Fig. 4, A and C). However, the evidence summarized below eliminates this trivial explanation of the effects of TPA.

In contrast to the large effect of TPA on the $[Ca^{2+}]_i$ response to antigen, TPA had no significant effect on maximal histamine release stimulated by antigen (Fig. 4 D). At antigen concentrations that caused half-maximal histamine release, low concentrations of TPA (<10 nM) caused a slight potentiation (<15%) of the response, whereas inhibition of the response by up to 30% was observed only at the highest TPA concentration used (100 nM). This inhibition was variable (negligible in some experiments); we note that it may represent a nonspecific action of TPA rather than a response to activated protein kinase C. These data may be contrasted with those of Sagi-Eisenberg et al. (34, 35), who found substantial inhibition of histamine release from 2H3 cells by 10–50 nM TPA. This difference may reflect a phenotypic drift in the property of 2H3 cells in culture noted by several workers. Alternatively, their data may reflect the synergistic effect of TPA and low concentrations of solvent (dimethylsulfoxide or ethanol), which have been found to cause potent inhibition of antigen-stimulated histamine release in 2H3 cells. In the present studies the effects of TPA (particularly at 50–100 nM) were compared at several solvent dilutions to exclude the possibility that the responses were due to the synergistic action of solvent with TPA. Under the conditions used in our experiments (i.e., with a final concentration of <0.1% dimethylsulfoxide) 50 nM TPA did not inhibit antigen-induced histamine release.

The possibility that protein kinase C activation by TPA prevents either the activation of the primary signal from the receptor or antigen binding to the receptor is eliminated by the observation that TPA with antigen stimulates histamine

release under conditions where there was little or no $[Ca^{2+}]_i$ response or release of inositol phosphates. Binding studies to estimate numbers of unoccupied receptors using ^{125}I -monomeric IgE in the presence of subsaturating concentrations of covalent oligomers of IgE, which results in direct aggregation of IgE receptors (27), confirmed that treatment with 50 nM TPA before addition of the IgE oligomers did not significantly affect IgE oligomer binding (<2%) to the receptors.

Taken together, the data showed that while any continuous activation of protein kinase C via the antigen must be very small compared with full activation obtained with TPA, histamine release in response to antigen is, at most, only slightly modulated by maximal activation of protein kinase C. The question therefore arises of whether any small activation of protein kinase C that may occur through antigen stimulation is sufficient to synergize with the $[Ca^{2+}]_i$ response to account for the release of histamine. To examine this further, the effects of TPA on histamine release and on the $[Ca^{2+}]_i$ increase in response to A23187 were investigated. The $[Ca^{2+}]_i$ response to A23187 was unaffected by pretreatment of the cells with any concentration of TPA up to 100 nM (Fig. 6 A) or by the addition of TPA (50 nM) when the $[Ca^{2+}]_i$ response was maximal. The inability of TPA to affect the $[Ca^{2+}]_i$ response to A23187 suggests that the plasma membrane proteins that normally regulate $[Ca^{2+}]_i$ in the absence of antigen are not significantly modulated by activated protein kinase C. This implies that protein kinase C blocks the $[Ca^{2+}]_i$ response to antigen by inhibiting, directly or otherwise, the Ca^{2+} signal influx pathway that is normally opened by the antigen receptor.

The effect of TPA on histamine release by A23187 is shown in Fig. 6 B. In the presence of 10 nM TPA, concentrations of A23187 up to 100 nM caused only a small increase in histamine release, but at 200 nM A23187 there was a marked synergistic effect of TPA that increased histamine release by five-

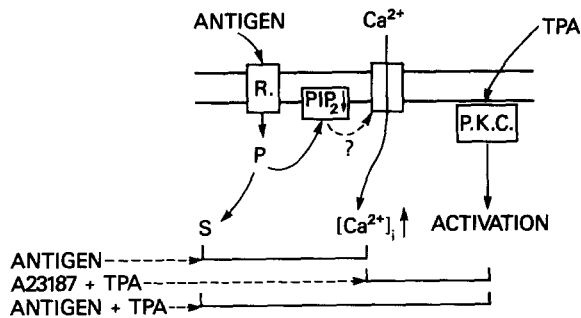


Figure 7. Synergistic signals that stimulate exocytosis in 2H3 cells. R, receptor; P, primary signal; S, unidentified signal; PIP_2 , phosphatidylinositol (4,5)-biphosphate; P.K.C., protein kinase C.

to sevenfold. The dose-response curve for TPA with 200 nM A23187 is shown in Fig. 6 C. The data show that the increase in histamine release is half-maximal at >5 nM and that the effect of low concentrations of TPA (<1 nM) would be very small. Effective synergism between the ionophore and TPA therefore requires much greater activation of protein kinase C than can be contributed by antigen (equivalent to <1 nM TPA). This is further supported by the observation that the stimulation of histamine release is much smaller in response to A23187 with high TPA concentrations than to antigen concentrations that cause the same increase in $[\text{Ca}^{2+}]_i$ (Fig. 6 D). The conclusion drawn from these data is that any activation of protein kinase C that may occur in response to antigen is much too small to synergize with the $[\text{Ca}^{2+}]_i$ response to account for the release of histamine from the cells when the $[\text{Ca}^{2+}]_i$ response is maximal.

Conclusions

The simplest model that accommodates the present data for exocytosis in 2H3 cells is summarized in Fig. 7.

(a) The combination of antigen with receptor in Ca^{2+} -containing medium generates a primary signal which leads to activation of polyphosphoinositide breakdown to release inositol phosphates. The release of inositol phosphates is normally closely correlated with the $[\text{Ca}^{2+}]_i$ increase, although the $[\text{Ca}^{2+}]_i$ increase can be inhibited by La^{3+} or removal of external Ca^{2+} with little or no effect on polyphosphoinositide breakdown. Whether the release of inositol phosphates is responsible for the $[\text{Ca}^{2+}]_i$ increase is unresolved (Fig. 6), since there is no evidence for release of Ca^{2+} from an intracellular pool in response to antigen in 2H3 cells, and there are no data at present to indicate whether the inositol phosphates can activate the Ca^{2+} signal influx pathway in the plasma membrane of 2H3 cells.

(b) Antigen together with TPA can stimulate significant histamine release under conditions where there is no detectable increase in $[\text{Ca}^{2+}]_i$ (i.e., an increase of <10 nM above the resting level in unstimulated cells of 105 nM). This suggests that there must be an unidentified signal in response to antigen, that is able to synergize with TPA-activated protein kinase C to cause exocytosis, since TPA alone does not cause histamine release. The signal is generated independently of both the $[\text{Ca}^{2+}]_i$ increase and the release of inositol phosphates. This implies that neither the $[\text{Ca}^{2+}]_i$ signal nor polyphosphoinositide breakdown constitutes the primary signal from the antigen receptor.

(c) The simplest hypothesis to account for exocytosis in response to antigen alone is that the signal is able to synergize with the $[\text{Ca}^{2+}]_i$ increase generated by Ca^{2+} entry through the influx pathway to provide the combination of signals required for the normal response. The model in Fig. 7 implies that the signal can synergize with either the $[\text{Ca}^{2+}]_i$ signal or with activated protein kinase C to cause exocytosis: the mechanistic function of the signal is therefore different from either of those responses. There is no evidence at present that the antigen receptor activates protein kinase C as an initiating response in the release of histamine from 2H3 cells.

(d) Exocytosis also occurs in response to TPA with A23187, indicating that activation of protein kinase C together with a large increase in $[\text{Ca}^{2+}]_i$ cause a synergistic release of histamine. This is consistent with previous data for a variety of cells stimulated by this combination of agents. TPA alone does not stimulate exocytosis, nor does A23187, except at concentrations that cause a very large increase in $[\text{Ca}^{2+}]_i$. Whether this occurs through activation of protein kinase C at very high $[\text{Ca}^{2+}]_i$, as noted in the protein phosphorylation studies on platelets described earlier, or by some other mechanism, is unresolved.

(e) The primary signal and the unidentified signal remain to be defined biochemically. If the primary signal is, for example, a GTP-activated protein or a protein kinase, it must have the capacity both to activate polyphosphoinositide breakdown and to generate the unidentified signal independently of that pathway. The 2H3 cells system provides access to characterization of the unidentified signal, which may be a cryptic component of the mechanism of exocytosis in other types of cell. Evidence has recently been obtained for the involvement of GTP-activated protein(s) at more than one stage in the exocytotic pathway in mast cells (13, 18).

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