Calcium Influx in a Rat Mast Cell (RBL-2H3) Line

USE OF MULTIVALENT METAL IONS TO DEFINE ITS CHARACTERISTICS AND ROLE IN EXOCYTOSIS*

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(Received for publication, April 25, 1991)

An increase in concentration of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(c\)) is associated with an accelerated influx of \(^{46}\)Ca\(^{2+}\) when cultured RBL-2H3 cells are stimulated with either antigen or analogs of adenosine although these agents act via different receptors and coupling proteins (Ali, H., Cunha-Melo, J. R., Saul, W. F., and Beaven, M. A. (1990) J. Biol. Chem. 265, 745-753). The same mechanism probably operates for basal Ca\(^{2+}\) influx in unstimulated cells and for the accelerated influx in stimulated cells. This influx had the following characteristics. 1) It was decreased when cells were depolarized with high external K\(^+\); 2) it was blocked by other cations (La\(^{3+}\) > Zn\(^{2+}\) > Cd\(^{2+}\) > Mn\(^{2+}\) = Co\(^{2+}\) > Ba\(^{2+}\) > Ni\(^{2+}\) > Sr\(^{2+}\)) either by competing with Ca\(^{2+}\) at external sites (e.g. La\(^{3+}\) or Zn\(^{2+}\)) or by co-traffic into the cell (e.g. Mn\(^{2+}\) or Sr\(^{2+}\)); and 3) the inhibition of influx by K\(^+\) and the metal ions had exactly the same characteristics whether cells were stimulated or unstimulated even though influx rates were different. The dependence of various cellular responses on influx of Ca\(^{2+}\) was demonstrated as follows. The stimulated influx of Ca\(^{2+}\), rise in [Ca\(^{2+}\)]\(c\), and secretion, could be blocked in a concentration-dependent manner by increasing the concentration of La\(^{3+}\), but concentrations of La\(^{3+}\) (>20 \(\mu\)M) that suppressed influx to below basal rates of influx markedly suppressed the hydrolysis of inositol phospholipids (levels of inositol 1,4,5-trisphosphate were unaffected). Some metal ions, e.g. Mn\(^{2+}\) and Sr\(^{2+}\), however, supported the stimulated hydrolysis of inositol phospholipid and some secretion in the absence of Ca\(^{2+}\). Thus a basal rate of influx of Ca\(^{2+}\) was required for the full activation of inositol phospholipid hydrolysis, but in addition an accelerated influx was necessary for exocytosis.

The exocytotic discharge of granules from mast cells, basophils, and cultured, cognate cell lines, in response to either immunological or nonimmunological stimuli, is associated with the mobilization of Ca\(^{2+}\) from extracellular (1-5) and intracellular (6-9) sources and an increase in concentration of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(c\)) of 4 (4, 10-13). These processes have been studied in detail in the RBL-2H3 cell, which exhibits frequently, but not invariably, oscillations in [Ca\(^{2+}\)]\(c\) (8). In this cell, the increase in [Ca\(^{2+}\)]\(c\) probably is due in part to release of intracellular Ca\(^{2+}\) by inositol 1,4,5-trisphosphate (14, 15), but the influx of Ca\(^{2+}\) is clearly essential for the sustained increase in [Ca\(^{2+}\)]\(c\) and exocytosis (16-18). Influx is independent of voltage-activated channels (19), and is enhanced in cholera toxin-treated cells (18, 20). Entry of other metal ions may also occur (see below). Nevertheless, there is no clear description to date of the mechanism of influx of Ca\(^{2+}\) or of the individual contributions of intracellular and extracellular Ca\(^{2+}\) pools to the stimulatory process in these cells.

The physiological trigger for the stimulation of these cells is the aggregation of receptors for IgE in the plasma membrane by multivalent binding of antigen to receptor-bound IgE (21). This aggregation leads to stimulation of phospholipase C, probably via a toxin-resistant G\(_i\)-like G-protein (18), to cause rapid and sustained hydrolysis of the inositol phospholipids (16). There is also extensive hydrolysis of other phospholipids (22), primarily phosphatidylinositol, through the action of phospholipase D (23), and the activation of phospholipase A\(_2\) to cause release of arachidonic acid (24). In RBL-2H3 cells, the same events are induced transiently by adenosine analogs through receptors for adenosine by a mechanism that is inhibited by both cholera toxin and pertussis toxin (18).

It is uncertain whether the accelerated influx of Ca\(^{2+}\) in stimulated cells is a consequence of these early events, i.e. mediated by intracellular messengers, or of the direct interaction of the aggregated IgE receptors with the cromolyn-binding protein (25-27) that is present in the plasma membrane of RBL-2H3 cells (28, 29). In support of this latter mechanism, it has been reported that Ca\(^{2+}\)-conducting channels can be reconstituted in lipid bilayers with purified IgE receptor, IgE, and the cromolyn-binding protein and then activated with the appropriate antigen (27, 30). A prediction from this model is that such channels would form only in the presence of antigen and be activated independently of the generation of intracellular messenger molecules.

In contrast, patch-clamp studies with mast cells indicate a

* The abbreviations used are: [Ca\(^{2+}\)]\(c\), the concentration of free cytosolic calcium; [Ca\(^{2+}\)], the concentration of external Ca\(^{2+}\); [K\(^+\)], the concentration of external K\(^+\); NECA, 5'-N-ethylcarboxamidoadenosine; IgE, immunoglobulin E; DNP, dinitrophenol; DNP-BSA, antigen with 24 molecules of dinitrophenol covalently linked to one molecule of bovine serum albumin; G-protein, any identified or unidentified protein that is analogous to those involved in the stimulation (G\(_i\)) or suppression (G\(_j\)) of thriophosphate; PIPES, 1,4-piperazine diethanesulfonic acid; EGTA, [ethylenebis(oxyethylene)nitrilo]tetraacetic acid; DTPA, diethyleneetriaminepentaacetic acid; GTP\(\gamma\)S, guanosine 5'-[3-O-thio]triphosphate.
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delay in the activation of Ca\^{2+} conductances, and this delay has been attributed to recruitment of intracellular molecules in activation of a non-voltage-gated influx mechanism (31, 32). In general, the process for influx of Ca\(^{2+}\) appears to be nonselective in that intact antigen-stimulated cells exhibit increased permeability to Na\(^{+}\) (33) and Sr\(^{2+}\) (34), as well as to Ca\(^{2+}\), and various multivalent cations either inhibit or support exocytosis in mast cells (1, 35, 36), basophils (5, 37), and RBL-2H3 cells (4). Of particular note, La\(^{3+}\) blocks exocytosis (4, 36) and the increase in [Ca\(^{2+}\)](4, 37). Moreover, Sr\(^{2+}\) can replace Ca\(^{2+}\) and by itself induces some exocytosis (1), but La\(^{3+}\) blocks antigen-induced exocytosis whether supported by Ca\(^{2+}\) or Sr\(^{2+}\) (35).

The uncertainty as to the necessity for Ca\(^{2+}\)-influx in initiating early stimulatory events in RBL-2H3 cells arises from the following seemingly contradictory observations. One set of observations suggest that the increase in [Ca\(^{2+}\)], hydrolysis of inositol phospholipids, and exocytosis are highly dependent on Ca\(^{2+}\) influx as blockade of Ca\(^{2+}\) entry by addition of EGTA or inhibitors of inositol lipases of La\(^{3+}\) suppresses all three responses by more than 95% (4, 17, 38). Another set of observations indicates release of intracellular Ca\(^{2+}\) in individual RBL-2H3 cells (8) and a substantial increase in [Ca\(^{2+}\)] in cell suspensions (19) when Ca\(^{2+}\) entry is blocked with high K\(^{+}\); the cells do not degranulate, but it is unknown how other other stimulatory events are affected. The studies point to differences in requirement for external Ca\(^{2+}\) in that low concentrations of La\(^{3+}\) preferentially suppress the increase in [Ca\(^{2+}\)], and exocytosis without inhibiting the phosphoinositide response in RBL-2H3 cells (4, 38).

In this paper we show that the uptake mechanism, in both stimulated and unstimulated RBL-2H3 cells, is nonselective as demonstrated by the inhibition of uptake of 45Ca\(^{2+}\) by various metal cations into quin2-loaded and nonloaded cells. The extent and time course of quenching of the fluorescence of the quin2/Ca\(^{2+}\) complex (4) by some of the metal cations also indicated that some cations block entry of Ca\(^{2+}\) at the cell surface, whereas others compete with Ca\(^{2+}\) for entry into the cell. Finally, the properties of the various metal ions and [K\(^{+}\)], reveal distinctive features of the influx mechanism and the exact requirements of the stimulatory responses for Ca\(^{2+}\) entry.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from the following sources. Nickel(II) chloride and cesium(II) chloride were from Aldrich; the chloride salts of other metals were of the highest available grade; \^4CaCl\(_2\), [1,2,4-C]hydroxytryptamine binoxalate, and [14]CeCl\(_3\) were purchased from Du Pont-New England Nuclear; myo[2-H]inositol was purchased from both Amersham Corp. and Du Pont/New England Nuclear. Other reagents were from the sources listed in our previous publications (14, 18, 39).

Preparation of Cells and Buffers—The preparation of cell cultures and the conditions for stimulation of the cells were exactly as described elsewhere (2). The cultures were incubated for an additional 60 min. The cultures were then washed, 0.2 ml of the indicated medium was added, and the cultures were incubated for 10 min, after which the buffer was replaced by buffer that contained 45Ca\(^{2+}\) (1 pCi/0.2 ml), the indicated metal ion, and 20 ng/ml DNP-specific IgE. The growth medium was then replaced with wash buffer, and the cultures were grossly interfered with the assay of the inositol trisphosphate. As described elsewhere, the content of heparin, chondroitin E, and other proteoglycans in extracts of RBL-2H3 cells, basophils, and mast cells grossly interfered with the assay of the inositol trisphosphate. Although the filtration caused some loss of the trisphosphate (up to 30%), the problem was circumvented by subjecting standards and samples to the same procedure through the assay. Measurement of Uptake of 45Ca\(^{2+}\)—In these experiments the uptake was determined in quin2-loaded cells to extend the period over which uptake was linear with time (18, 40) as well as in cultures that did not contain quin2. Cultures were prepared in 24-well cluster plates exactly as described above and incubated overnight at 57 °C with 0.5 mg/ml DNP-specific IgE. The growth medium was then replaced with warm (37 °C) growth medium that contained 30 mM quin2 acetoxymethylester or vehicle (dimethyl sulfoxide, 0.1%). The cultures were incubated for an additional 60 min. The cultures were then washed, 0.2 ml of the indicated medium was added, and the cultures were incubated for 10 min, after which the buffer was replaced by buffer that contained 45Ca\(^{2+}\) (1 pCi/0.2 ml), the indicated metal ion, and 20 ng/ml DNP-BSA. All solutions contained 1.0 mM CaCl\(_2\). The cultures were incubated for 5 min at 37 °C. The reactions were terminated by removal of the medium and washing the cultures twice with ice-cold medium. These reactions were done in 0.5 ml of a mixture of chloroform and methanol for the separation of water-soluble and chloroform-soluble [3H]inositol metabolites. Intracellular [3H]inositol was separated from [3H]inositol on columns of Dowex-1 formate and assayed as a percent of the maximal uptake in the absence of metal ion. Curve fitting (second order polynomial) was done by computer program.
RESULTS

Suppression of Uptake of "Ca" by Various Metal Ions and High [K'].—Both quin2-loaded and nonloaded cells were used to monitor uptake of "Ca". As in previous studies (18, 39), the uptake of "Ca" into nonloaded cells occurred at rapidly diminishing rates to give no further net increase by 5-6 min. In quin2-loaded cells, the rate of uptake did not markedly diminish until 5 min after the addition of stimulant (data not shown). Under either condition, multivalent metal ions inhibited uptake, in a concentration-dependent manner, in antigen-stimulated and unstimulated cells (Fig. 1). The rank order of inhibitory potencies was La3+ > Ce3+ > Cd2+ > Mn2+ > Co2+ > Ba2+ > Ni2+ > Sr2+ (Table I). The discrimination in values among Mn2+, Co2+, and Ba2+ was not as complete as that observed with La3+ (Fig. 2). The concentration of influx of Ca2+ was not as complete as that observed with La3+ (Fig. 2).

The most significant observation was the similarity in the pattern of inhibition by the various metal ions in unstimulated and antigen-stimulated cells, even though uptake of Ca2+ was accelerated by 3-4-fold in antigen-stimulated cells (Table I; compare also panels A and B in Fig. 1). Also of interest was the marked diminution of the inhibitory effects of La3+ in quin2-loaded cells (compare panels A and C in Fig. 1) probably due to chelation of the La3+ by quin2 (41) that leaks from RBL-2H3 cells (4). A concentration of La3+ of 1 µM was required to suppress uptake of "Ca" by 50% (IC50) in quin2-loaded cells whereas 0.5 µM La3+ was sufficient to inhibit uptake by 50% in nonloaded cells (Table I). The concentration of external quin2 was between 1 and 2 µM in these studies. Other metal ions were not affected by the external quin2, presumably because they inhibit uptake only at greater than micromolar concentrations. Thus external quin2 would have only a trivial effect at these concentrations.

As reported by others (19, 33), the acceleration of influx of "Ca" in antigen-stimulated cells was substantially reduced by high [K']. In contrast to the previous reports (19, 33), however, influx was reduced in unstimulated cells as well. In either case, however, the impairment of uptake by high [K'] was not as complete as that observed with La3+ (Fig. 2).

Therefore, the only difference between antigen-stimulated and unstimulated cells in these experiments was in the rate of influx of Ca2+. Both the IC50 values and the Hill coefficients for inhibition by metal ions and the effects of high [K'], were virtually independent of stimulation.

Selectivity of the Ca2+ Influx Pathway.—To determine whether metal ions blocked "Ca" uptake at the sites of entry on the cell surface or competed with Ca2+ for entry, experiments were conducted in quin2-loaded cells. Entry of Mn2+, Zn2+, and La3+ could be assessed because all three metal ions had higher affinity for quin2 than Ca2+ and quenched quin2 fluorescence (41). These experiments demonstrated that Mn2+, but not Zn2+ or La3+, entered the cells after taking into account the spontaneous release of quin2 from the cells at an approximate rate of 1%/min.

Typical experiments (e.g. Fig. 3), indicated an immediate

The extent of uptake of Mn2+, Zn2+, and La3+ (Table I)3 provided the prospect of determining the kinetics of its binding on RBL-2H3 cells. There was, however, a high degree of nonspecific binding to cells, RSA in the medium, and even some types of plastic ware, and estimates of saturable binding were suspiciously high to give an apparent 70 x 106 sites/cell.

Table I

| Metal ions | Quin 2-loaded | Nonloaded |
|------------|---------------|-----------|
| La3+      | 1.1 (0.8)     | 1.4 (0.8) |
| Ce3+      | 0.6 (1.0)     | 1.2 (0.8) |
| Cd2+      | 252 (0.9)     | 155 (1.4) |
| Mn2+      | 440 (1.6)     | 290 (1.6) |
| Co2+      | 394 (0.95)    | 375 (1.1) |
| Ba2+      | 389 (0.6)     | 560 (0.6) |
| Ni2+      | 1076 (1.4)    | 653 (1.1) |
| Sr2+      | 5660 (0.8)    | 7650 (0.9) |

*Calculated Hill coefficient.

Fig. 1. Inhibition of "Ca" influx in quin2-loaded and nonloaded cells by various metal ions. Cultures were loaded with quin2 (panels A and B) or left unloaded (panel C). The cultures were washed before addition of fresh buffer (37°C) that contained 1 mM Ca2+, the indicated concentration of metal ion and antigen (20 mg/ml DNP-BSA) (panels A and C), or no antigen (panel B). The reaction was terminated 5 min later. Values (mean ± S.E., three cultures) indicate uptake in the presence of metal ion as a percent of uptake of "Ca" in the absence of metal ion in either antigen-stimulated (748 ± 65 and 404 ± 37 pmol of Ca2+/culture, for, respectively, panels A and C) or unstimulated (195 ± 14 pmol of Ca2+/culture for panel B) cultures. Data from this and additional experiments are summarized in Table I. The various metal ions were compared in Table I, and the Hill coefficients for inhibition by metal ions and the effects of high [K'], were virtually independent of stimulation.

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quenching of fluorescence of external quin2 upon addition of Mn^{2+}, Zn^{2+}, or La^{3+}. In unstimulated cells, there was then a slow, steady decline in fluorescence which could be due to continuous leakage of the quin2 and its quenching by the metal ions. However, because the decay in fluorescence was more rapid (>2.5%/min) in the presence of Mn^{2+} (Fig. 3A) than in the presence of La^{3+} (~1%/min) and Zn^{2+} (1–1.5%/min) (Fig. 3, B and C), Mn^{2+} probably entered the cells but at a very slow rate.

In antigen-stimulated cells, the addition of Mn^{2+} resulted in an immediate decline in fluorescence due to external quin2 and then a decline to a level lower than that observed in unstimulated cells (Fig. 3A), a probable indication that influx of Mn^{2+} was enhanced in stimulated cells. In contrast, after the addition of Zn^{2+} or La^{3+} (Fig. 3, B and C), the fluorescence never declined to levels below that in unstimulated cells. The conclusion drawn from these experiments was that, after antigen stimulation, additional Mn^{2+} but not La^{3+} or Zn^{2+} was taken up irreversibly by the cells.

The entry of Mn^{2+} and lack of entry of La^{3+} and Zn^{2+} in antigen-stimulated cells was further demonstrated by the effect of chelating agents. The addition of DTPA, which has been used to chelate selectively Mn^{2+} (42), resulted in only partial restoration of fluorescence to levels less than those observed in the absence of Mn^{2+} in antigen-stimulated cells (Fig. 3A). Therefore, a fraction of the Mn^{2+} was now inaccessible to the chelator. In contrast, the addition of EDTA or EGTA to cell suspensions that contained Zn^{2+} (Fig. 3B) or La^{3+} (Fig. 3C) resulted in complete restoration of fluorescence to the intensities observed in the absence of metal ions. These data indicated that all of the Zn^{2+} and La^{2+} remained accessible for binding to EDTA or EGTA.

**Effects of Metal Ions and High [K+]c on the Increase in [Ca^{2+}].**—As in our previous studies (4), La^{3+} at concentrations of 1–2 μM attenuated the increase in [Ca^{2+}] by 50% in antigen-treated cells. At concentrations >10 μM, La^{3+} suppressed the increase in [Ca^{2+}] by more than 90% (i.e. increases in [Ca^{2+}]; of <50 nM versus increases of >500 nM in the absence of La^{3+}). The increase in [Ca^{2+}] was also substantially reduced (80%) by 100 μM Zn^{2+} (data not shown but see Ref. 4). The increase in [Ca^{2+}] was partially attenuated by high [K^+], and more so by omission of external Ca^{2+}. Moreover, addition of 0.1 mM EGTA gave increases in [Ca^{2+}] of <30 nM compared with increases of >250 nM in high [K^+], and Ca^{2+} (Fig. 4A).

Similar experiments with NECA-stimulated cells indicated that the increase in [Ca^{2+}], was reduced to the same extent as in antigen-stimulated cells by high [K^+], and EGTA (Fig. 4B) and was barely detectable (<50 nM) in the presence of the same concentrations of metal ions (data not shown). The studies thus indicated that the same impediments to Ca^{2+} influx resulted in markedly diminished increases in [Ca^{2+}], in response to antigen or NECA and that high [K^+], was less effective than La^{3+} or EGTA in suppressing the mobilization of Ca^{2+} in these cells. Also the cells were dependent on a non-
The importance of Ca\textsuperscript{2+} influx in promoting stimulatory and secretory responses in RBL-2H3 cells—Advantage was taken of the ability to manipulate influx of Ca\textsuperscript{2+}, by use of high [K\textsuperscript{+}], or different concentrations of metal ions, to determine the precise requirement of each response on influx. In either chloride or glutamate buffers, high [K\textsuperscript{+}], did not suppress breakdown of the inositol phospholipids, but it did suppress exocytosis (Fig. 7). Although this suppression was never complete, and varied from 50 to 80% in different experiments, it did indicate that a diminution of stimulated influx (i.e. Fig. 2) and of the increase in [Ca\textsuperscript{2+}], (i.e. Fig. 4A) will impair exocytosis but not necessarily the phosphoinositide response.

A partial uncoupling of responses was also evident in studies with differen concentrations of various metal ions. Exocytosis appeared to be more susceptible to inhibition than hydrolysis of the phospholipids with most metal ions. The exceptions were Sr\textsuperscript{2+}, which inhibited neither response, and Ba\textsuperscript{2+}, which suppressed exocytosis to a limited extent (Fig. 8). These exceptions were not unexpected because, as noted above, both these ions were reported to enter mast cells and substitute for Ca\textsuperscript{2+} to support hydrolysis of inositol phospholipids totally, and exocytosis partially, whereas other metal ions, by exclusion of Ca\textsuperscript{2+}, inhibited antigen-induced responses.

FIG. 5. Ability of metal ions to promote hydrolysis of inositol phospholipids and exocytosis in the absence of Ca\textsuperscript{2+}. Ca\textsuperscript{2+} (1 mM) in the medium was replaced with 1 mM of the indicated metal ions. In some cultures the medium contained no added cation (−) or, for controls, 1 mM Ca\textsuperscript{2+} (not shown). The cells were stimulated with antigen (20 ng/ml DNP-BSA) for 30 min or left unstimulated (−) for measurement of release of inositol phosphates (IPs, open bars) and secretion of [\textsuperscript{3}H]5-hydroxytryptamine (5HT, solid bars) in the same cultures. Values were expressed (mean ± S.E. for three cultures) as percent of the responses obtained with antigen in normal medium (i.e. with 1 mM Ca\textsuperscript{2+}; 49.8 ± 0.5% release of [\textsuperscript{3}H]inositol phosphates and 68.2 ± 1.9% release of [\textsuperscript{3}H]-5-hydroxytryptamine). The effects of high concentrations (10 mM) of metal ions that appeared to support or partially support stimulatory responses are shown in the inset. Similar results were obtained in two other experiments.

They probably acted by interfering with entry of the residual Ca\textsuperscript{2+} that remained in the Ca\textsuperscript{2+}-free medium (no EGTA was added in these experiments). These results established that Mn\textsuperscript{2+} and Sr\textsuperscript{2+} entered the cells and could substitute for Ca\textsuperscript{2+} to support hydrolysis of inositol phospholipids totally, and exocytosis partially, whereas other metal ions, by exclusion of Ca\textsuperscript{2+}, inhibited antigen-induced responses.

The ability of metal ions to substitute for Ca\textsuperscript{2+} in supporting cellular responses—Because the influx mechanism conducted Mn\textsuperscript{2+}, and probably other metal ions, in addition to Ca\textsuperscript{2+}, the various metal ions were tested for their ability to substitute for Ca\textsuperscript{2+} in supporting antigen-induced responses in intact cells (Fig. 5). As noted previously (4, 17, 38), hydrolysis of inositol phospholipids was much reduced and exocytosis blocked by the omission of Ca\textsuperscript{2+}. The presence of 1 mM Mn\textsuperscript{2+} or Sr\textsuperscript{2+}, however, partially restored both responses to antigen although the extent of exocytosis was still small when compared with the response in 1 mM Ca\textsuperscript{2+}. At a concentration of 10 mM, Sr\textsuperscript{2+} now promoted substantial secretion and a full phosphoinositide response (Fig. 5, inset). The presence of 10 mM Ba\textsuperscript{2+} also resulted in a small increase in secretion (6%) but it should be noted that high concentrations of this ion are generally required to replace the need for Ca\textsuperscript{2+}, e.g., >10 mM with antigen-stimulated rat mast cells (1).

The presence of other metal ions (Cd\textsuperscript{2+}, Zn\textsuperscript{2+}, Co\textsuperscript{2+}, and La\textsuperscript{3+}) suppressed the residual antigen-induced hydrolysis of inositol phospholipids in the intact cell (Fig. 5). Because none of these metal ions, at concentrations up to 50 μM, suppressed antigen-induced responses in permeabilized cells (Fig. 6), they probably acted by interfering with entry of the residual Ca\textsuperscript{2+} that remained in the Ca\textsuperscript{2+}-free medium (no EGTA was added in these experiments). Similar results were obtained in two other experiments.
of myosin (46), the activation of protein kinase C may provide the second synergistic signal for exocytosis in RBL-2H3 cells. There are, however, indications of an additional synergistic signal that is independent of protein kinase C (44, 47) as well as evidence for substantial hydrolysis of phosphatidylcholine by a phospholipase D enzyme, which may supply messenger

DISCUSSION

Although the increase in [Ca2+]i provides an essential signal for the exocytotic release of granules from rat mast cells (43) and RBL-2H3 cells (16), additional synergistic signals are clearly necessary. Low concentrations of the Ca2+-specific ionophores, for example, can induce substantial increases in [Ca2+]i, without stimulating RBL-2H3 cells to secrete (44). Because antigen stimulation results in the translocation of protein kinase C from the cytosol to the membrane fraction (45) as well as protein kinase C-dependent phosphorylation
molecules (23) in addition to those generated by the hydrolysis of the inositol phospholipids (16). Despite the uncertainty of the relationship of the various stimulatory events to exocytosis, the full expression of all stimulatory responses and exocytosis in RBL-2H3 cells and their variants are, with exception, highly dependent on the presence of external Ca^{2+} and, presumably, the influx of Ca^{2+} (16).

The influx of Ca^{2+} into RBL-2H3 cells can be slowed by La^{3+} (4) or depolarization of the plasma membrane with high K^{+}. (19). As in mast cells (48, 49), influx is not blocked by organic blockers of voltage activated Ca^{2+}-channels (48, 49). As reported here, the process allows entry of Mn^{2+}, Sr^{2+}, and possibly other divalent cations. It is blocked competitively by Zn^{2+} and La^{3+} outside the cell. The interaction of some metal ions with the influx mechanism exhibit a pseudo-Hill coefficient of greater than one to indicate possible cooperative actions at the site of uptake. Finally, the characteristics of the influx mechanism are the same in unstimulated and stimulated cells. This could indicate that other additional channels (or carriers) are recruited or the opening times of existing channels are extended in stimulated cells. It is highly unlikely, therefore, that the influx mechanisms operate by the interaction of the antigen-IgE-receptor complex with a membrane protein (26, 30). In any case, the same influx mechanism appears to be activated by NECA, which acts independently of IgE receptors (18).

The earlier work with rat peritoneal mast cells provides sufficient description to suggest that mast cells and RBL-2H3 cells utilize similar mechanisms of uptake. La^{3+} and Mn^{2+}, for example, inhibit Ca^{2+}-dependent histamine release in a reversible, competitive manner in stimulated mast cells to give values for IC_{50} that are comparable with those reported here for inhibition of uptake of Ca^{2+} (35). In addition, Sr^{2+} and Ca^{2+} compete for uptake in unstimulated mast cells (34), and Sr^{2+} inhibits Ca^{2+}-dependent release of histamine in stimulated mast cells to give a value for IC_{50} of about 4 mM (1). The data, in fact, show remarkable qualitative, and even quantitative, similarities.

Our intention was to utilize the metal ions to obtain a kinetic description of the uptake mechanism in RBL-2H3 cells and, thereby, provide an experimental model for comparison with uptake mechanisms in other types of cells. Of specific interest was the non-voltage-gated, second messenger-operated channel because of the presumed ubiquitous distribution of these channels in cells (50). This category of channels, as opposed to voltage-gated or receptor-operated channels, has not been well defined, however, because of the lack of selective and diagnostic blockers of these channels. We thought instead that the metal ions could be used in view of their well defined kinetics of inhibition of influx.

The evidence for non-voltage-activated influx of cations in tissue cells, including mast cells, comes from studies with patched-clamped cells and from studies with metal ions in intact cells. Electrophysiological studies indicate that discrete cation channels can be activated in at least three cellular systems, namely T lymphocytes (51), neutrophils (52), and rat peritoneal mast cells (31). These channels, whose activation is independent of membrane potential, are of low conductance (5–10 pico siemens) and become apparent several seconds after the addition of stimulant. In mast cells and T lymphocytes the channels carry Ba^{2+} and are blocked by Cd^{2+}. In neutrophils, the channels are permeable to Na^{+} and K^{+}. Nonselective cation channels of high conductance (50 pS) are also activated in stimulated rat mast cells (32). All these studies suggest the possible involvement of messenger molecules or G-proteins in that the opening of the channels is delayed and the same channel activities or conductances can be activated by inositol 1,4,5-trisphosphate, Ca^{2+}, or GTPyS.

Studies with intact cells also indicate that the production of the inositol phosphates and the mobilization of intracellular Ca^{2+} are often associated with the activation of nonselective, non-voltage-activated influx of cations (50). Some patterns emerge that are analogous to the present studies. The trivalent lanthanides block uptake of Ca^{2+} not only in mast cells (9) but in many other types of cells (Ref. 53 and citations therein). The alkaline metal ions Sr^{2+} and Ba^{2+} can permeate cells and activate responses or substitute for Ca^{2+} in many cell types (Ref. 53 and citations therein) in addition to mast cells. In two types of cells, receptor-stimulated influx of these metal ions occurs (53, 54), and in one instance Sr^{2+}, but not Ba^{2+}, was shown to enter intracellular stores of Ca^{2+} (53). Of the transition metal ions, Mn^{2+} has been widely used to study...
influx of cations because this ion quenches fluorescence of intracellular probes for Ca\textsuperscript{2+} (42). The effects of Ni\textsuperscript{2+} and Co\textsuperscript{2+} are variable, but Ni\textsuperscript{2+} does not permeate lachrimal acinar cells and blocks entry of Ca\textsuperscript{2+} (53). Most studies provide only qualitative descriptions of the effects of the metal ions, and because of this, the influx mechanism cannot be directly compared with that in mast cells. In addition there may be different mechanisms of uptake even within the same cell (42).

The indications from patch-clamp studies with mast cells of the involvement of second messengers in cation influx are consistent with all that is known about the stimulatory events in RBL-2H3 cells. For example, a transient stimulation of hydrolysis of the inositol phospholipids in RBL-2H3 cells via adenosine receptors is associated with a similarly transient influx of Ca\textsuperscript{2+} and rise in [Ca\textsuperscript{2+}]\textsubscript{i}, whereas these responses are sustained in antigen-stimulated RBL-2H3 cells (18). In addition all manipulations that depress or enhance stimulated breakdown of inositol phospholipids invariably result in identical changes in the magnitude of the increase in [Ca\textsuperscript{2+}]\textsubscript{i} (16, 17) and influx of Ca\textsuperscript{2+} (39).

Even though stimulated breakdown of the inositol phospholipids was not nearly as dependent on influx of Ca\textsuperscript{2+} as exocytosis, there was a threshold for rate of influx below which this breakdown could no longer be sustained. This threshold appeared to be equal to or possibly slightly lower than that in unstimulated cells (basal influx). Thus high [K\textsuperscript{+}], did not inhibit stimulated Ca\textsuperscript{2+} influx to less than basal rates of influx (Fig. 2), nor did it inhibit the stimulated breakdown of the phospholipids. Both the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, and exocytosis, however, were impaired (Figs. 4 and 7). In contrast, suppression to below basal rates of influx with >10 \(\mu\text{M}\) La\textsuperscript{3+} (Fig. 2) caused significant impairment of hydrolysis of the phospholipids (Fig. 9). These rates might have been insufficient for maintenance of adequate levels of Ca\textsuperscript{2+} in intracellular stores and cytosol.

In contrast to the hydrolysis of inositol phospholipids in total, the generation of inositol 1,4,5-trisphosphate is less dependent on external Ca\textsuperscript{2+} and probably accounts for the transient increases in [Ca\textsuperscript{2+}] in the absence of external Ca\textsuperscript{2+} in individual RBL-2H3 cells (8). The data add support to the previous suggestion that much of the hydrolysis of the inositol phospholipids is not dependent on the formation of inositol 1,4,5-trisphosphate (14). Whether the extensive hydrolysis of inositol phospholipids in the presence of Ca\textsuperscript{2+} is due to a change in specificity of phospholipase C with elevated [Ca\textsuperscript{2+}] or to recruitment of additional isozymes of phospholipase C is unknown.

We conclude that full activation of early stimulatory events requires external Ca\textsuperscript{2+} and a basal influx of Ca\textsuperscript{2+}, but that an acceleration of influx is necessary for exocytosis. The homeostasis of Ca\textsuperscript{2+} is important, not only in stimulated RBL-2H3 cells to maintain high [Ca\textsuperscript{2+}] (4), but may be equally critical in unstimulated cells to maintain [Ca\textsuperscript{2+}], high enough to support activation mechanisms and low enough not to activate exocytosis. Here too the synergistic signals come into play, as increases of 0.1 \(\mu\text{M}\) free Ca\textsuperscript{2+} will elicit substantial exocytosis in permeabilized RBL-2H3 cells when this coincides with the activation of phospholipase C, but without this activation concentrations of free Ca\textsuperscript{2+} >1 \(\mu\text{M}\) are necessary for exocytosis (55).
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