Depolarization of Rat Basophilic Leukemia Cells Inhibits Calcium Uptake and Exocytosis

F. Charles Mohr and Clare Fewtrell
Department of Pharmacology, New York State College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

Abstract. We have investigated the unusual observation that depolarization of rat basophilic leukemia cells in high potassium not only fails to induce secretion, but also inhibits the secretion induced when receptors for IgE are aggregated by antigen. Antigen-stimulated 45Ca uptake and the rise in cytoplasmic free ionized calcium measured with the fluorescent indicator quin2 were both inhibited in depolarized cells. 45Ca efflux, on the other hand, was unaffected, which confirms that IgE receptor activation was not impaired in high potassium. Unlike the large increase in total cell calcium seen when cells in normal saline solution were stimulated with antigen, there was a decrease in total cell calcium when depolarized cells were stimulated. This is consistent with our finding that 45Ca uptake was inhibited while 45Ca efflux was unaffected. Inhibition of 45Ca uptake and secretion closely paralleled the decrease in membrane potential, and could be overcome by increasing the extracellular calcium concentration. We conclude that changes in the electrochemical gradient for calcium are important in determining calcium influx and the magnitude of antigen-stimulated secretion from rat basophilic leukemia cells, while the release of calcium from intracellular stores is unaffected.

Rat basophilic leukemia (RBL) cells, like mast cells and basophils, secrete a variety of chemical mediators of inflammation. Secretion is the final outcome of stimulus-secretion coupling and is dependent on a rise in cytoplasmic free calcium (10). This increase in cytoplasmic calcium is a consequence of the aggregation of receptors for IgE, but secretion can also be induced in the absence of receptor aggregation using calcium-carrying ionophores (9). Molecular events occurring at the plasma membrane upon stimulation are clearly important in understanding the early events of stimulus-secretion coupling. Many such events have been documented in basophils and mast cells. These include phospholipid methylation, phospholipase activation, release of arachidonic acid derivatives (7), activation of calcium-activated kinases (13), adenylate cyclase (16), phosphatidylinositol metabolism (3) and cellular depolarization (19, 29, 30). The relationship between these early events and the elevation of cytoplasmic Ca2+ is unclear. However, it is known that receptor activation results in an increase in plasma membrane permeability to Ca2+ and a possible candidate for the calcium channel has recently been identified in RBL cells (23).

In a variety of cell types, e.g., neuronal, muscular, and endocrine, depolarization of the cell leads to cellular activation. The depolarizing stimulus opens voltage-regulated calcium channels, which leads to an influx of calcium down a steep electrochemical gradient. One method of depolarizing cells is to suspend them in a saline solution containing a high level of K+. Since K+ is the major permeant ion in most mammalian cells, the resting membrane potential is generally close to the equilibrium potential for potassium. Thus, an increase in extracellular K+ will lead to depolarization. RBL cells are no exception, having a resting membrane potential somewhere in the region of −55 mV (29) to −94 mV (19). As predicted, they depolarize when they are exposed to high concentrations of K+, but, in contrast to excitable cells, they are not stimulated to secrete. Furthermore, we and others (20) have found that secretion does not occur even when K+-depolarized RBL cells are stimulated with antigen. We report here a series of experiments designed to elucidate the biological mechanism behind this latter observation.

Materials and Methods

Cells

All experiments were performed with a secreting subline (2H3) of RBL cells (2) maintained in monolayer culture as described (34).

Saline Solutions

The standard isotonic saline solution (NaK saline) was a modified Tyrode's solution containing 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 0.05% gelatin, and 10 mM Hepes adjusted to pH 7.4 with NaOH or Tris base. Depolarizing saline solution (K saline) contained the above components, except that the NaCl was completely replaced with KCl, while Na saline contained 140 mM NaCl and no KCl. In experi-

1. Abbreviations used in this paper: Bis-oxonol, bis-(1,3-diethylthiobarbiturate) trimethineoxonol; RBL, rat basophilic leukemia.
elements demonstrating calcium dependence, CaCl₂ was left out of the saline solutions and added separately to the cells. When concentrations of calcium or other agents were high enough to affect tonicity, adjustments were made in the NaCl or KCl concentration to keep all solutions osmotically equivalent. In experiments using the calcium ionophore A23187, the pH of the saline solutions was increased to 8 to optimize the secretory response (9).

**Reagents**

[³H]serotonin (5-[1,2-³H(N)]-hydroxytryptamine bisnitolate), [³H]EDTA, and ⁴⁵CaCl₂ were purchased from New England Nuclear (Boston, MA). Quin2 acetoxymethyl ester, PMA, and protonicidin were obtained from Sigma Chemical Co. (St. Louis, MO); A23187 and ionomycin from Calbiochem (La Jolla, CA); and bis-(1,3-diethylthioubarbiturate) trimethineoxonol (bis-oxonol) from Molecular Probes (Junction City, OR). Stock solutions of graminidcin, A23187, and ionomycin were prepared in ethanol; bis-oxonol, quin2 acetoxymethyl ester, and PMA were dissolved in dimethylsulfoxide.

Cells were exposed to 1% or less of ethanol and 0.1% or less of dimethylsulfoxide. Purified mononuclear mouse IgE directed against dinitrophenyl hapten (17, 22) was a gift from Barbara Baird and David Holowka, Department of Chemistry, Cornell University. The antigen used was bovine gamma globulin to which an average of 15 dinitrophenyl groups per molecule had been coupled (8).

**Secretion Assays**

Secretion was assessed by measuring the release of serotonin or β-hexosaminidase. Whenever possible the release of both markers was measured. In all experiments described here they paralleled one another. All secretion assays were run in duplicate.

**[³H]Serotonin Release**

Serotonin secretion was measured by release of incorporated [³H]serotonin in a standard tube assay (35). Monolayer cultures were loaded overnight with [³H]serotonin (1) before trypsinization and sensitization with mouse IgE (1 μg/ml). After sensitization the cells were resuspended in one of the saline solutions described above to give a final concentration of 2 × 10⁶ cells/ml after all additions had been made. Cell viability was always >95% as assessed by trypan blue exclusion. The experiments were run at 37°C. The time between suspension in the relevant saline solution and stimulation with antigen (0.1 μg/ml) or Ca²⁺ ionophore (0.25 or 0.5 μM) was 10–15 min. After 50 min of stimulation the reaction was quenched by the addition of ice-cold saline solution (either the solution in which the reaction was run or a simplified saline solution containing 135 mM NaCl, 5 mM KCl, and 10 mM Hepes, pH 7.4). An aliquot of the supernatant was counted for [³H] and the counts were expressed as a percentage of the total [³H]serotonin content of the cell suspension before centrifugation.

**β-Hexosaminidase Release**

β-Hexosaminidase was assayed fluorometrically using 4-methylumbelliferyl-N-acetyl β-d-glucosaminide (Sigma Chemical Co.) as the substrate (5). Release was expressed as a percentage of the total β-hexosaminidase activity of the cells.

**Calcium Determinations**

In all experiments involving calcium movements duplicate samples were analyzed.

**⁴⁵Ca Uptake**

This was measured as described (7) with a modification of the quenching saline solution. 10⁶ cells in 3 ml culture medium were seeded into each well of several multiwell plates (Falcon Labware, Oxnard, CA) 2 days before the experiment. The cells in each well were sensitized with IgE (0.6 μg in 1 ml NaK saline/well for 60 min) and then washed twice with the relevant saline solution (warmed to 37°C). Approximately 10 min after suspension in the relevant solution, ⁴⁵Ca uptake was initiated by the addition of ⁴⁵Ca (5 or 10 μCi/ml) with or without antigen (1 μg/ml). At indicated time points each well was washed twice with 4 ml of ice-cold quenching solution containing 135 mM NaCl, 5 mM KCl, 10 mM Hepes, 1.8 mM CaCl₂, and 1 mM MgCl₂. Duplicate aliquots were taken after quenching to determine [³H]serotonin or β-hexosaminidase release, and each well was then washed twice with the same quenching solution. The cells were solubilized in 10% Triton X-100 and an aliquot from each well was counted for ⁴⁵Ca. Separate wells were used to determine cell number.

**⁴⁵Ca Movements at Isotopic Equilibrium**

This measurement was performed as above except that RBL cell monolayers in multiwell plates were cultured overnight with ⁴⁵Ca (10 μCi/ml culture medium containing 1.8 mM CaCl₂). ⁴⁵Ca was maintained at the same specific activity throughout the experiment until the final quenching and washing with ice-cold quenching solution (see above).

**⁴⁵Ca Efflux**

Cells in monolayer culture were trypsinized and then suspended in NaK saline. They were loaded with ⁴⁵Ca (50 μCi/ml) for 90 min. Sensitization with mouse IgE was performed simultaneously. The cells were then centrifuged and resuspended in the appropriate saline solution at 37°C (zero time). 15 min after resuspension, the cells were stimulated with antigen (1 μg/ml final concentration). At appropriate time intervals, 100-μl aliquots were removed and the cells centrifuged through oil (dibutyl phthalate/bis-ethylhexyl phthalate, 6:4 vol/vol) in 400-μl microtube tubes. An aliquot of the supernatant was assayed for β-hexosaminidase, and the tips of the tubes containing the cell pellets were incubated overnight with 10% Triton X-100 to solubilize the cells before counting. Residual ⁴⁵Ca associated with the cells was expressed as a fraction of the ⁴⁵Ca present in the cells 5 min after resuspension in a ⁴⁵Ca-free saline solution.

**Cell Volume**

This was determined in a similar manner to the calcium efflux experiments using [³H]EDTA as a total volume marker. There was no change in the cell volume of nonstimulated or stimulated cells suspended in NaK saline (0.83 ± 0.09 and 0.90 ± 0.10 μl/10⁶ cells, respectively, after 60 min) in these experiments. The increase in cell volume was noted (1.36 ± 0.38 μl/10⁶ cells after 60 min) in cells suspended in K saline, commencing 15–30 min after suspension and continuing throughout the course of the experiment (60 min). However, if these cells were stimulated with antigen 15 min after suspension no swelling occurred (0.88 ± 0.21 μl/10⁶ cells after 60 min).

**Cytoplasmic Free Ionized Calcium**

Changes in cytoplasmic Ca²⁺ were monitored using the fluorescent indicator quin2 (36). All saline solutions for these experiments contained 0.1% bovine serum albumin (instead of gelatin) to maximize uptake and retention of quin2 by the cells (4). Sensitized RBL cells (10⁶ cells/ml) were incubated at 37°C with 5 μM quin2 acetoxymethyl ester for 60 min. These conditions resulted in optimal uptake of the acetoxymethyl ester and its intracellular conversion to the free acid, quin2. After loading with quin2 the cells were resuspended at the same concentration and 3-ml aliquots were transferred to quartz cuvettes maintained at 37°C and constantly stirred. Quin2 fluorescence (excitation 339 nm, emission 429 nm) was recorded with a fluorescence spectrophotometer (LS-5, Perkin-Elmer Corp., Norwalk, CT). The quin2 fluorescence signal was calibrated as described by Hesketh et al. (44). At the end of the experiment, 30 μl 10% Triton X-100 was added to each cuvette to solubilize the cells and to saturate quin2 with calcium. Subsequent addition of 1 mM manganese chloride quenches all fluorescence due to quin2. Calcium-sensitive fluorescence was assumed to be 84% of the total fluorescence (ΔF) due to quin2 (14). The effective dissociation constant (Kᵢ) for calcium binding to quin2 in the presence of 1 mM Mg²⁺ is 115 nM (36). The intracellular free ionized calcium concentration was calculated using the relationship

$$ Ca^{2+} = K_i \frac{F}{1 - F} \quad (reference \ 36), $$

where

$$ F = \frac{(1 - 0.16 \Delta F)}{0.84 \Delta F} \quad (reference \ 14). $$

F is the fraction of quin2 that has bound calcium, and I is the measured quin2 fluorescence intensity after correcting for extracellular quin2. The intracellular quin2 concentration was between 1 and 3 nM, a concentration range that does not affect secretion from RBL cells (4, Fewtrell, C., and P. Millard, unpublished observations). Fluorescence due to extracellular quin2 was determined by adding manganese chloride to aliquots of the cells.
at the beginning and end of the experiment. Between 10 and 20% of the quin2 was extracellular at the beginning of an experiment and this increased to ~30–40% by the end (~40 min). The rate of quin2 leakage was constant throughout an experiment and was comparable in the different saline solutions and with stimulated and unstimulated cells. The extracellular quin2 at any time point during an experiment was therefore determined by extrapolation.

**Membrane Potential**

Changes in membrane potential were measured using the fluorescent dye bis-oxonol (27). Bis-oxonol (100 nM final) was added to sensitized RBL cells in isotonic saline solution (106 cells/ml). Aliquots (3 ml) were placed in quartz cuvettes in the fluorometer (excitation 540 nm, emission 580 nm) with constant stirring. For the determinations shown in Fig. 9, aliquots of the cells were resuspended in saline solutions containing the required KCI concentrations. To normalize the responses, the fluorescence at each KCI concentration was divided by the fluorescence of the same cell suspension in the presence of gramicidin (1 μM), which completely depolarizes the cells (27, 24a). The fluorescence response of oxonol dyes appears to be approximately linear with membrane potential (37).

**Cell Viability**

Release of the cytoplasmic enzyme lactate dehydrogenase was assayed spectrophotometrically (34). Cellular viability was also assessed by trypan blue exclusion. No significant decrease in cell viability was detected in any of the experiments.

**Scintillation Counting**

[3H]serotonin, 45Ca, and 3H2O were measured in a scintillation counter (LS 1800; Beckman Instruments, Inc., Palo Alto, CA) using scintillation cocktail (ACS; Amersham Corp., Arlington Heights, IL).

**Results**

**Antigen-induced Secretion Is Inhibited by High Concentrations of K+**

When RBL cells were depolarized by resuspension in an isotonic saline solution in which Na+ was replaced with K+, there was no increase in the spontaneous release of either [3H]serotonin or β-hexosaminidase (Fig. 1). Furthermore, addition of the C-kinase activator PMA, which alone has no effect but which markedly potentiates calcium ionophore–induced secretion (see Fig. 3), failed to increase the spontaneous release of serotonin from K+-depolared RBL cells.

It has been shown, using the membrane potential probe tetraphenylphosphonium+, that RBL cells do depolarize in a high K+ saline solution (20, 30) and we have confirmed this finding using the negatively charged, potential-sensitive fluorescent probe bis-oxonol (27). Addition of gramicidin to RBL cells suspended in NaK saline led to an increase in bis-oxonol fluorescence, which corresponded to depolarization of the cells. In contrast, gramicidin had no effect on the fluorescence of cells suspended in K saline, thus demonstrating that the cells were already fully depolarized (24a).

Although spontaneous secretion was unaffected, antigen-stimulated secretion was substantially reduced when RBL cells were depolarized in K saline (Fig. 1). This was not simply due to Na+ removal, since replacing NaCl with 270 mM glucose had no effect on secretion (Fig. 1). This effect was reversible, since resuspension of K+ depolarized cells in NaK saline restored their ability to secrete in response to antigen. RBL cells were incubated for 30 min in K saline, after which one aliquot of the cells was suspended in NaK saline while the other was resuspended in K saline. 15 min later the cells were stimulated with antigen. Secretion from cells in K saline was inhibited, as expected, whereas the secretory response of cells resuspended in NaK saline was completely restored (102 ± 6% of that seen with control cells that were maintained in NaK saline throughout).

An interesting, but as yet unexplained finding was that complete removal of K+ from the saline solution also inhibited secretion (Fig. 1). Again this effect was reversible; resuspension of cells in NaK saline led to a complete recovery of the antigen-induced secretory response.

**Calcium Movements in Depolarized Cells**

Since calcium is known to be a linking messenger between IgE receptor stimulation and the ultimate event of secretion in RBL cells, we compared calcium movements in cells bathed in a depolarizing saline solution (K saline) with cells in normal saline solution (NaK saline). Four different methods were employed: early 45Ca uptake and 45Ca efflux studies designed to study unidirectional calcium movements, changes in total cell calcium measured with cells at isotopic equilibrium with 45Ca, and measurements of cytosolic free ionized calcium using the fluorescent probe quin2.

**45Ca Uptake Is Inhibited in Depolarized Cells**

When cells are depolarized the electrical component of the electrochemical gradient for calcium will be abolished. Thus, a large part of the driving force for Ca2+ entry into the cell is removed, and this should be reflected by a decrease in 45Ca uptake. Fig. 2 shows that this was indeed the case with antigen-stimulated cells. Resting cells in both normal
Figure 2. The initial rate of spontaneous and antigen-induced uptake of \(^{45}\text{Ca}\) by RBL cells in normal (NaK) saline and depolarizing (K) saline. The cells were preincubated in the relevant saline solution for 10 min before the addition of \(^{45}\text{Ca}\) and antigen (1 \(\mu\text{g/ml}\)). Antigen-stimulated cells in NaK saline (solid triangle) and K saline (solid square). Spontaneous uptake was the same in both saline solutions (dashed line); the data points for spontaneous uptake have been omitted for clarity.

In experiments with rat peritoneal mast cells there has been considerable concern that measurements of \(^{45}\text{Ca}\) uptake merely reflected an increase in \(\text{Ca}^{2+}\) binding sites on the plasma membrane due to exocytosis of granules (11, 12). Thus, when secretion was prevented, \(^{45}\text{Ca}\) uptake would automatically be reduced. Although RBL cells contain very few secretory granules and therefore do not undergo the profound morphological changes seen with stimulated peritoneal mast cells, it is possible that an increase in \(\text{Ca}^{2+}\) binding sites could also explain the inhibition of \(^{45}\text{Ca}\) uptake that we saw with K' depolarized cells. However, if this were the case, then any manipulation that increased secretion should also cause a parallel increase in \(^{45}\text{Ca}\) uptake, and this clearly does not occur. Fig. 3 shows that secretion induced by the calcium ionophore A23187 was markedly increased by the C-kinase activator, PMA, whereas \(^{45}\text{Ca}\) uptake was completely unaffected. This confirms that \(^{45}\text{Ca}\) uptake is not an artifact of exocytosis but is likely to be a measure of true calcium influx into RBL cells.

\(^{45}\text{Ca}\) Efflux Is Not Affected by Depolarization

In contrast to the dramatic effects of depolarizing (K) saline on stimulated \(^{45}\text{Ca}\) uptake, \(^{45}\text{Ca}\) efflux from RBL cells was completely unaffected. Spontaneous and antigen-stimulated \(^{45}\text{Ca}\) efflux curves are shown in Fig. 4, and it is clear that these were unchanged when cells were resuspended in K saline or Na saline instead of the usual NaK saline. These results clearly demonstrate that receptors for IgE were not inactivated in Na saline or K saline, since they were still able to elicit a response (i.e., \(^{45}\text{Ca}\) efflux) when they were aggregated by antigen.

Total Cell Calcium Decreased When Depolarized Cells Were Stimulated with Antigen

Changes in total cell calcium were assessed using RBL cells that had been cultured overnight in the presence of \(^{45}\text{Ca}\) to allow intracellular calcium pools to reach isotopic equilibrium. The cells were then bathed in NaK saline or K saline containing \(^{45}\text{Ca}\) at the same specific activity. Net changes in
total cell calcium were determined from the changes in cell-associated $^{45}$Ca.

Cells bathed in NaK saline showed a net increase in total cell calcium upon stimulation with antigen (Fig. 5). This peaked at ~10-15 min, and then there was a gradual fall in total cell calcium to near resting levels 60 min after stimulation. Total cell calcium was unchanged when resting cells were incubated in K saline for as long as an hour. However, when cells in K saline were stimulated with antigen there was a significant (~50%) drop in total cell calcium (Fig. 5). This observation was consistent with our finding (above) that $^{45}$Ca uptake was inhibited in K saline while $^{45}$Ca efflux was unaffected. Thus, a net loss of calcium such as that shown in Fig. 5 would be expected.

**The Stimulated Rise in Cytoplasmic Ca$^{2+}$ Is Attenuated in Depolarized Cells**

Cytoplasmic free ionized calcium was measured using RBL cells loaded with the fluorescent calcium indicator, quin2. The antigen-induced increase in cytoplasmic Ca$^{2+}$ in cells suspended in NaK saline is shown in Fig. 6a. There was a rapid rise in Ca$^{2+}$, which peaked within 2 min and then declined to a stable, but elevated level that was maintained for at least 40 min (not shown). Fig. 6b shows the calculated Ca$^{2+}$ concentrations at representative points during the first 10 min. When cells were resuspended in K saline, the resting cytoplasmic Ca$^{2+}$ level was virtually unchanged ($82 \pm 4$ nM in NaK saline; $91 \pm 7$ nM in K saline; results are means plus or minus standard deviations from six paired experiments). The initial rise in cytoplasmic Ca$^{2+}$ in response to antigen-stimulation followed the same time course but was significantly attenuated in K saline (Fig. 6, c and d). The degree of attenuation varied somewhat from experiment to experiment, but in all cases the signal rapidly returned to near basal levels after the first few minutes (Fig. 6, c and d). This is in marked contrast to the signal generated by stimulated RBL cells in NaK saline, which remained elevated (Fig. 6, a and b). As expected, release of β-hexosaminidase or [$^3$H]serotonin measured directly from the cuvettes was almost completely inhibited in depolarized cells (not shown).

**Increasing the Extracellular Calcium Concentration Can Overcome the Inhibition Seen in Depolarized Cells**

If inhibition of secretion is related to a decreased uptake of calcium due to the abolition of the electrical component of the gradient for calcium, we reasoned that by increasing the extracellular calcium concentration, sufficient amounts of calcium may be able to enter the depolarized cells and overcome the inhibition. This was indeed the case; both antigen-stimulated $^{45}$Ca uptake (Fig. 7) and [$^3$H]serotonin secretion (Fig. 8) from depolarized RBL cells were restored by increasing the extracellular Ca$^{2+}$ concentration. In each case the calcium dose-response curves obtained with RBL cells in K saline were shifted to the right, but paralleled those for

---

Figure 4. Spontaneous and antigen-induced β-hexosaminidase release (a) and $^{45}$Ca efflux (b) from RBL cells suspended in isotonic saline solutions of different compositions. NaK saline (open triangle, solid triangle); K saline (open square, solid square); Na saline (open circle, solid circle). Open symbols represent resting cells while solid symbols represent cells stimulated with antigen (1 μg/ml).

Figure 5. Effect of K$^+$ depolarization on total cell calcium in resting (dashed line) and antigen-stimulated (solid line) RBL cells. (Solid triangle) Cells in NaK saline; (solid square) cells in K saline. (Solid triangle) Cells in NaK saline; (solid square) cells in K saline.
cells in NaK saline. Thus, in NaK saline the half-maximal secretory response was seen at 0.2 mM Ca\(^{2+}\) while in K saline this required 3 mM Ca\(^{2+}\) (Fig. 8). Nevertheless, at 10 mM extracellular Ca\(^{2+}\) the inhibition in K saline was largely overcome, and if the Ca\(^{2+}\) concentration was increased to 30 mM, recovery was 97% complete (not shown). Since a substantial reduction in NaCl or KCl concentration was necessary to maintain the correct osmolarity when 30 mM Ca\(^{2+}\) was used, these data were not included in Figs. 7 and 8.

The inhibition seen with cells suspended in Na saline (i.e., in the complete absence of K\(^+\)) was clearly different since it could not be overcome to any significant extent by increasing the extracellular calcium concentration (Fig. 8).

**Inhibition of Calcium Uptake and Secretion Closely Follows Depolarization**

If calcium uptake and secretion are regulated by the magnitude of the electrochemical gradient for calcium, then it would follow that progressive depolarization should lead to a parallel decrease in calcium uptake and secretion. This is shown in Fig. 9. These experiments were carried out in 0.5 mM Ca\(^{2+}\) to ensure that the extracellular Ca\(^{2+}\) concentration would be limiting the secretory response even in NaK saline (see Fig. 8). As the KCl concentration was increased, the cells became more depolarized until complete depolarization was reached between 100 and 140 mM KCl (Fig. 9). As predicted, the inhibition of \(^{45}\)Ca uptake and [\(^{3}H\)]serotonin release closely paralleled the reduction in the membrane potential of the cells.

We have no explanation, at present, for our finding that complete removal of extracellular K\(^+\) inhibited both secretion and \(^{45}\)Ca influx (Figs. 1 and 9). However, this was not due to depolarization of the cells, since the membrane potential of cells in Na saline was not significantly lower than that of cells in NaK saline (Fig. 9).

Since cells suspended in a solution in which all the NaCl was replaced with isotonic glucose secreted normally (Fig.

---

**Figure 6.** Measurement of the antigen-stimulated rise in quin2 fluorescence in cells suspended in NaK saline (a) and K saline (c). Cells were preincubated for 10 min before stimulation with antigen (1 \(\mu g/ml\)). Separate cuvettes of cells were used to measure fluorescence changes in unstimulated cells (lower traces in a and c). The calculated rise in free cytoplasmic calcium at selected time points from the corresponding traces in a and c are shown in b and d.

**Figure 7.** Effect of increasing the extracellular Ca\(^{2+}\) concentration on the initial rate of antigen-induced \(^{45}\)Ca uptake by RBL cells in NaK (solid triangle) and K (solid square) saline. The cells were preincubated in the relevant saline solution for 10 min before the addition of \(^{45}\)Ca and antigen (1 \(\mu g/ml\)). \(^{45}\)Ca uptake was measured 4 min after stimulation.

**Figure 8.** Effect of increasing the extracellular Ca\(^{2+}\) concentration on antigen-induced secretion from RBL cells in different isotonic saline solutions. The cells were resuspended in the relevant saline solution 10 min before the addition of antigen (0.1 \(\mu g/ml\)). Spontaneous release of [\(^{3}H\)]serotonin (15–20%) has been subtracted.
we expected that the membrane potential would be unaffected. In agreement with this we found that the bisoxonol fluorescence of cells in the isotonic glucose-containing solution was indistinguishable from that of cells in normal (NaK) saline (not shown).

**Secretion Induced by Calcium Ionophores Is Inhibited in Depolarized Cells**

The calcium ionophore A23187 is a carboxylic acid that forms a neutral complex with calcium (25). Since it exchanges Ca\(^{2+}\) for Mg\(^{2+}\) or 2H\(^+\), it is an electrically neutral, passive transporter and should be unaffected by the membrane potential. We therefore expected that ionophore-induced calcium uptake and the subsequent secretion of serotonin from RBL cells would be unaffected by depolarization in K saline. This was not the case. At physiological concentrations of calcium, ionophore-induced calcium uptake and secretion were almost completely inhibited when RBL cells were depolarized in K saline (Fig. 10). This effect was not due to a lack of sodium, since replacing NaCl with glucose (270 mM) had no effect on A23187-induced secretion (not shown). However, secretion and \(^{45}\)Ca uptake were partially (>50%) inhibited in Na saline (not shown). As with antigen (see Figs. 7 and 8) there was a shift to the right in the calcium dose-response curves for both calcium uptake and secretion in K\(^+\)-depolarized cells (Fig. 10). Furthermore, the secretory response of depolarized cells was completely restored at 10 mM Ca\(^{2+}\) (Fig. 10 b). Thus, \(^{45}\)Ca uptake and \([^{3}H]\)serotonin release at 10 mM Ca\(^{2+}\) in K saline were comparable to those seen at the normal physiological Ca\(^{2+}\) concentration (1.8 mM) in NaK saline. A23187-induced \(^{45}\)Ca efflux was completely unaffected when cells were depolarized in K saline (not shown), which is again similar to our findings with antigen-stimulated cells (Fig. 4).

The quin2 fluorescence response to the calcium ionophore ionomycin (Fig. 11) was also reminiscent of our findings with antigen-stimulated cells (cf. Figs. 6 and 11). Thus, the initial increase in cytoplasmic Ca\(^{2+}\), which we think is due, in part, to the release of Ca\(^{2+}\) from intracellular stores, was only partially reduced in K saline. In contrast, the prolonged elevation in cytoplasmic Ca\(^{2+}\), which appears to be due to Ca\(^{2+}\) influx into the cells and is required for the initiation of secretion, was almost completely abolished in K saline.

**Discussion**

Many secretory cells, including pituitary cells (33), pancreatic \(\beta\) cells (21), and neuronal cells (32) are stimulated to secrete upon exposure to high K\(^+\) (>20 mM). Both this response (which is dependent on extracellular calcium) and the physiological response to secretagogues can be inhibited by classical calcium antagonists such as verapamil, D600, and the dihydropyridine antagonists. This suggests that these cells have voltage-sensitive calcium channels that open when the cells depolarize in high K\(^+\) or in response to secretagogues.

Mast cells, basophils, and, as we confirm here, RBL cells appear to be different in this respect, since these cells fail to secrete when they are depolarized in high K\(^+\) (6, 18, 20). Since RBL cells secrete rather poorly when cytoplasmic Ca\(^{2+}\) is increased using the ionophore A23187 (9), we thought it was possible that depolarization might be opening Ca\(^{2+}\) channels, but that the rise in cytoplasmic Ca\(^{2+}\) alone might not be sufficient to cause secretion. The C-kinase activator PMA, which alone has no effect on secretion from RBL cells, markedly potentiates Ca\(^{2+}\) ionophore-induced secretion (Fig. 3 and reference 31). However, PMA was without effect on spontaneous secretion from K\(^+\)-depolarized RBL cells (Fewtrell, C., unpublished observations). Furthermore, spontaneous \(^{45}\)Ca uptake is not increased when RBL cells are suspended in K saline (Fig. 2 and reference 20), which suggests that these cells do not possess voltage-sensitive calcium channels. In agreement with this it has been shown that antigen-induced secretion from basophils (24) and mast cells (28) is unaffected by organic calcium antagonists.

Although high K\(^+\) did not affect spontaneous secretion, it had a profound effect on antigen-induced secretion and calcium movements in RBL cells (reference 20 and this paper). We show here that as Na\(^+\) was replaced with K\(^+\), antigen-stimulated calcium uptake and secretion were increasingly inhibited and were almost completely abolished when all the Na\(^+\) was replaced with K\(^+\) (Fig. 9). This was not a cytotoxic effect of high K\(^+\) nor was it due to Na\(^+\) deprivation, since

---

**Figure 9.** Effect of increasing concentrations of K\(^+\) on antigen-induced \(^{45}\)Ca uptake, \([^{3}H]\)serotonin release, and resting membrane potential. All saline solutions contained 0.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 5.6 mM glucose, 0.05% gelatin, and 10 mM Tris Hepes, pH 7.4. To maintain isotonicity, the NaCl concentration was decreased so that the sum of the NaCl and KCl concentrations was maintained at 140 mM in all cases. All parameters are expressed as a fraction of the control (NaK) saline (i.e., 5 mM KCl). In this saline solution antigen-stimulated \([^{3}H]\)serotonin secretion was 33% and \(^{45}\)Ca uptake was 1265 pmol/10\(^6\) cells per 3 min. Spontaneous \(^{45}\)Ca uptake (90 pmol/10\(^6\) cells per 3 min) and \([^{3}H]\)serotonin release (5%) remained constant at all KCl concentrations and were subtracted from the stimulated values. The standard deviation never exceeded 0.02 U for \(^{45}\)Ca uptake and 0.05 U for secretion. Resting membrane potentials were measured in a separate experiment using 100 mM bis-oxonol. The decrease in fluorescence at the different K\(^+\) concentrations (relative to the fluorescence of cells completely depolarized in 140 mM KCl) was expressed as a fraction of the decrease seen with cells in NaK saline. These values (with standard deviation bars) represent the average of three determinations.
secretion was unaltered when NaCl was replaced with glucose rather than with KCl.

We and others (20) have therefore proposed that the inhibition of secretion observed in K saline is a direct consequence of RBL cell depolarization and is presumably due to the abolition of the electrical component of the electrochemical gradient for calcium. In agreement with this, we found that $^{45}$Ca uptake and the increase in cytoplasmic free Ca$^{2+}$ seen in response to antigen-stimulation were both dramatically reduced in K$^+$-depolarized cells (Figs. 2 and 6). $^{45}$Ca efflux, on the other hand, was unchanged (Fig. 4), which confirmed that IgE receptor activation was not prevented. Since the release of calcium from intracellular stores should not be affected by changes in the membrane potential, this result is also consistent with our model. If $^{45}$Ca uptake is inhibited but $^{45}$Ca efflux is unchanged, there should be a net loss of calcium from depolarized cells in response to antigen. This is shown in Fig. 5 and is in marked contrast to the severalfold increase in total cell calcium seen with antigen-stimulated cells in normal (NaK) saline.

The antigen-stimulated rise in cytoplasmic free ionized calcium occurs in two phases (Fig. 6): an initial rise that peaks after about 2 min and which then declines to a somewhat lower but still elevated level that is maintained while secretion occurs (at least 40 min). Since this latter phase is almost completely inhibited in K$^+$-depolarized cells, it is likely to be due to the influx of extracellular calcium. However, the initial rise in cytoplasmic Ca$^{2+}$ is somewhat attenuated, but by no means abolished, in depolarized cells, which suggests that it may be due, at least in part, to the release of calcium from intracellular stores. Similar findings were observed when the cells were stimulated with the calcium ionophore ionomycin (Fig. 11). It is interesting to note that this transient increase in cytoplasmic Ca$^{2+}$ in depolarized cells is not sufficient for the initiation of secretion. Since stimulated secretion is prevented when the late phase of the Ca$^{2+}$ signal is inhibited, it seems that it is the sustained influx of extracellular calcium and not the transient release of calcium from intracellular stores that is important in generating the message that eventually leads to exocytosis in RBL cells.

If the inhibition of secretion and calcium uptake in K$^+$-depolarized cells is indeed due to the abolition of the electrical component of the calcium gradient, it should be possible to overcome this effect by increasing the chemical gradient for calcium. As predicted, $^{45}$Ca uptake and [$^3$H]serotonin secretion from depolarized RBL cells were restored by increasing the extracellular calcium concentration (Figs. 7 and 8). Furthermore, there should also be a good correlation between the extent of K$^+$-induced depolarization and the subsequent $^{45}$Ca uptake and secretion in response to antigen, and this was indeed the case (Fig. 9).

Very little is known about the calcium permeability pathway in mast cells and basophils, although it is generally

Figure 10. Effect of increasing the extracellular Ca$^{2+}$ concentration on the initial rate of $^{45}$Ca uptake (a) and [$^3$H]serotonin release (b) in response to 0.5 μM A23187. Conditions were the same as those in Figs. 6 and 7 except that $^{45}$Ca uptake was measured 3 min after the addition of ionophore.

Figure 11. Measurement of the calcium ionophore-induced rise in quin2 fluorescence in NaK saline (a) and K saline (c). This experiment was performed as described in Fig. 6 except that after quin2 loading the saline solutions contained 0.05% gelatin instead of bovine serum albumin and the cells were stimulated with 0.25 μM ionomycin. The calculated rise in free cytoplasmic calcium at selected time points from the corresponding traces in a and c are shown in b and d.
assumed to be a channel that is somehow open when receptors for IgE are aggregated. The recent isolation, purification, and reconstitution of a cromolyn-binding protein that appears to behave as a calcium-selective channel has provided the first direct evidence for such a mechanism (23).

Both channel lifetime and conductance are independent of the applied voltage, which is consistent with our finding that depolarization of RBL cells does not lead to calcium influx. It is also in agreement with our observation that depolarization inhibits antigen-induced Ca\(^{2+}\) influx, since abolition of the membrane potential should substantially reduce the calcium current flowing through the channel.

Although the findings we have discussed so far are consistent with the idea that the inhibition of antigen-induced secretion in K saline is due to the abolition of the electrical component of the calcium gradient, our results with the ionophore A23187 are less easy to reconcile. Since A23187 is an electrically neutral exchange diffusion carrier (25), we predicted that \(^{45}\text{Ca}\) uptake and the secretion induced by A23187 would be unaltered in K\(^+\) depolarized cells, but it is clear from our results that both were profoundly inhibited. A similar inhibition of the sustained phase of the increase in cytoplasmic Ca\(^{2+}\) was also observed in quin2-loaded cells. It was, however, possible to overcome the inhibition of calcium uptake and secretion by increasing the extracellular Ca\(^{2+}\) concentration as we had been able to do for antigen-induced secretion (cf. Figs. 7, 8, and 10).

One possible explanation for these results is that the local extracellular calcium concentration sensed by the antigen-stimulated permeability pathway and a membrane-associated ionophore molecule is reduced when RBL cells are depolarized. It is well established that the surface concentration of divalent cations, and in particular Ca\(^{2+}\), can be considerably higher than the bulk concentration, due to the negative surface potential (reference 15, Chapter 13). Part of this is due to the charge on the membrane capacitor, which would be dissipated when the cells are depolarized (reference 15, p. 320). However, the relative contribution of the surface potential due to fixed negative charges on the external surface of the plasma membrane may be much greater than that due to the membrane capacitance and this contribution should not change when the cells are depolarized.

Another somewhat unexpected result was our finding that complete removal of extracellular K\(^+\) also inhibited antigen-induced secretion and \(^{45}\text{Ca}\) uptake. However, several features of this inhibition suggest that it occurs by a very different mechanism from the one we have proposed for K\(^+\)-depolarized cells. Thus, we have shown that secretion from cells in K\(^-\)free solution (Na saline) cannot be restored by increasing the extracellular calcium concentration, as was the case with cells in K saline (Fig. 8). Consistent with this is our finding that cells in Na saline are not depolarized (Fig. 9). Furthermore, completely replacing NaCl with glucose drastically alters the ionic strength of the solution but has no effect on antigen-induced secretion (Fig. 1). Thus, we feel that these differences are unique and that the effects we see are not simply due to nonspecific alterations in the ionic composition of the extracellular solution.

In summary, we have investigated the intriguing observation that depolarization of RBL cells in high potassium, rather than inducing secretion, actually inhibits antigen-induced exocytosis. We suggest that when the cells are depolarized the driving force for calcium entry is markedly reduced, since the electrical component of the electrochemical gradient for calcium is abolished. Furthermore, in cells with calcium channels that are not regulated by voltage, there will be no increase in calcium conductance to offset the effects of a reduction in the electrochemical gradient and so calcium uptake will be inhibited. If this is indeed the correct interpretation of our results, then similar findings could be predicted for other cell types that rely on extracellular Ca\(^{2+}\), but apparently lack voltage-sensitive calcium channels.

We are grateful to Dr. Paul Millard for writing the program used to analyze the quin2 fluorescence data. Dr. Michael Beaven and Dr. Henry Metzger kindly made references 3 and 4, and 20, respectively, available to us before publication. We thank Drs. David Holowka, Gregory Weiland, and Paul Millard for critical reading of this manuscript. This work was supported by National Institutes of Health grants BRS4 08-ST805462F-22 and Al 1990, and in part by a grant from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation and a consortium of industries.

Received for publication 16 September 1985, and in revised form 20 October 1986.

Note Added in Proof: An attractive explanation for our finding that K\(^+\)-induced depolarization inhibits calcium ionophore-induced Ca\(^{2+}\) influx in RBL cells is raised by the recent demonstration of Ca\(^{2+}\)-activated nonselective cation channels in neutrophils (36a). These channels are activated when intracellular Ca\(^{2+}\) is increased in response to calcium ionophores or by the release of calcium from intracellular stores in response to chymotryptic peptides. Unlike most other nonspecific cation channels, these channels are highly permeable to Ca\(^{2+}\) and may therefore constitute the physiological pathway for Ca\(^{2+}\) influx. If similar channels exist in RBL cells (and there is recent evidence to suggest that they do in peritoneal mast cells (21x)), they should be activated in response to both antigen and calcium ionophores. Furthermore, since the driving force for Ca\(^{2+}\) entry through these channels will depend on the membrane potential, both antigen and ionophore-induced Ca\(^{2+}\) influx should be inhibited in K\(^+\)-depolarized cells, which is indeed what we observed.

References


cromolyn binding protein constitutes the Ca\(^{2+}\) channel of basophils opening upon immunological stimulus. Agents Actions. 8:338–346.


The Journal of Cell Biology, Volume 104, 1987 792