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.

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.
The Journal of Cell Biology, Volume 104, 1987

The measurement of 

This was performed as described above except that RBL cell monolayers in multiwell plates were cultured overnight with 

Cells in monolayer culture were trypsinized and then suspended in NaK saline. They were loaded with 

This was determined in a similar manner to the calcium efflux experiments using 

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 in 5 mM quin2 acetoxyethyl ester for 60 min. These conditions resulted in optimal uptake of the acetoxyethyl 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 Heesketh et al. (14). At the end of the experiment, 10 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 (K₅) 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

\[
Ca^{++} = \frac{K_5 F}{1 - F}
\]

where \( F = \left( \frac{1 - 0.16 \Delta F}{0.84 \Delta F} \right) \) (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 twice with the same quenching solution. The cells were solubilized in 10% Triton X-100 and an aliquot from each well was counted for 

4Ca Movements at Isotopic Equilibrium

This measurement was performed as described above except that RBL cell monolayers in multiwell plates were cultured overnight with 

Cells in monolayer culture were trypsinized and then suspended in NaK saline. They were loaded with 

Cell Volume

This was determined in a similar manner to the calcium efflux experiments using 

Calcium Determinations

In all experiments involving calcium movements duplicate samples were analyzed.

4Ca 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, 4Ca uptake was initiated by the addition of 

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 

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.

4H]Serotonin Release

Serotonin secretion was measured by release of incorporated 

Reagents

[4H]Serotonin (5-[1,2-3H(N)]-hydroxytryptamine bisinolate), H₂O, and 

4CaCl₂ were purchased from New England Nuclear (Boston, MA). Quin2 acetoxyethyl ester, PMA, and gramicidin were obtained from Sigma Chemical Co. (St. Louis, MO); A23187 and ionomycin from Calbiochem (La Jolla, CA); and bis-(1,3-diethylthiobarbiturate) trimethineoxonol (bis-oxonol) from Molecular Probes (Junction City, OR). Stock solutions of gramicidin, A23187, and ionomycin were prepared in ethanol; bis-oxonol, quin2 acetoxyethyl ester, and PMA were dissolved in dimethylsulfoxide. Cells were exposed to 1% or less of ethanol and 0.1% or less of dimethylsulfoxide. Purified monoclonal mouse IgE directed against diinitrophenyl 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 diinitrophenyl groups per molecule had been coupled (8).

LaCl₃. Duplicate aliquots were taken after quenching to determine [3H]-serotonin secretion was measured by release of incorporated 

10 mM Hepes, pH 7.4). An aliquot of the supernatant was counted for 3H activity of the cells.

Calcium Determinations

In all experiments involving calcium movements duplicate samples were analyzed.

4Ca 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, 4Ca uptake was initiated by the addition of 

\[ Ca^{++} = K_s \frac{F}{1 - F} \] (reference 36),

where \( F = \left( \frac{1 - 0.16 \Delta F}{0.84 \Delta F} \right) \) (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 twice with the same quenching solution. The cells were solubilized in 10% Triton X-100 and an aliquot from each well was counted for 4Ca. Separate wells were used to determine cell number.

4Ca Movements at Isotopic Equilibrium

This measurement was performed as described above except that RBL cell monolayers in multiwell plates were cultured overnight with 4Ca (10 µCi/ml culture medium containing 1.8 mM CaCl₂). 4Ca was maintained at the same specific activity throughout the experiment until the final quenching and washing with ice-cold quenching solution (see above).

4Ca Efflux

Cells in monolayer culture were trypsinized and then suspended in NaK saline. They were loaded with 4Ca (10 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 (dibutyrylphosphate/bis-2-ethylhexylphosphate, 6:4 vol/vol) in 400-µl microfuge tubes. An aliquot of this 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 4Ca associated with the cells was expressed as a fraction of the 4Ca present in the cells 5 min after resuspension in a 4Ca-free saline solution.

Cell Volume

This was determined in a similar manner to the calcium efflux experiments using 

H₂O as a total volume marker. There was no change in the cell volume of nonstimulated or stimulated cells suspended in NaK saline (0.08±0.09 and 0.90±0.10 µl/10⁶ cells, respectively, after 60 min.) In three separate experiments an 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 mM quin2 acetoxyethyl ester for 60 min. These conditions resulted in optimal uptake of the acetoxyethyl 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 Heesketh et al. (14). At the end of the experiment, 10 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 (K₅) 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

\[ Ca^{++} = K_s \frac{F}{1 - F} \] (reference 36),

where \( F = \left( \frac{1 - 0.16 \Delta F}{0.84 \Delta F} \right) \) (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 (10^6 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 KCl concentrations. To normalize the responses, the fluorescence of each KCl concentration was divided by the fluorescence of the same cell suspension in the presence of gramicidin (1 nM), 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, 4Ca, and H2O 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⁺-depolarized 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 4Ca uptake and 4Ca efflux studies designed to study unidirectional calcium movements, changes in total cell calcium measured with cells at isotopic equilibrium with 4Ca, and measurements of cytosolic free ionized calcium using the fluorescent probe quin2.

**4Ca 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 Ca⁺⁺ entry into the cell is removed, and this should be reflected by a decrease in 4Ca uptake. Fig. 2 shows that this was indeed the case with antigen-stimulated cells. Resting cells in both normal

**Figure 1.** Spontaneous and antigen-induced (0.1 μg/ml) release of[^3H]serotonin and β-hexosaminidase from RBL cells suspended in isotonic solutions of different compositions. All solutions contained 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.05% gelatin, and 10 mM Tris-Hepes, pH 7.4. The solutions were made isotonic with the following: 135 mM NaCl and 5 mM KCl (NaK); 140 mM KCl (K); 140 mM NaCl (Na) or 270 mM glucose and 5 mM KCl (Glu).
Figure 2. The initial rate of spontaneous and antigen-induced uptake of $^{45}$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 $^{45}$Ca and antigen (1 ng/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.

Figure 3. Effect of PMA on spontaneous (solid circle, open circle) and 0.25 μM A23187-induced (solid triangle, open triangle) β-hexosaminidase (a) and $^{45}$Ca uptake (b) in RBL cells suspended in NaK saline. The cells were preincubated for 30 min in the absence (solid symbols) or presence (open symbols) of 50 nM PMA. 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 $^{45}$Ca uptake was completely unaffected. This confirms that $^{45}$Ca uptake is not an artifact of exocytosis but is likely to be a measure of true calcium influx into RBL cells.

$^{45}$Ca Efflux is Not Affected by Depolarization

In contrast to the dramatic effects of depolarizing (K) saline on stimulated $^{45}$Ca uptake, $^{45}$Ca efflux from RBL cells was completely unaffected. Spontaneous and antigen-stimulated $^{45}$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., $^{45}$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 $^{45}$Ca to allow intracellular calcium pools to reach isotopic equilibrium. The cells were then bathed in NaK saline or K saline containing $^{45}$Ca at the same specific activity. Net changes in
Figure 4. Spontaneous and antigen-induced \(\beta\)-hexosaminidase release (a) and \({\text{\(^{40}\)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 \(\mu\)g/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 circle) Cells in Na saline; (open circle) cells in NaK saline. (Open square) cells in K saline. (Open triangle) cells in Na saline.

**total cell calcium** were determined from the changes in cell-associated \({\text{\(^{40}\)Ca}}\).

Cells bathed in NaK saline showed a net increase in total cell calcium upon stimulation with antigen (Fig. 5). This peaked at \(\sim \)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 (\(\sim 50\%\)) drop in total cell calcium (Fig. 5). This observation was consistent with our finding (above) that \({\text{\(^{40}\)Ca}}\) uptake was inhibited in K saline while \({\text{\(^{40}\)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 ± 4 nM in NaK saline; 91 ± 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. 5, a and b). 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 \(\beta\)-hexosaminidase or \([\text{\(^{3}\)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 \({\text{\(^{40}\)Ca}}\) uptake (Fig. 7) and \([\text{\(^{3}\)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
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 [\(\text{H}\)]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. 9), we conclude that the NaCl concentration is more critical than the Na\(^{+}\) concentration in determining the secretory response.

**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 [\(\text{H}\)]serotonin (15–20%) has been subtracted.
changes 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 physiologic 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...
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 45Ca uptake and the increase in cytoplasmic free Ca2+ seen in response to antigen-stimulation were both dramatically reduced in K+-depolarized cells (Figs. 2 and 6). 45Ca 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 45Ca uptake is inhibited but 45Ca 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 Ca2+ 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 Ca2+ in depolarized cells is not sufficient for the initiation of secretion. Since stimulated secretion is prevented when the late phase of the Ca2+ signal is inhibited, it seems that 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, 45Ca uptake and [3H]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 45Ca 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 Ca2+ concentration on the initial rate of 45Ca uptake (a) and [3H]serotonin release (b) in response to 0.5 μM A23187. Conditions were the same as those in Figs. 6 and 7 except that 45Ca 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 opened 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\textsuperscript{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 \textsuperscript{4}Ca uptake and the secretion induced by A23187 would be unaltered in K\textsuperscript{+} 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{+} also inhibited antigen-induced secretion and \textsuperscript{4}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\textsuperscript{+}-depolarized cells. Thus, we have shown that secretion from cells in K\textsuperscript{+}-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\textsuperscript{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 Wetland, and Paul Millard for critical reading of this manuscript. This work was supported by National Institutes of Health grants BRSG 08-STCR05462F-22 and AI 19900, 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\textsuperscript{+}-induced depolarization inhibits calcium ionophore-induced Ca\textsuperscript{2+} influx in RBL cells is raised by the recent demonstration of Ca\textsuperscript{2+}-activated nonselective cation channels in neutrophils (36a). These channels are activated when intracellular Ca\textsuperscript{2+} is increased in response to calcium ionophores or by the release of calcium from intracellular stores in response to chemotactic peptides. Unlike most other nonselective cation channels, these channels are highly permeable to Ca\textsuperscript{2+} and may therefore constitute the physiological pathway for Ca\textsuperscript{2+} influx. If similar channels exist in RBL cells (and there is recent evidence to suggest that they do in peritoneal mast cells [21b]), they should be activated in response to both antigen and calcium ionophores. Furthermore, since the driving force for Ca\textsuperscript{2+} entry through these channels will depend on the membrane potential, both antigen and ionophore-induced Ca\textsuperscript{2+} influx should be inhibited in K\textsuperscript{+} depolarized cells, which is indeed what we observed.

References

1. Baird, B. D., S. Sajewski, and S. Madvin. 1983. A microtiter plate assay using cellulose acetate filters for measuring cellular [H]serotonin release. J. Immunol. Method. 64:365-375.
2. Barsumian, E. L., C. Isersky, M. G. Petrino, and R. P. Siraganian. 1981. IgE-induced histamine release from rat basophilic leukemia cell lines—Isolation of releasing and nonreleasing clones. Eur. J. Immunol. 11:317-323.
3. Beaven, M. A., J. P. Moore, G. A. Smith, T. R. Hesketh, and J. C. Metcalfe. 1984. The calcium signal and phosphatidylinositol breakdown in 2H3 cells. J. Biol. Chem. 259:7129-7142.
4. Beaven, M. A., J. Rogers, T. R. Hesketh, G. A. Smith, and J. C. Metcalfe. 1984. The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. J. Biol. Chem. 259:7129-7136.
5. Brog, F. E., G. H. Glaser, K. J. Rozon, W. S. Sly, and F. D. Stahl. 1974. In vitro correction of deficient human fibroblasts by β-glucuronidase from different human sources. Biochem. Biophys. Res. Commun. 57:1-8.
6. Cochrane, D. E., and W. W. Douglas. 1976. Histamine release by exocytosis from rat mast cells on reduction of extracellular sodium: a secretory response inhibited by calcium, strontium, barium, or magnesium. J. Physiol. 257:433-448.
7. Crews, F. T., Y. Morita, A. McGivney, F. Hirata, R. P. Siraganian, and J. Axelrod. 1981. IgE-mediated histamine release in rat basophilic leukemia cells: cell receptor activation, phospholipid methylation, Ca\textsuperscript{2+} flux, and release of arachidonic acid. Arch. Biochem. Biophys. 212:561-571.
8. Eisent, H. N., M. Kern, W. T. Newton, and E. Helmreich. 1959. A study of the distribution of 2,4-dinitrobenzenesensitive betas between isolated lymph node cells and extracellular medium in relation to induction of contact skin sensitivity. J. Exp. Med. 110:187-206.
9. Fewtrell, C., D. Lagouf, and H. Metzger. 1981. Secretion from rat basophilic leukemia cells induced by calcium ionophores. Effect of pH and metacacid inhibition. Biochem. Biophys. Acta. 648:363-368.
10. Fewtrell, C., and H. Metzger. 1981. Stimulus-secretion coupling in rat basophilic leukemia cells. In Biochemistry of the Allergic Reactions.
cromolyn binding protein constitutes the Ca$^{2+}$ channel of basophils opening

The Journal of Cell Biology, Volume 104, 1987 792

smooth muscle calcium antagonists on human basophil histamine release. **Proc. Natl. Acad. Sci. USA.** 1986. Evaluation of calcium entry blockers in several models of immediate hypersensitivity. **J. Pharmacol. Exp. Ther.** 229:690–695.

30. Sagi-Eisenberg, R., and I. Pecht. 1984. Resolution of cellular compartments involved in membrane potential changes accompanying IgE-mediated degranulation of rat basophilic leukemia cells. **EMBO (Eur. Mol. Biol. Organ.) J.** 3:497–500.

31. Sagi-Eisenberg, R., and I. Pecht. 1984. Protein kinase C, a coupling element between stimulus and secretion of basophils. **Immunol. Lett.** 8:237–241.

32. Takahashi, M., and A. Ogura. 1983. Dihydropyridines as potent calcium channel blockers in neuronal cells. **FEBS (Fed. Eur. Biochem. Soc.) Lett.** 152:191–194.

33. Tan, K.-N., and A. H. Tashjian, Jr. 1984. Voltage-dependent calcium channels in pituitary cells in culture. II. Participation in thyrotropin-releasing hormone action on prolactin release. **J. Biol. Chem.** 259:427–434.

34. Taurog, J. D., C. Fewtrell, and E. L. Becker. 1979. IgE-mediated triggering of rat basophil leukemia cells: lack of evidence for serine esterase activation. **J. Immunol.** 122:2150–2153.

35. Taurog, J. D., G. R. Mendoza, W. A. Hook, R. P. Sigranigan, and H. Metzger. 1977. Noncytotoxic IgE-mediated release of histamine and serotonin from murine mastocytoma cells. **J. Immunol.** 119:1757–1761.

36. Tien, R. Y., T. Pozzan, and T. J. Rink. 1982. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. **J. Cell Biol.** 94:325–334.

36a. von Tscharner, V., B. Prodhom, M. Baggio, and H. Reuter. 1986. Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. **Nature (Lond.)** 324:369–372.

37. Wilson, H. A., and T. M. Chused. 1983. Lymphocyte membrane potential and Ca$^{2+}$-sensitive potassium channels described by oxonol dye fluorescence measurements. **J. Cell. Physiol.** 125:72–81.