Quantitative Analysis of the Cytosolic Free Calcium Dependency of Exocytosis from Three Subcellular Compartments in Intact Human Neutrophils

P. Daniel Lew, Antoinette Monod, Francis A. Waldvogel, Beatrice Dewald, Marco Baggiolini, and Tullio Pozzan

Division of Infectious Diseases, University Hospital, 1211 Geneva 4, Switzerland; Theodor-Kocher-Institut, University of Bern, 3000 Bern 9, Switzerland; and Istituto di Patologia Generale, 35131 Padova, Italy

Abstract. Cytosolic free calcium concentration, \([\text{Ca}^{2+}]_i\), and exocytosis of azurophil granules (\(\beta\)-glucuronidase), specific granules (vitamin B12-binding protein), and secretory vesicles (gelatinase) were measured concomitantly in intact human neutrophils under steady state \([\text{Ca}^{2+}]_i\). The cells were loaded with the fluorescent calcium indicator quin2 in the presence or absence of extracellular Ca\(^{2+}\), and steady state \([\text{Ca}^{2+}]_i\) levels ranging from 20 to >2,000 nM were obtained by adding the Ca\(^{2+}\) ionophore ionomycin at various concentrations of extracellular calcium.

The extent of exocytosis from the three granule populations was found to be a function of \([\text{Ca}^{2+}]_i\). The minimal \([\text{Ca}^{2+}]_i\) that caused significant release (threshold \([\text{Ca}^{2+}]_i\)) was ~200–300 nM and was similar for all three compartments. Marked differences, however, were found when the \([\text{Ca}^{2+}]_i\) for half-maximal exocytosis (EC\(_{50}\)) was determined. In the absence of cytochalasin B the EC\(_{50}\) was 1,100 ± 220 nM and 1,600 ± 510 nM for specific granules and secretory vesicles, respectively, and ~6,000 nM for azurophil granules. Cytochalasin B did not affect the threshold \([\text{Ca}^{2+}]_i\), but decreased the EC\(_{50}\) and enhanced the rate of exocytosis. In the presence of cytochalasin B the EC\(_{50}\) was ~600 nM both for secretory vesicles and specific granules, and ~2,600 nM for azurophil granules.

The addition of the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine dramatically changed the \([\text{Ca}^{2+}]_i\) dependency of granule secretion: It decreased the threshold \([\text{Ca}^{2+}]_i\), to <20 and <50 nM, and the EC\(_{50}\) to 50 and 200 nM for specific and azurophil granules, respectively, and it significantly increased the rate of exocytosis. Thus, the additional signal(s) provided by receptor activation markedly lower(s) the Ca\(^{2+}\) requirement of the exocytic process.

Furthermore, these results indicate that the secretion from three different granule populations within the same cell type are differently modulated by \([\text{Ca}^{2+}]_i\).

The Journal of Cell Biology, Volume 102, June 1986 2197-2204

© The Rockefeller University Press, 0021-9525/86/06/2197/08 $1.00
The Journal of Cell Biology, Volume 102, 1986 2198

...dependency of exocytosis in intact human neutrophils. The cells were loaded with the Ca²⁺ chelator and indicator quin2, steady state [Ca²⁺]ᵢ's were established with ionomycin and appropriate concentrations of extracellular Ca²⁺, and exocytosis from three distinct subcellular storage organelles was assessed. The results show that the specific granules and the secretory vesicles containing gelatine have different [Ca²⁺], requirements for exocytosis than do the azurophil granules. In addition, receptor stimulation substantially decreases the [Ca²⁺], dependency for inducing exocytosis, but the difference between specific and azurophil granules is retained.

Materials and Methods

Special reagents were obtained from the indicated sources: N-formyl-methionyl-leucyl-phenylalanine (FMLP), cytochalasin B, 4-methylumbelliferyl-β-D-glucoronide (Sigma Chemical Co., St. Louis, MO); quin2 acetoxymethyl tetraester (quin2/AM), cyano[STCo]cobalamin, 10-20 µCi/µg (The Radiochemical Centre, Amersham, England); dextran T500 and Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden); diisopropyl-phosphofluoridate (Fluka, Buchs, Switzerland); [3H]lactate anhydride, 50 mCi/mmol (New England Nuclear, Dreieich, Federal Republic of Germany). Ionomycin was a kind gift of C. M. Liu of Hoffmann-La Roche, Nutley, NJ.

Preparation of Human Neutrophils

Neutrophils were prepared from fresh blood samples (usually 90 ml) obtained from healthy volunteers. The cells were purified by dextran sedimentation and subsequent centrifugation through a layer of Ficoll-Paque as described previously (9, 16). The granulocyte pellet was washed in saline buffer and contaminating erythrocytes were eliminated by hypotonic shock. The preparation obtained contained 95% neutrophils. The cells were resuspended in a modified Kreb's-Ringer bicarbonate medium containing 138 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 1 mM NaHPO₄, 5 mM NaHCO₃, 5.5 mM glucose, 20 mM Hepes, pH 7.4, and kept in this medium at room temperature until use.

Measurement of [Ca²⁺]

Quin2 loading was performed as described previously (16). Suspensions of 5 × 10⁷ cells/ml were equilibrated at 37°C for 5 min. Quin2/AM was added to a final concentration of 10 to 40 µM from a 10 µM stock solution in dimethylsulfoxide. Control cells were treated with dimethylsulfoxide (<0.5%) only. In most cases the loading with quin2/AM was performed in the Kreb's-Ringer bicarbonate buffer medium described above. In a few cases (see test) the loading was performed in Kreb's-Ringer bicarbonate buffer that contained 1 mM EGTA and no CaCl₂ to obtain [Ca²⁺]₀ below resting level (10). After 10 min at 37°C the cells were diluted to 1 × 10⁷ cells/ml with warm medium containing 0.5% bovine serum albumin and incubated for another 50 min at 37°C. Loaded cells were kept at room temperature. Before use, an aliquot of the suspension was centrifuged and the cells were resuspended in the medium indicated. Most fluorescence measurements were performed with a Perkin-Elmer LS-3 fluorimeter (Perkin-Elmer Corp., Norwalk, CT). For the experiment shown in Fig. 7 a Fluorolog 2 model F2C from Spex International, Edison, NJ, which allows higher resolution of fast reactions, was used. The fluorimeter cuvette holder was thermostatted at 37°C and equipped with a magnetic stirring device. Excitation and emission wavelengths were 339 ± 5 and 492 ± 10 nm, respectively. To minimize light scattering artifacts, two cut-off filters, UV D25 and UV 35 for excitation and emission, respectively, were used. Quin2 fluorescence in terms of [Ca²⁺], was calibrated as described previously (19).

Experimental Protocol

For the analysis of exocytosis induced by ionomycin at stable steady state [Ca²⁺]ᵢ levels (Figs. 1, 2, 4 and Table I) the neutrophils were loaded with moderately high concentrations of quin2 (which functions as a cytosolic Ca²⁺ buffer as well as an indicator (16)). Loading was performed with 20-40 µM quin2/AM and 5 × 10⁶ cells/ml, yielding intracellular quin2 concentrations ([quin2]) of 0.8-1.0 nmol/10⁶ cells. The [quin2]ᵢ is critical for experiments of this type. At low [quin2] the release of Ca²⁺ from intracellular stores causes a marked transient [Ca²⁺]ᵢ rise, which is followed by a lower steady state [Ca²⁺].

On the other hand, high [quin2] dampens the calcium responses, and steady state [Ca²⁺]ᵢ levels are reached only very slowly. After loading, aliquots of the suspension (usually 10⁷ cells) were centrifuged, and the cells were resuspended in 3.2 ml of Ca²⁺-free buffer medium at 37°C. The extracellular Ca²⁺ concentration, [Ca²⁺]₀, was then adjusted, and where indicated cytochalasin B (5 µg/ml) was included. After 3 min ionomycin (500 nM) was added. 150-250-µl aliquots were withdrawn from the fluorimeter cuvette at various times for the assessment of exocytosis. Controls showed that removal of up to 750 µl did not affect the fluorescence measurements. In principle it should also be possible to establish different steady state levels of [Ca²⁺], by varying the concentration of ionomycin at a fixed extracellular Ca²⁺ concentration (10). In our experiments this approach was less reproducible, particularly at low ionophore concentrations. We have also found that it is important to measure [Ca²⁺], and granule content release in the same reaction mixture rather than in parallel incubations (i.e., [Ca²⁺], in the cuvette and exocytosis in the test tube). For the study of the Ca²⁺ dependency of FMLP-induced exocytosis (Figs. 5, 6, and 7), the cells were loaded with 10-20 µM quin2/AM, resulting in [quin2] of 0.1-0.3 nmol/10⁶ cells.

![Figure 1. Establishment of steady state cytosolic free calcium, [Ca²⁺], with ionomycin. 10⁷ neutrophils loaded with quin2 (0.8 nmol quin2/10⁶ cells) were suspended in 3.2 ml buffer with increasing concentrations of CaCl₂ as indicated. Ionomycin (Iono; 500 nM) was added as indicated. Fluorescence tracings are shown at the bottom. The relationship between the steady state [Ca²⁺], obtained and the calcium concentration of the extracellular medium, [Ca²⁺]₀, is shown on top. In neutrophils pretreated with cytochalasin B virtually identical results were obtained.](image-url)
Determination of Marker Release

Samples withdrawn from the cuvette were cooled on ice and centrifuged at 800 g for 10 min. In the experiments shown in Fig. 7 special care was taken to cool rapidly the samples to ensure instant termination of the reaction. Samples withdrawn from the fluorimeter cuvette were immediately mixed with an equal volume of ice-cold buffer and further cooled in ice before centrifugation. β-Glucuronidase, vitamin B₁₂-binding protein, gelatinase, and lactate dehydrogenase were then assayed in the supernatants. Release was calculated as percent of the initial cellular content. Background release by unstimulated controls was determined for each cell batch and subtracted from the values obtained with stimulated cells.

Statistical Analysis

Values are expressed as mean ± SD. Statistical analysis was performed by the Student's t-test (two-tail probability).

Results

As shown in Fig. 1, ionomycin induced a rapid rise in [Ca⁺⁺]ᵢ up to a plateau which depended on [Ca⁺⁺]₀. The plateau remained constant for ~10 min, indicating that steady state levels ranging from 120 nM to >2,000 nM can be established by the experimental protocol adopted. The upper graph shows the existence of a logarithmic relationship between [Ca⁺⁺]ᵢ and [Ca⁺⁺]₀. Due to the poor indicator sensitivity of quin2 at [Ca⁺⁺]₀ above 2 μM, calibration at higher values was not attempted.

Exocytosis as a Function of Steady State [Ca⁺⁺]

Fig. 2 shows that release of the specific granule marker, vitamin B₁₂-binding protein, increased with increasing [Ca⁺⁺]ᵢ levels. The release was much faster and more pronounced in cells that had been pretreated with cytochalasin B. Under the latter conditions the response was almost complete after 5–6 min at both [Ca⁺⁺]ᵢ levels. Without cytochalasin B treatment it continued beyond the 10 min duration of the experiment. The experiment shown in Fig. 3 illustrates an interesting aspect of the action of cytochalasin B. For all three markers, β-glucuronidase (azurophil granules), vitamin B₁₂-binding protein (specific granules), and gelatinase (secretory vesicles), the release was much larger in cytochalasin B-treated cells at extracellular calcium levels up to 1–2 mM. At higher [Ca⁺⁺]₀, however, exocytosis reached a similar maximum level both in cytochalasin B-treated and nontreated neutrophils. Thus, provided the extracellular calcium concentration is high enough, maximum release is reached independently of cytochalasin B. In these experiments [Ca⁺⁺]₀ was not determined because the levels were above the limits of accurate measurement with quin2. The release obtained at high [Ca⁺⁺]₀ was not due to nonspecific leakage from damaged cells since even at 5 mM [Ca⁺⁺]₀ liberation of the cytoplasmic marker lactate dehydrogenase was only slightly enhanced (from 5 to 9% of the total cellular activity). In Fig. 4 the relationship between [Ca⁺⁺]₀ and the extent of exocytosis is shown. Minimal release was observed when [Ca⁺⁺]₀ was <200–250 mM. Above this level release occurred, albeit to different extents, from all three storage compartments. Threshold [Ca⁺⁺]₀, defined as the intercept of the extrapolated slope of the [Ca⁺⁺]₀ dependency curve with the abscissa, were determined from three to six experiments of this type. These results are presented in Table I. For exocytosis from the specific

Figure 3. Effect of increasing concentrations of extracellular calcium on exocytosis. Neutrophils suspended at various [Ca⁺⁺]₀ were incubated for 3 min in the presence or absence of cytochalasin B (cyto B; 5 μg/ml) and then treated with 500 nM ionomycin. After 5 min (cells with cytochalasin B) or 6 min (cells without cytochalasin B) aliquots were withdrawn, and the release of β-glucuronidase (azurophil granules, top), vitamin B₁₂-binding protein (specific granules, middle), and gelatinase (secretory vesicles, bottom) was determined.

Figure 2. Time course of release of vitamin B₁₂-binding protein at two [Ca⁺⁺]₀ levels. Steady state [Ca⁺⁺]₀ of 500 and 1,000 nM were established as shown in Fig. 1. Left, + cytochalasin B. Right, – cytochalasin B. 250-μl samples were withdrawn from the fluorimeter cuvette at various times after addition of ionomycin, and the amount of vitamin B₁₂-binding protein released was determined in the cell-free medium. Where indicated the cells were pretreated with cytochalasin B (5 μg/ml) for 5 min. Release is expressed as percent of the initial cellular content as described in Materials and Methods.
Fig. 4. Exocytosis as a function of steady state [Ca²⁺]i. Experimental conditions as in Fig. 1. Where indicated the cells were pretreated with cytochalasin B (cyto B; 5 μg/ml) for 5 min. Samples for the determination of marker release were withdrawn from the fluorimeter cuvette 5 min (with cytochalasin B) or 6 min (without cytochalasin B) after addition of ionomycin (500 nM). The broken lines on top of the graphs indicate the level of release obtained under identical conditions at 5 mM extracellular calcium (maximal release as shown in Fig. 3).

Table I. [Ca²⁺]i Threshold and EC₅₀ for Exocytosis from Azurophil Granules, Specific Granules, and Secretory Vesicles

| Subcellular marker | Cytochalasin B | [Ca²⁺]i (nM) Threshold | EC₅₀ (nM) |
|--------------------|----------------|------------------------|-----------|
| β-Glucuronidase     | −              | ND                     | 6,000 ± 1,500* (5) |
| +                  | 280 ± 40 (6)   | 2,600 ± 480* (4)       |
| Vitamin B12-binding protein | −              | 220 ± 60 (5)          | 1,100 ± 220 (5)   |
| +                  | 240 ± 60 (4)   | 610 ± 170 (4)         |
| Gelatinase          | −              | 190 ± 35 (3)          | 1,600 ± 510 (3)   |
| +                  | 235 ± 30 (4)   | 650 ± 130 (4)         |

The experimental conditions were the same as in Fig. 4. Values are means ± SD, number of experiments in brackets. ND, not determined.
* Values estimated by extrapolation on the basis of the relationship between [Ca²⁺]i and [Ca²⁺] as shown in Fig. 1.

granules and the secretory vesicles similar threshold [Ca²⁺]i values were obtained that ranged between 190 and 240 nM, independent of cytochalasin B pretreatment of the cells. For azurophil granule exocytosis, the threshold concentration in the absence of cytochalasin B could not be determined because the amounts released at [Ca²⁺]i below 2,000 nM were too low for accurate calculation. In the presence of cytochalasin B, the threshold was 280 nM, a value slightly higher but not statistically different from that obtained for the specific granules and the secretory vesicles. In view of the well-established fact that cytochalasin B enhances the rate and extent of exocytosis (2), it is interesting to note that pretreatment with cytochalasin B did not appreciably affect the [Ca²⁺]i threshold levels. From the same experiments, the [Ca²⁺]i that gave half-maximal release of the three markers (EC₅₀) was calculated (Fig. 4 and Table I). In neutrophils pretreated with cytochalasin B, these values were 610 and 650 nM for vitamin B12-binding protein and gelatinase, respectively (no statistical difference). The EC₅₀ for β-glucuronidase release was 2,600 nM, which is much higher than the EC₅₀ for release from specific granules and secretory vesicles (P < 0.001). Threshold [Ca²⁺]i and EC₅₀ for exocytosis from specific granules and secretory vesicles were similar. The rate of release, however, was higher for the gelatinase-containing organelles (not shown). In addition, a nearly complete release of gelatinase was observed (Fig. 4), whereas maximum release of vitamin B12-binding protein did not exceed 50% of the total cellular content. In neutrophils not pretreated with cytochalasin B, the rate of exocytosis, as pointed out earlier, was much lower. Maximal release was reached after 20–30 min only, making an accurate calculation of the EC₅₀ values difficult. In fact, [Ca²⁺]i tended to decrease during incubations exceeding 10 min possibly due to a redistribution of the ionophore within the cells. We noted, however, that in the presence of cytochalasin B the EC₅₀ was about the same whether it was determined during progression or after completion of release (not shown). Thus, in the absence of cytochalasin B, we used the extent of marker release at 6 min after ionomycin addition to calculate the EC₅₀. As shown in Table I the EC₅₀ values in the absence of cytochalasin B were much higher than in its presence. For the specific granules and the secretory vesicles values between 1,100 and 1,600 nM were found (no statistical difference). The release of β-glucuronidase in untreated cells is very low. However, as already indicated in Fig. 3, progressive release was observed when the extracellular calcium concentration was raised above 2 mM. An EC₅₀ of ~6,000 nM could be estimated on the basis of the data shown in Fig.
Figure 5. Experimental protocol for studying the [Ca$^{2+}$]$_i$ dependency of exocytosis induced by fMLP. (A) 10$^7$ cells were loaded with a low concentration of quin2 in calcium-containing medium (0.12 nmol/quin2 per 10$^6$ cells) and then resuspended in calcium-free medium. EGTA (1 mM) and ionomycin (Iono; 500 nM) were added where indicated. Cytochalasin B (5 μg/ml) was added 3 min before ionomycin. The arrows on top of the graph designate the time points corresponding to different [Ca$^{2+}$]$_i$ levels at which 1 μM fMLP was added to parallel samples (i.e., each sample received fMLP at a different time point). In cells not pretreated with ionomycin, fMLP increased [Ca$^{2+}$]$_i$ to approximately the same level as the ionophore, whereas fMLP added after ionomycin did not modify the [Ca$^{2+}$]$_i$ transient (16, 19). (B) Conditions as in A except that higher [quin2]$_i$ was used (0.6-1 nmol quin2/10$^6$ cells) and both loading and the subsequent incubation were performed in calcium-free medium containing 1 mM EGTA as described (10).

3, and the relationship between the extracellular and intracellular calcium concentration shown in Fig. 1.

Since no significant differences were observed in the calcium dependency of exocytosis from specific granules and secretory vesicles, in further studies described below, only release from the azurophil and specific granules was compared.

Effect of Receptor Activation on the [Ca$^{2+}$]$_i$ Dependency of Exocytosis

It is now generally accepted that Ca$^{2+}$-mobilizing stimuli generate in addition to a [Ca$^{2+}$]$_i$ rise other intracellular messengers, in particular diacylglycerol, an activator of the phospholipid and Ca$^{2+}$-dependent protein kinase C (3). Diacylglycerol and the tumor-promoting phorbol esters are believed to increase the Ca$^{2+}$ affinity of this enzyme. In turn, protein kinase C-dependent phosphorylation(s) is (are) thought to increase the Ca$^{2+}$ sensitivity of other Ca$^{2+}$-dependent processes (13, 18). Recent evidence suggests that in neutrophils a number of stimuli act via this bifurcating signal transduction mechanism. The most widely used among these stimuli is fMLP.

Fig. 5 shows the protocol that was used in an attempt to estimate the [Ca$^{2+}$]$_i$ threshold and the EC$_{50}$ of fMLP-induced exocytosis. The protocol described in Fig. 1 for ionomycin was not practical for the following two reasons: The [Ca$^{2+}$]$_i$ rise induced by fMLP is transient and therefore no steady state [Ca$^{2+}$]$_i$ can be obtained. The interpretation of an effect of fMLP at steady state [Ca$^{2+}$]$_i$ levels established with ionomycin in the presence of extracellular Ca$^{2+}$ appeared impossible in view of the stimulatory effect of the ionophore alone. We thus applied ionomycin in Ca$^{2+}$-free medium. Under these conditions, the ionophore does not appreciably stimulate exocytosis (19) but releases Ca$^{2+}$ from internal stores, leading to a transient elevation of [Ca$^{2+}$]$_i$. We then added fMLP at different times after ionomycin and determined the extent of release of granule markers. fMLP added after the ionophore does not modify [Ca$^{2+}$]$_i$, but is a potent stimulator of exocytosis (16, 19). The protocol of this experiment is illustrated in Fig. 5A, and curves relating fMLP-dependent exocytosis to [Ca$^{2+}$]$_i$ are shown in Fig. 6. After stimulation with the chemotactic peptide, exocytosis is very rapid and reaches completion within 30 s. It appears therefore justified to calculate [Ca$^{2+}$]$_i$ thresholds and EC$_{50}$ values on the basis of the [Ca$^{2+}$]$_i$ levels determined at the time of fMLP addition. Note that
high [Ca$^{2+}$], elevations were very transient (Fig. 5A), and consequently the exocytosis responses at these levels were less reproducible than at lower [Ca$^{2+}$]. [Ca$^{2+}$]'s below resting level were obtained by loading the cells with quin2 in Ca$^{2+}$-free medium (10). Under these conditions the Ca$^{2+}$ stores are depleted, and basal [Ca$^{2+}$] is decreased 3- to 10-fold below the normal resting level, depending on the quin2 concentration (Fig. 5B). Fig. 6 shows the results of experiments performed according to the protocols described in Fig. 5, A and B. In the presence of cytochalasin B the threshold [Ca$^{2+}$], for fMLP-induced release of vitamin B12-binding protein was found to be $<$20 nM ($n = 12$) and for $\beta$-glucuronidase $<$50 nM ($n = 5$). These values are about one order of magnitude lower than those obtained with ionomycin alone. An $EC_{50}$ of 200 ± 60 nM ($n = 3$) was calculated for azurophil and of 60 ± 20 nM ($n = 3$) ($P < 0.001$) for specific granule exocytosis. Due to the transient nature of the [Ca$^{2+}$]$_{i}$ elevation under these conditions reliable values for the threshold and $EC_{50}$ in the absence of cytochalasin B could not be determined.

It is well established that in neutrophils exocytosis in response to receptor stimulation is much faster than that induced by Ca$^{2+}$ ionophores (2). A quantitative comparison of the time course of [Ca$^{2+}$]$_{i}$ elevation and exocytosis in cytochalasin B-treated neutrophils upon stimulation with fMLP or ionomycin is shown in Fig. 7. In these experiments the cells were loaded with a low quin2 concentration in order to reach rapid maximal [Ca$^{2+}$] levels. Although the rise in [Ca$^{2+}$] was similarly fast with fMLP and ionomycin, the rate of exocytosis differed considerably. After stimulation with fMLP the release from specific and azurophil granules had already been completed in ~30 s. By contrast, exocytosis induced by ionomycin started with a lag of ~15 s and proceeded at a much lower rate for $>$1 min. This occurred despite the fact that similar elevations of [Ca$^{2+}$] (700 nM for fMLP at [Ca$^{2+}$]$_{o} = 1.0$ mM, 800 nM for ionomycin at [Ca$^{2+}$]$_{o} = 0.4$ mM) were observed. The lag phase after ionophore treatment could not be shortened by increasing [Ca$^{2+}$] ($>2 \mu M$ for ionomycin at [Ca$^{2+}$]$_{o} = 1$ mM). In this experiment the rate of release of vitamin B12-binding protein obtained with ionomycin was clearly higher than in the experiment shown in Fig. 2. This apparent discrepancy is due to the difference in [quin2], (0.24 nmol/10$^6$ cells in this experiment vs 0.8 nmol/10$^6$ cells in Fig. 2). It has been shown previously that increasing [quin2] increases the time needed to reach the maximal [Ca$^{2+}$] (see also Fig. 1) and decreases the rate of marker release (16).

**Discussion**

The present work aimed at a quantitative assessment of the [Ca$^{2+}$], requirement for exocytosis in intact neutrophils. In other experimental systems this problem has been studied by using cells permeabilized with digitonin, ATP, or high voltage clamp in the presence of Ca$^{2+}$ buffers (1, 12, 17). Alternatively, clamping of the [Ca$^{2+}$] has been achieved using high concentrations of Ca$^{2+}$ ionophores (15). Permeabilized cells have one important advantage. They permit control not only of [Ca$^{2+}$], but also of other cytosolic components, and studies of the effects of compounds such as drugs and certain peptides that normally do not cross the plasma membrane can be performed. On the other hand, permeabilization has some drawbacks: (a) While leading to the entry of Ca$^{2+}$, it also causes the loss of small solutes (e.g., ions, peptides, cyclic nucleotides, and other products of receptor activation) that might be normally involved in the exocytosis process. (b) It markedly alters the transmembrane potential. In addition, receptor function and receptor-activated reactions may be disturbed by the relatively drastic manipulations needed for permeabilizing the plasma membrane. The “ionophore clamp” approach overcomes some of these problems since it manipulates [Ca$^{2+}$], more selectively. However, several factors, i.e., Ca$^{2+}$ release from intracellular stores, Ca$^{2+}$ buffering, and the effect of pH gradients across the membrane must be

![Figure 7. Comparison of the time course of the [Ca$^{2+}$], changes and exocytosis in response to stimulation with fMLP or ionomycin.](image)
taken into consideration, and reliable information can be obtained only if \([\text{Ca}^{2+}]_\text{i}\) is monitored. It must be noted that ionophore clamping assumes that the ionophore can completely overcome the \(\text{Ca}^{2+}\) buffering mechanisms of the cell, so that \([\text{Ca}^{2+}]_\text{o} = 2\Delta \text{pH}[\text{Ca}^{2+}]_\text{i}\). We observed that with 0.5–1.0 \(\mu\text{M}\) ionomycin \([\text{Ca}^{2+}]_\text{i}\) was still \(\sim 1000\)-fold higher than \([\text{Ca}^{2+}]_\text{i}\), and the equilibrium \([\text{Ca}^{2+}]_\text{i}\) = 2 \(\Delta \text{pH}[\text{Ca}^{2+}]_\text{i}\) was not reached even at 10 \(\mu\text{M}\) ionomycin. A similar discrepancy between the assumed and measured value for \([\text{Ca}^{2+}]_\text{i}\) has recently been shown in adrenal glomerulosa cells (5, 13).

The approach used in this study is related to the "ionophore clamp" method. The high affinity \(\text{Ca}^{2+}\) chelator quin2 served two purposes, i.e., to allow manipulation of \([\text{Ca}^{2+}]_\text{i}\) and monitoring of these levels. Human neutrophils were selected as the model cell because they contain three biochemically well-defined storage compartments with different reactivity to exocytotic stimuli (9, 25, 26). We found that the \([\text{Ca}^{2+}]_\text{i}\) requirement for half-maximal exocytosis (EC50) of specific granules and secretory vesicles was significantly lower than that for azurophil granules. In the presence of ionomycin similar threshold values were obtained for the three granule populations. Values in a comparable range were reported by Baker et al. (1) and Knight et al. (14) for exocytosis by platelets and adrenal medulla cells permeabilized by high voltage discharge, whereas higher values were observed for human neutrophils permeabilized with saponin (23). It is thus reasonable to conclude that high voltage discharge is less disturbing to the membrane organization than is detergent treatment. The finding that receptor stimulation decreases the \([\text{Ca}^{2+}]_\text{i}\) requirement for exocytosis is not new, as shown by observations in different experimental systems (1, 10, 14). To our knowledge, however, this is the first quantitative analysis performed in intact cells. We observed that receptor stimulation caused an \(\sim 10\)-fold decrease of the \([\text{Ca}^{2+}]_\text{i}\) threshold and EC50. Again, the \([\text{Ca}^{2+}]_\text{i}\) requirement for exocytosis was lower for specific granules than for azurophils. In cytochalasin B–treated cells, marked exocytosis of specific granules was obtained already at resting \([\text{Ca}^{2+}]_\text{i}\) levels. In addition, receptor stimulation increased the rate of exocytosis. A problem that we have not addressed in this study is whether \(\text{Ca}^{2+}\) is the direct trigger of exocytosis or whether its effects are indirect and dependent on processes related to receptor stimulation. It must be emphasized that even the \([\text{Ca}^{2+}]_\text{i}\) dependency assessed in the presence of ionomycin reflects the overall requirement of \([\text{Ca}^{2+}]_\text{i}\) for exocytosis and does not necessarily indicate a direct stimulatory action of \(\text{Ca}^{2+}\) on the effector system. In fact, a rise in \([\text{Ca}^{2+}]_\text{i}\) could induce exocytosis also by producing other intracellular messengers. For instance, elevated \([\text{Ca}^{2+}]_\text{i}\) could activate phospholipase A2 with consequent liberation of arachidonic acid (22). Some of the lipoxygenase products, in particular leukotriene B4, could then act via specific receptors. In addition, experiments with rabbit neutrophils may be taken to suggest that a rise in \([\text{Ca}^{2+}]_\text{i}\) could cause polyphosphoinositide breakdown (7), thus mimicking the effect of receptor stimulation. Consistent with the possibility that the effect of \([\text{Ca}^{2+}]_\text{i}\) on secretion is indirect are the following observations made in our neutrophil system: The time course and extent of the \([\text{Ca}^{2+}]_\text{i}\) rise after ionomycin and fMLP were found to be very similar, yet exocytosis induced by ionomycin occurred after a lag phase, at a time when the fMLP-induced exocytosis was almost completed (Fig. 7). In the absence of extracellular calcium only minor amounts of granule contents were released by ionomycin despite a large transient rise in \([\text{Ca}^{2+}]_\text{i}\) (Fig. 5). These findings could be explained if the ionomycin-induced \([\text{Ca}^{2+}]_\text{i}\) rises caused exocytosis by triggering the formation of one or more additional second messengers, which in turn would take time to accumulate to the stimulatory level. In this case, the rise in \([\text{Ca}^{2+}]_\text{i}\) could either serve as an amplification pathway or act as a synergistic stimulus (e.g., for the activation of protein kinase C).

On the other hand the similarity of the \([\text{Ca}^{2+}]_\text{i}\) dependency of granule exocytosis with the in vitro \(\text{Ca}^{2+}\) activation curve of calcium-activated proteins such as calmodulin and gelsolin (6, 24) suggests a possible direct control of some steps of the secretory process by \([\text{Ca}^{2+}]_\text{i}\). In this respect the present data offer some clues to the mechanism of the stimulatory action of cytochalasin B and thus to the involvement of cytoskeletal proteins in exocytosis. Cytochalasin B has been shown to interact directly with actin filaments (8), and rupture of the cytoskeleton network has been assumed to be necessary for easier access of granules to the plasma membrane (24). It has been proposed that actin-binding proteins such as gelsolin, villin, and fragmin control exocytosis in a similar manner, i.e., by binding to actin filaments and splitting them into shorter filaments upon rises in \([\text{Ca}^{2+}]_\text{i}\). The finding that cytochalasin B lowers the calcium requirement for induction of the exocytotic process is consistent with the hypothesis that cytochalasin B could replace some of these physiological actin regulatory proteins.

Various examples exist of cells with different granule populations that can release their contents independently. Selective secretion of serotonin and of N-acetyl-\(\beta\)-glucosaminidase has been reported in platelets (14). Similarly, pituitary cells can store different hormones in different secretory vesicles (11). For human neutrophils previous reports have shown that some stimuli can elicit selective exocytosis of specific granules and secretory vesicles (9, 26). Corresponding observations have also been made in vivo during inflammation (25). Our data suggest that a different \([\text{Ca}^{2+}]_\text{i}\) sensitivity of the various different granule populations could, at least in part, explain selective secretion. Obviously it remains to be established whether such a model can be extended to other cellular systems.

This investigation was supported by grants 3.990.0.84 and 3.353.0.82 from the Swiss National Science Foundation and by a grant from the Italian Ministry of Public Education (60%) to T. Pozzan. P. D. Lew is the recipient of a Max Cloetta career development award.

Received for publication 12 November 1985, and in revised form 23 December 1985.

References

1. Baker, P. F., D. E. Knight, and J. A. Umbach. 1985. Calcium clamp of the intracellular environment. Cell Calcium. 6:5–14.

2. Bennett, J. P., S. Cokcroft, and B. D. Gomperz. 1980. Use of cytochalasin B to distinguish between early and late events in neutrophil activation. Biochim. Biophys. Acta. 601:584–591.

3. Berriode, M. J., and R. F. Irvine. 1984. Inositol triphosphate, a novel second messenger in cellular signal transduction. Nature (Lond.). 312:315–321.

4. Campbell, A. K. 1983. Intracellular Calcium. Its Universal Role as Regulator. John Wiley & Sons Ltd., New York. 554 pp.

5. Capponi, A. M., P. D. Lew, L. Jornot, and M. B. Vallotton. 1984. Correlation between cytosolic free calcium and aldosterone production in bovine adrenal glomerulosa cells. J. Biol. Chem. 259:8865–8869.
6. Cheung, W. Y. 1980. Calmodulin plays a pivotal role in cellular activation. Science (Wash. DC). 207:19-25.
7. Cokcroft, S., J. P. Bennett, and B. D. Gomperts. 1981. The dependence on Ca^{2+} of phosphatidylinositol breakdown and enzyme secretion in rabbit neutrophils stimulated by formylmethionyl-leucylphenylalanine or ionomycin. Biochem. J. 200:501–508.
8. Cribbs, D. H., J. R. Glenney, and P. Kaulfus. 1982. Interaction of cytochalasin B with actin filaments nucleated or fragmented by villin. J. Biol. Chem. 257:395–399.
9. Dewald, B., U. Bretz, and M. Baggiolini. 1982. Release of gelatinase from a novel secretory compartment of human neutrophils. J. Cell Biol. 97:518–525.
10. Di Virgilio, F., P. D. Low, and T. Pozzan. 1984. Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca^{2+} levels. Nature (Lond.). 310:691–693.
11. Fumagalli, G., and A. Zanini. 1985. In cow anterior pituitary growth hormone and prolactin can be packed in separate granules of the same cell. J. Cell Biol. 100:2019–2024.
12. Gomperts, B. D. 1983. Involvement of guanine nucleotide binding in the gating of Ca^{2+} by receptors. Nature (Lond.). 306:64–66.
13. Kojima, I., H. Lippes, K. Kojima, and H. Rasmussen. 1983. Aldosterone secretion: effect of phorbol ester and A23187. Biochem. Biophys. Res. Commun. 16:555–562.
14. Knight, D. E., T. J. Hallam, and M. C. Scrutton. 1982. Agonist selectivity and second messenger concentration in calcium mediated secretion. Nature (Lond.). 296:256–257.
15. Lew, V. L., and J. Garcia-Sancho. 1985. Use of the ionophore A23187 to measure and to control cytoplasmic Ca^{2+} levels in intact red cells. Cell Calcium 6:15–24.
16. Lew, P. D., C. B. Wollheim, F. A. Waldvogel, and T. Pozzan. 1984. Modulation of cytosolic-free calcium transients by changes in intracellular calcium-buffering capacity: correlation with exocytosis and O_2 production in human neutrophils. J. Cell Biol. 99:1212–1220.
17. Nilson, S. P., and N. Kirchner. 1983. Calcium evoked secretion from digitonin permeabilized adrenal medullary chromaffin cells. J. Biol. Chem. 258:4994–5000.
18. Nishizuka, Y. 1984. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature (Lond.). 308:693–698.
19. Pozzan, T., P. D. Lew, C. B. Wollheim, and R. Y. Tsien. 1983. Is cytosolic ionized calcium regulating neutrophil activation? Science (Wash. DC). 221:1413–1415.
20. Rink, T. J., S. W. Smith, and R. Y. Tsien. 1982. Cytosplasmic free Ca^{2+} in human platelets: Ca^{2+} thresholds and Ca^{2+}-independent activation for shape-change and secretion. FEBS (Fed. Eur. Biochem. Soc.) Lett. 148:21–26.
21. Romeo, D., G. Zabucchi, N. Miani, and F. Rossa. 1975. Ion movement across leukocyte plasma membrane and excitation of their metabolism. Nature (Lond.). 235:542–545.
22. Samuelsson, B. 1983. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. Science (Wash. DC). 220:568–575.
23. Smolen, J. E., and S. J. Stoehr. 1985. Micromolar concentrations of free calcium provoke secretion of lysozyme from human neutrophils permeabilized with saponin. J. Immunol. 134:1859–1863.
24. Southwick, F. S., and T. P. Stossel. 1983. Contractile proteins in leukocyte function. Semin. Haematol. 20:305–321.
25. Wright, D. G., and J. I. Gallin. 1979. Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. J. Immunol. 123:285–294.
26. Wright, D. G., D. A. Bralove, and J. I. Gallin. 1977. The differential mobilization of human neutrophil granules. Am. J. Pathol. 87:273–281.