

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

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**Abstract.** Cytosolic free calcium concentration,  $[Ca^{2+}]_i$ , 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  $[Ca^{2+}]_i$ . The cells were loaded with the fluorescent calcium indicator quin2 in the presence or absence of extracellular  $Ca^{2+}$ , and steady state  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$ . The minimal  $[Ca^{2+}]_i$  that caused significant release (threshold  $[Ca^{2+}]_i$ ) was  $\sim 200$ – $300$  nM and was similar for all three compartments. Marked differences, however, were found when the  $[Ca^{2+}]_i$  for half-maximal exocytosis ( $EC_{50}$ ) was determined. In the absence of cytochalasin B the  $EC_{50}$  was  $1,100 \pm 220$  nM and  $1,600 \pm 510$  nM for specific granules and secretory vesicles,

respectively, and  $\sim 6,000$  nM for azurophil granules. Cytochalasin B did not affect the threshold  $[Ca^{2+}]_i$  but decreased the  $EC_{50}$  and enhanced the rate of exocytosis. In the presence of cytochalasin B the  $EC_{50}$  was  $\sim 600$  nM both for secretory vesicles and specific granules, and  $\sim 2,600$  nM for azurophil granules.

The addition of the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine dramatically changed the  $[Ca^{2+}]_i$  dependency of granule secretion: It decreased the threshold  $[Ca^{2+}]_i$  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 exocytotic process.

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

**T**HE term exocytosis is commonly used to indicate the process by which hydrophilic cellular products (e.g., proteins, peptides, and neurotransmitters) segregated within intracellular vesicles are discharged into the extracellular fluid. This process is triggered by stimulation of the cell and consists of several discrete events including the movement of the storage organelles to the plasma membrane, the fusion between the two membranes, and the liberation of the stored contents.

More than 20 years ago, it was discovered that  $Ca^{2+}$  plays a pivotal role in regulated exocytosis (4). In particular it has been demonstrated that exocytosis often depends on the presence of  $Ca^{2+}$  in the extracellular medium and that it can be induced by ionophores that selectively transport  $Ca^{2+}$  across natural and artificial membranes (15, 21). Additional support for the involvement of  $Ca^{2+}$  has been provided by the discovery of the inhibitory action of  $Ca^{2+}$  antagonists and the role

of  $Ca^{2+}$ -regulated proteins (6, 21, 24). In recent years two technical developments have led to important progress in this field, namely the introduction of permeabilized cells that retain their capacity to secrete (1, 12, 17), and the use of fluorescent  $Ca^{2+}$  chelators for manipulating and measuring cytosolic free calcium concentration  $[Ca^{2+}]_i$  (19, 20). The quantitative relationship between  $[Ca^{2+}]_i$  and secretion has been mainly investigated in permeabilized cells, but, except for a preliminary report by Rink et al. (20), who used platelets, validation of such data on intact cells has not yet been attempted.

In this paper, we present an investigation of the  $[Ca^{2+}]_i$

1. *Abbreviations used in this paper:*  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_o$ , cytosolic free calcium and extracellular  $Ca^{2+}$  concentration, respectively;  $EC_{50}$ ,  $[Ca^{2+}]_i$  required for half-maximal exocytosis; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine;  $[quin2]_i$ , intracellular quin2 concentration; quin2/AM, quin2 acetoxymethyl tetraester.

dependency of exocytosis in intact human neutrophils. The cells were loaded with the  $\text{Ca}^{2+}$  chelator and indicator quin2, steady state  $[\text{Ca}^{2+}]_i$  were established with ionomycin and appropriate concentrations of extracellular  $\text{Ca}^{2+}$ , and exocytosis from three distinct subcellular storage organelles was assessed. The results show that the specific granules and the secretory vesicles containing gelatinase have different  $[\text{Ca}^{2+}]_i$  requirements for exocytosis than do the azurophil granules. In addition, receptor stimulation substantially decreases the  $[\text{Ca}^{2+}]_i$  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- $\beta$ -D-glucuronide (Sigma Chemical Co., St. Louis, MO); quin2 acetoxyethyl tetraester (quin2/AM), cyano $^{57}\text{Co}$ cobalamin, 10–20  $\mu\text{Ci}/\mu\text{g}$  (The Radiochemical Centre, Amersham, England); dextran T500 and Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden); diisopropyl-phosphofluoridate (Fluka, Buchs, Switzerland);  $^3\text{H}$ lactic 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 Krebs's Ringer bicarbonate medium containing 138 mM NaCl, 6 mM KCl, 1 mM  $\text{MgSO}_4$ , 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{NaH}_2\text{PO}_4$ , 5 mM  $\text{NaHCO}_3$ , 5.5 mM glucose, 20 mM Hepes, pH 7.4, and kept in this medium at room temperature until use.

### Measurement of $[\text{Ca}^{2+}]_i$

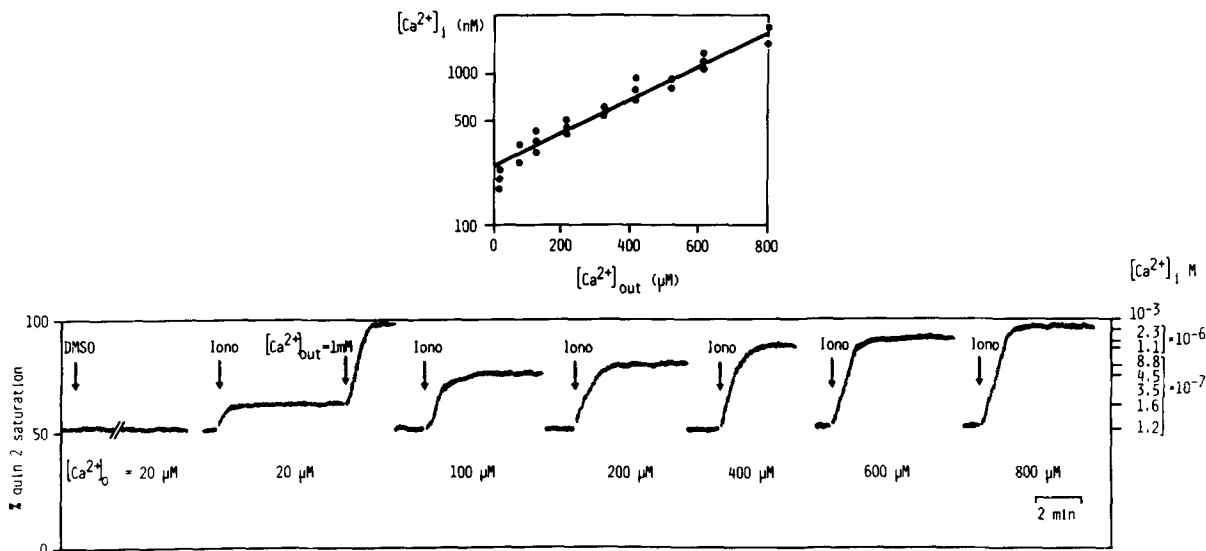
Quin2 loading was performed as described previously (16). Suspensions of  $5 \times 10^7$  cells/ml were equilibrated at 37°C for 5 min. Quin2/AM was added to a final concentration of 10 to 40  $\mu\text{M}$  from a 10 mM stock solution in dimethylsulfoxide. Control cells were treated with dimethylsulfoxide (<0.5%) only. In

most cases the loading with quin2/AM was performed in the Krebs's-Ringer bicarbonate buffer medium described above. In a few cases (see text) the loading was performed in Krebs's-Ringer bicarbonate buffer that contained 1 mM EGTA and no  $\text{CaCl}_2$  to obtain  $[\text{Ca}^{2+}]_i$  below resting level (10). After 10 min at 37°C the cells were diluted to  $1 \times 10^7$ /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  $339 \pm 5$  and  $492 \pm 10$  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  $[\text{Ca}^{2+}]_i$  was calibrated as described previously (19).

### Experimental Protocol

For the analysis of exocytosis induced by ionomycin at stable steady state  $[\text{Ca}^{2+}]_i$  levels (Figs. 1, 2, 4 and Table 1) the neutrophils were loaded with moderately high concentrations of quin2 (which functions as a cytosolic  $\text{Ca}^{2+}$  buffer as well as an indicator [16]). Loading was performed with 20–40  $\mu\text{M}$  quin2/AM and  $5 \times 10^7$  cells/ml, yielding intracellular quin2 concentrations ( $[\text{quin2}]_i$ ) of 0.8–1.0 nmol/ $10^6$  cells. The  $[\text{quin2}]_i$  is critical for experiments of this type. At low  $[\text{quin2}]_i$ , the release of  $\text{Ca}^{2+}$  from intracellular stores causes a marked transient  $[\text{Ca}^{2+}]_i$  rise, which is followed by a lower steady state  $[\text{Ca}^{2+}]_i$ . On the other hand, high  $[\text{quin2}]_i$  dampens the calcium responses, and steady state  $[\text{Ca}^{2+}]_i$  levels are reached only very slowly.

After loading, aliquots of the suspension (usually  $10^7$  cells) were centrifuged, and the cells were resuspended in 3.2 ml of  $\text{Ca}^{2+}$ -free buffer medium at 37°C. The extracellular  $\text{Ca}^{2+}$  concentration,  $[\text{Ca}^{2+}]_o$ , was then adjusted, and where indicated cytochalasin B (5  $\mu\text{g}/\text{ml}$ ) was included. After 3 min ionomycin (500 nM) was added. 150–250- $\mu\text{l}$  aliquots were withdrawn from the fluorimeter cuvette at various times for the assessment of exocytosis. Controls showed that removal of up to 750  $\mu\text{l}$  did not affect the fluorescence measurements. In principle it should also be possible to establish different steady state levels of  $[\text{Ca}^{2+}]_i$  by varying the concentration of ionomycin at a fixed extracellular  $\text{Ca}^{2+}$  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  $[\text{Ca}^{2+}]_i$  and granule content release in the same reaction mixture rather than in parallel incubations (i.e.,  $[\text{Ca}^{2+}]_i$  in the cuvette and exocytosis in the test tube). For the study of the  $\text{Ca}^{2+}$  dependency of fMLP-induced exocytosis (Figs. 5, 6, and 7), the cells were loaded with 10–20  $\mu\text{M}$  quin2/AM, resulting in  $[\text{quin2}]_i$  of 0.1–0.3 nmol/ $10^6$  cells.



**Figure 1.** Establishment of steady state cytosolic free calcium,  $[\text{Ca}^{2+}]_i$ , with ionomycin.  $10^7$  neutrophils loaded with quin2 (0.8 nmol quin2/ $10^6$  cells) were suspended in 3.2 ml buffer with increasing concentrations of  $\text{CaCl}_2$  as indicated. Ionomycin (Iono; 500 nM) was added as indicated. Fluorescence tracings are shown at the bottom. The relationship between the steady state  $[\text{Ca}^{2+}]_i$  obtained and the calcium concentration of the extracellular medium,  $[\text{Ca}^{2+}]_o$ , is shown on top. In neutrophils pretreated with cytochalasin B virtually identical results were obtained.

## 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.  $\beta$ -Glucuronidase, vitamin B<sub>12</sub>-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  $\pm$  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  $[Ca^{2+}]_i$  up to a plateau which depended on  $[Ca^{2+}]_o$ . The plateau remained constant for  $\sim$ 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  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_o$ . Due to the poor indicator sensitivity of quin2 at  $[Ca^{2+}]_i$  above 2  $\mu$ M, calibration at higher values was not attempted.

### Exocytosis as a Function of Steady State $[Ca^{2+}]_i$

Fig. 2 shows that release of the specific granule marker, vitamin B<sub>12</sub>-binding protein, increased with increasing  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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,  $\beta$ -glucuronidase (azurophil granules), vitamin B<sub>12</sub>-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  $[Ca^{2+}]_o$ , however, exocytosis reached a similar maxi-

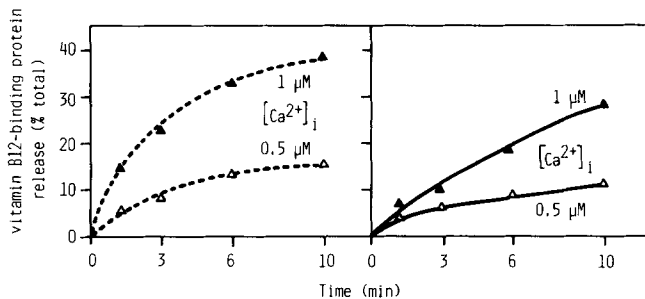


Figure 2. Time course of release of vitamin B<sub>12</sub>-binding protein at two  $[Ca^{2+}]_i$  levels. Steady state  $[Ca^{2+}]_i$  of 500 and 1,000 nM were established as shown in Fig. 1. *Left*, + cytochalasin B. *Right*, - cytochalasin B. 250- $\mu$ l samples were withdrawn from the fluorimeter cuvette at various times after addition of ionomycin, and the amount of vitamin B<sub>12</sub>-binding protein released was determined in the cell-free medium. Where indicated the cells were pretreated with cytochalasin B (5  $\mu$ g/ml) for 5 min. Release is expressed as percent of the initial cellular content as described in Materials and Methods.

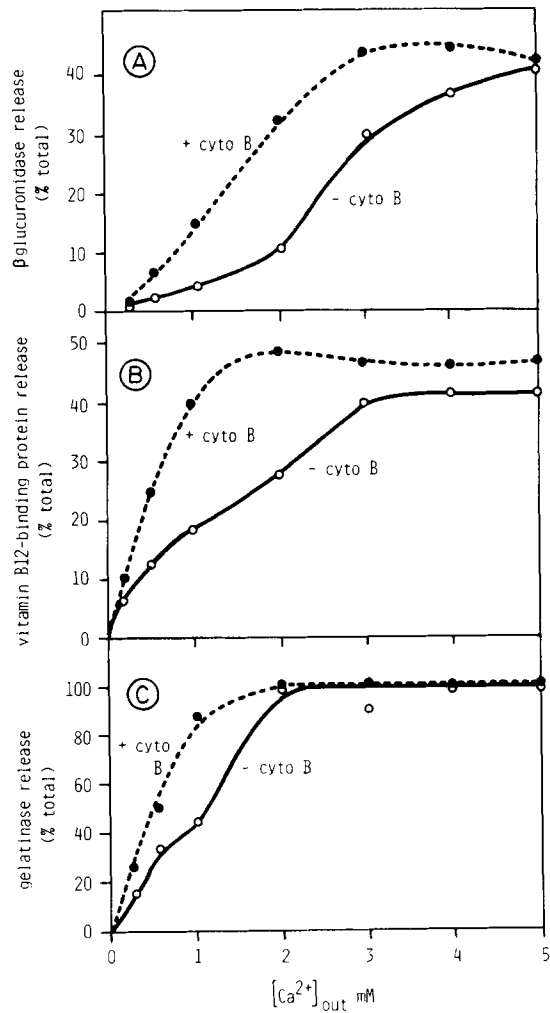


Figure 3. Effect of increasing concentrations of extracellular calcium on exocytosis. Neutrophils suspended at various  $[Ca^{2+}]_o$  were incubated for 3 min in the presence or absence of cytochalasin B (*cyto B*; 5  $\mu$ g/ml) and then treated with 500 nM ionomycin. After 5 min (cells with cytochalasin B) or 6 min (cells without cytochalasin B) aliquots were withdrawn, and the release of  $\beta$ -glucuronidase (azurophil granules, *top*), vitamin B<sub>12</sub>-binding protein (specific granules, *middle*), and gelatinase (secretory vesicles, *bottom*) was determined.

imum 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  $[Ca^{2+}]_i$  was not determined because the levels were above the limits of accurate measurement with quin2. The release obtained at high  $[Ca^{2+}]_o$  was not due to nonspecific leakage from damaged cells since even at 5 mM  $[Ca^{2+}]_o$  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  $[Ca^{2+}]_i$  and the extent of exocytosis is shown. Minimal release was observed when  $[Ca^{2+}]_i$  was  $<200$ –250 nM. Above this level release occurred, albeit to different extents, from all three storage compartments. Threshold  $[Ca^{2+}]_i$ , defined as the intercept of the extrapolated slope of the  $[Ca^{2+}]_i$  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

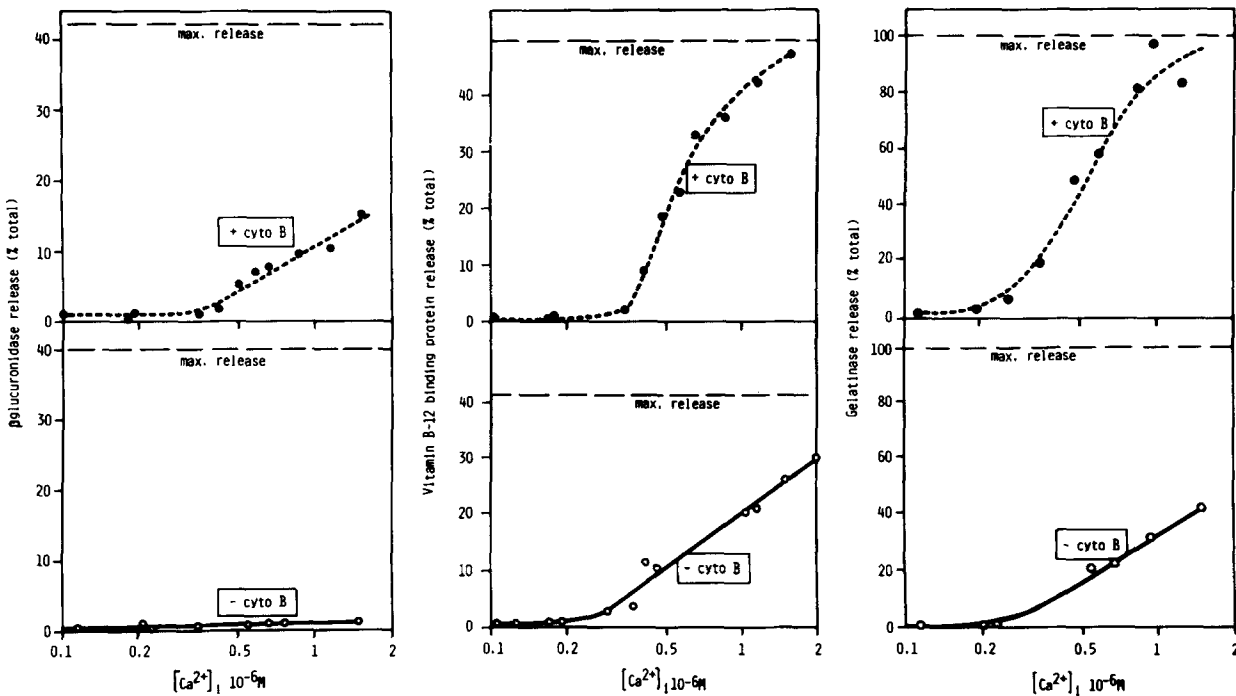


Figure 4. Exocytosis as a function of steady state  $[Ca^{2+}]_i$ . Experimental conditions as in Fig. 1. Where indicated the cells were pretreated with cytochalasin B (cyto B; 5  $\mu$ g/ml) for 5 min. Samples for the determination of marker release were withdrawn from the fluorimeter cuvette 5 min (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.  $[Ca^{2+}]_i$  Threshold and  $EC_{50}$  for Exocytosis from Azurophil Granules, Specific Granules, and Secretory Vesicles

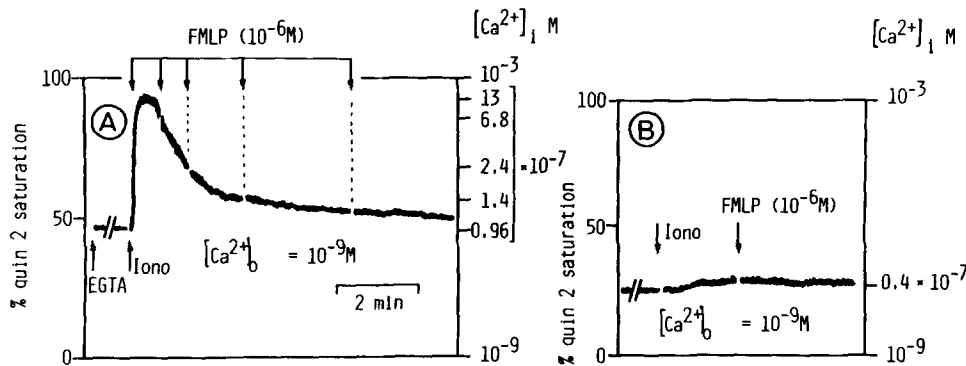
Subcellular marker	Cytochalasin B	$[Ca^{2+}]_i$ (nM)		
		Threshold	$EC_{50}$	
$\beta$ -Glucuronidase	-	ND	$6,000 \pm 1,500^*$	(5)
	+	$280 \pm 40$ (6)	$2,600 \pm 480^*$	(4)
Vitamin B12-binding protein	-	$220 \pm 40$ (5)	$1,100 \pm 220$	(5)
	+	$240 \pm 60$ (4)	$610 \pm 170$	(4)
Gelatinase	-	$190 \pm 35$ (3)	$1,600 \pm 510$	(3)
	+	$235 \pm 30$ (4)	$650 \pm 130$	(4)

The experimental conditions were the same as in Fig. 4. Values are means  $\pm$  SD, number of experiments in brackets. ND, not determined.

\* Values estimated by extrapolation on the basis of the relationship between  $[Ca^{2+}]_o$  and  $[Ca^{2+}]_i$  as shown in Fig. 1.

granules and the secretory vesicles similar threshold  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  threshold levels. From the same experiments, the  $[Ca^{2+}]_i$  that gave half-maximal release of the three markers ( $EC_{50}$ ) 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  $EC_{50}$  for  $\beta$ -glucuronidase release was 2,600 nM, which is much higher than the  $EC_{50}$  for release from specific granules and secretory vesicles ( $P < 0.001$ ). Threshold  $[Ca^{2+}]_i$  and  $EC_{50}$  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  $EC_{50}$  values difficult. In fact,  $[Ca^{2+}]_i$  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  $EC_{50}$  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  $EC_{50}$ . As shown in Table I the  $EC_{50}$  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  $\beta$ -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  $EC_{50}$  of  $\sim 6,000$  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  $\mu$ g/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  $\mu$ M 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]<sub>i</sub> 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).

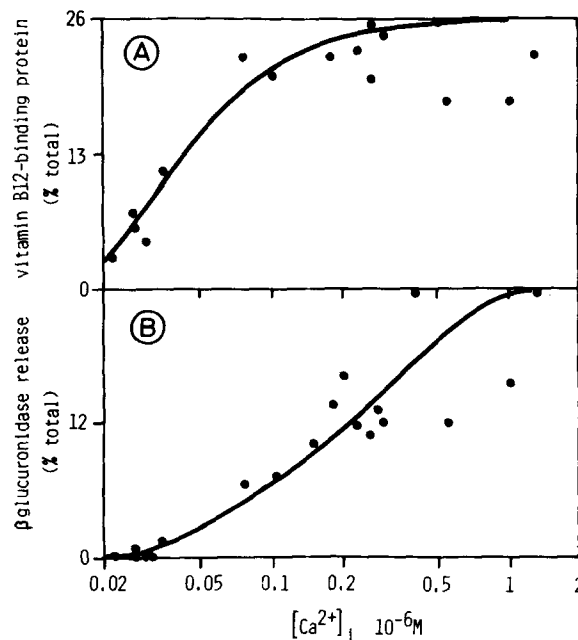
3, and the relationship between the extracellular and intracellular calcium concentration shown in Fig. 1.

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



**Figure 6.**  $[Ca^{2+}]_i$  dependency of fMLP-induced exocytosis. Conditions were as described in Fig. 5. Cytochalasin B (5  $\mu$ g/ml) was added 3 min before ionomycin (Iono; 500 nM).  $[Ca^{2+}]_i$  is the value measured at the time of fMLP addition. Samples for the determination of marker release were collected from the fluorimeter cuvette 5 min after fMLP was added. Release is expressed as percent of the initial cellular content minus the value obtained for ionomycin alone. Results are from two experiments. The respective values for ionomycin alone were 14 and 13% for vitamin B12-binding protein, and 5 and 4% for  $\beta$ -glucuronidase. The corresponding values for unstimulated cells were 8 and 7% for vitamin B12-binding protein, and 3 and 3% for  $\beta$ -glucuronidase.

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

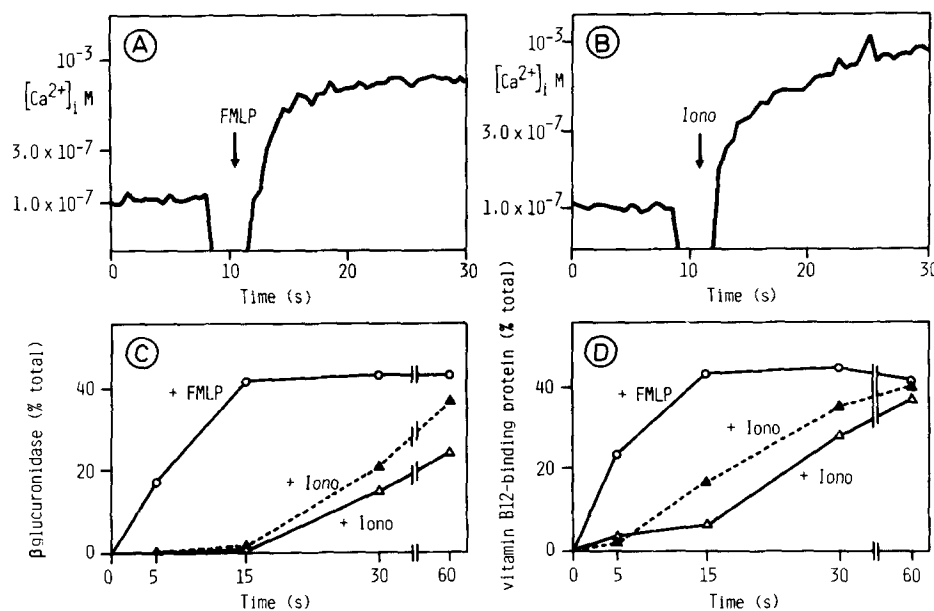
high  $[Ca^{2+}]_i$  elevations were very transient (Fig. 5A), and consequently the exocytosis responses at these levels were less reproducible than at lower  $[Ca^{2+}]_i$ .  $[Ca^{2+}]_i$ 's below resting level were obtained by loading the cells with quin2 in  $Ca^{2+}$ -free medium (10). Under these conditions the  $Ca^{2+}$  stores are depleted, and basal  $[Ca^{2+}]_i$  is decreased 3- to 10-fold below the normal resting level, depending on the quin2 concentration used (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  $[Ca^{2+}]_i$  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  $200 \pm 60$  nM ( $n = 3$ ) was calculated for azurophil and of  $60 \pm 20$  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  $[Ca^{2+}]_i$  levels. Although the rise in  $[Ca^{2+}]_i$  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  $\sim 30$  s. By contrast, exocytosis induced by ionomycin started with a lag of  $\sim 15$  s and proceeded at a much lower rate for  $>1$  min. This occurred despite the fact that similar elevations of  $[Ca^{2+}]_i$  (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  $[Ca^{2+}]_i$  ( $>2$   $\mu$ 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  $[quin2]_i$  (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  $[quin2]_i$  increases the time needed to reach the maximal  $[Ca^{2+}]_i$  (see also Fig. 1) and decreases the rate of marker release (16).

## Discussion

The present work aimed at a quantitative assessment of the  $[Ca^{2+}]_i$  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 discharge in the presence of  $Ca^{2+}$  buffers (1, 12, 17). Alternatively, clamping of the  $[Ca^{2+}]_i$  has been achieved using high concentrations of  $Ca^{2+}$  ionophores (15). Permeabilized cells have one important advantage. They permit control not only of  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  changes and exocytosis in response to stimulation with fMLP or ionomycin. Neutrophils were loaded with 0.24 nmol quin2 per  $10^6$  cells, much less than in the experiments shown in Figs. 1, 2, and 4 and Table I. Cytochalasin B (5  $\mu$ g/ml) was added 3 min before fMLP (1  $\mu$ M) or ionomycin (500 nM). Samples for the determination of granule marker release were withdrawn at the times indicated after stimulus addition from the fluorimeter cuvette and immediately mixed with an equal volume of ice-cold buffer. (A and B)  $[Ca^{2+}]_i$  changes after stimulation with fMLP (A) or ionomycin (B), both at  $[Ca^{2+}]_o = 1$  mM. (C and D) Release of  $\beta$ -glucuronidase (C) and vitamin B12-binding protein (D).  $[Ca^{2+}]_o$  was 1.0 mM for fMLP ( $\circ$ ), 0.4 mM ( $\Delta$ ) or 1.0 mM ( $\blacktriangle$ ) for ionomycin, resulting in maximal  $[Ca^{2+}]_i$  of 700, 800, and  $>2,000$  nM, respectively.

taken into consideration, and reliable information can be obtained only if  $[Ca^{2+}]_i$  is monitored. It must be noted that ionophore clamping assumes that the ionophore can completely overcome the  $Ca^{2+}$  buffering mechanisms of the cell, so that  $[Ca^{2+}]_i = 2\Delta pH [Ca^{2+}]_o$ . We observed that with 0.5–1.0  $\mu M$  ionomycin  $[Ca^{2+}]_o$  was still  $\sim 1,000$ -fold higher than  $[Ca^{2+}]_i$ , and the equilibrium  $[Ca^{2+}]_i = 2 \Delta pH [Ca^{2+}]_o$  was not reached even at 10  $\mu M$  ionomycin. A similar discrepancy between the assumed and measured value for  $[Ca^{2+}]_i$  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  $Ca^{2+}$  chelator quin2 served two purposes, i.e., to allow manipulation of  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  requirement for half-maximal exocytosis ( $EC_{50}$ ) 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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  threshold and  $EC_{50}$ . Again, the  $[Ca^{2+}]_i$  requirement for exocytosis was lower for specific granules than for azurophil. In cytochalasin B-treated cells, marked exocytosis of specific granules was obtained already at resting  $[Ca^{2+}]_i$  levels. In addition, receptor stimulation increased the rate of exocytosis. A problem that we have not addressed in this study is whether  $Ca^{2+}$  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  $[Ca^{2+}]_i$  dependency assessed in the presence of ionomycin reflects the overall requirement of  $[Ca^{2+}]_i$  for exocytosis and does not necessarily indicate a direct stimulatory action of  $Ca^{2+}$  on the effector system. In fact, a rise in  $[Ca^{2+}]_i$  could induce exocytosis also by producing other intracellular messengers. For instance, elevated  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  could cause polyphosphoinositide breakdown (7), thus mimicking the effect of receptor stimulation. Consistent with the possibility that the effect of  $[Ca^{2+}]_i$  on secretion is indirect are the following observations made in our neutrophil system: The time course and extent of the  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  (Fig. 5). These findings could be explained if the ionomycin-induced  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  dependency of granule exocytosis with the *in vitro*  $Ca^{2+}$  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  $[Ca^{2+}]_i$ . 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  $[Ca^{2+}]_i$ . 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  $[Ca^{2+}]_i$  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.

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