Modulation of Cytosolic-free Calcium Transients by Changes in Intracellular Calcium-buffering Capacity: Correlation with Exocytosis and O_2^- Production in Human Neutrophils

P. DANIEL LEW,* CLAES B. WOLLHEIM,‡ FRANCIS A. WALDVOGEL,* and TULLIO POZZAN§

Division of Infectious Diseases and ‡Institut de Biochimie Clinique, University Hospital, CH-1211 Geneva 4, Switzerland; and §Institute of General Pathology, CNR Unit for the Study of Physiology of Mitochondria, University of Padova, Italy

ABSTRACT The intracellularly trapped fluorescent calcium indicator, quin 2, was used not only to monitor changes in cytosolic-free calcium, $[Ca^{2+}]_i$, but also to assess the role of $[Ca^{2+}]_i$ in neutrophil function. To increase cytosolic calcium buffering, human neutrophils were loaded with various quin 2 concentrations, and $[Ca^{2+}]_i$ transients, granule content release as well as superoxide $[O_2^-]$ production were measured in response to the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) and the calcium ionophore ionomycin.

Receptor-mediated cell activation induced by fMLP caused a rapid rise in $[Ca^{2+}]_i$. The extent of $[Ca^{2+}]_i$ rise and granule release were inversely correlated with the intracellular concentration of quin 2, $[quin\ 2]_i$. These effects of $[quin\ 2]_i$ were more pronounced in the absence of extracellular Ca^{2+}. The initial rate and extent of fMLP-induced O_2^- production were also inhibited by $[quin\ 2]_i$. The rates of increase of $[Ca^{2+}]_i$ and granule release elicited by ionomycin were also inversely correlated with $[quin\ 2]_i$ in Ca^{2+}-containing medium. As the effects of ionomycin, in contrast to those of fMLP, are sustained, the final increase in $[Ca^{2+}]_i$ and granule release were not affected by $[quin\ 2]_i$. A further reduction of fMLP effects was seen when intracellular calcium stores were depleted by incubating the cells in Ca^{2+}-free medium with ionomycin.

The specificity of quin 2 effects on cellular calcium were confirmed by loading the cells with Anis/AM, a structural analog of quin 2 with low affinity for calcium which did not inhibit granule release. In addition, functional responses to phorbol myristate acetate (PMA), which stimulates neutrophils without raising $[Ca^{2+}]_i$, were not affected by $[quin\ 2]_i$. The findings indicate that rises in $[Ca^{2+}]_i$ control the rate and extent of granule exocytosis and O_2^- generation in human neutrophils exposed to the chemotactic peptide fMLP.

Until recently the major obstacle in understanding the role of calcium in the activation of cellular processes has been the lack of suitable techniques for measuring directly cytosolic-free calcium, $[Ca^{2+}]_i$ in small mammalian cells. The introduction of a new generation of Ca^{2+} indicators of high affinity and selectivity, the tetracarboxylates, and of their intracellularly hydrolyzable alkyl-esters, has made possible the measure of $[Ca^{2+}]_i$ in small intact mammalian cells (1–3). Although quin 2, the more useful of the tetracarboxylate Ca^{2+} indicators, has become quite fashionable in the last two years, in most cases it has been used either nonquantitatively or simply to demonstrate $[Ca^{2+}]_i$ rises as a consequence of certain stimuli (4–7). Very little attention has been paid to the use of quin 2 as a high-affinity Ca^{2+} chelator to buffer and control intracellular Ca^{2+} transients while simultaneously monitoring the actual values of $[Ca^{2+}]_i$ (8–10).

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By loading neutrophils with increasing concentrations of quin 2 we have examined the effect of buffering calcium transients on two functional responses of these cells: granule content release and O_2^- production.

We have previously demonstrated that to explain the stimulation of these responses by the chemotactic peptide, formyl-methyonyl-leucyl-phenylalanine (fMLP[1]), it is necessary to invoke the generation of another signal by a receptor-mediated mechanism at least, in addition to a $[Ca^{2+}]_i$ rise (11). Here we show that the $[Ca^{2+}]_i$ rise, which follows the interaction of fMLP with its surface receptors is a necessary component in the sequence of events that leads to both degranulation and superoxide production.

MATERIALS AND METHODS

Cytochrome c (type VI), superoxide dismutase (SOD), cytochalasin B, fMLP, and phorbol myristate acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO); Dextran T500 and Ficoll-Paque were from Pharmacia Fine Chemicals (Upsaala, Sweden). Quin 2 acetoxy-methylesther (quin 2/AM) was purchased from Lancaster Synthesis Ltd. White Lund (Lancashire, England). Anis/AM was kindly provided by Dr. Roger Tsien (University of California, Berkeley). The structures of quin 2/AM, quin 2, Anis/AM, and Anis have been previously published (2, 4, 10: see also APDA in reference 4). 4-Methylumbelliferyl substrates were purchased from Koch Laboratories (Haverhill, England). Ionomycin was a kind gift from C. M. Liu of Hoffman la Roche (Nutley, NJ).

Cell Preparations and Measurement of Cytosolic-free Calcium, $[Ca^{2+}]_i$: Neutrophils were prepared from fresh blood samples (usually 90 ml) obtained from healthy volunteers. Fresh neutrophils were purified by dextran sedimentation followed by centrifugation through a layer of Ficoll-Hypaque as described previously (12, 13). The granulocyte pellet was washed in saline, and the contaminating erythrocytes were eliminated by hypotonic shock. Preparations obtained in this manner contained 95% neutrophils. The cells were suspended in a medium containing 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 100 μM EGTA, 1 mM $NaHPO_4$, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES, pH 7.4.

Quin 2 loading was performed essentially as described previously (11). Cells, washed and suspended at a concentration of 5×10^7 cells/ml in the same medium as above were equilibrated at 37°C for 5 min; quin 2/AM usually at final concentrations of 30, 60, or 100 μM from a 20-mM stock solution in dimethylsulfoxide was added and the cells were left for 60 min at 37°C. Control cells were incubated in 0.5% dimethylsulfoxide. 10 min after quin 2/AM or dimethylsulfoxide addition, the cells were diluted to 1×10^7/ml with warm medium plus 0.5% BSA. After loading, the cells were kept at room temperature until used. Before use, an aliquot of the cells was centrifuged and suspended in the same medium as above without BSA.

Fluorescent measurements were performed with a Perkin-Elmer fluorimeter (LS3; Perkin-Elmer Corp., Norwalk, CT). The fluorimetric cuvette holder was thermostated (37°C) and magnetically stirred. The cell number was $2–2.5 \times 10^6$ cells/ml. Excitation and emission wavelengths were 339 ± 3 and 492 ± 10, respectively. To minimize light scattering artifacts two cut-off filters UV D25 and UV 35 for excitation and emission, respectively, were used. Quin 2 calibration of fluorescence as a function of $[Ca^{2+}]_i$ were performed as previously described (11). Intracellular quin 2 concentrations were determined by comparing the Ca^{2+}-dependent fluorescence of quin 2–loaded cells, which had been treated with Triton 0.1%, with the fluorescence of a standard solution of quin 2 free acid in the presence of unloaded cells, which had been treated with Triton 0.1% in Ca^{2+} medium (2). The trapping efficiency of quin 2/AM was rather constant among various batches of cells (~60%). (Thus when the statistical analysis of the effect of quin 2 loading on metabolic responses is reported, the total added quin 2/AM rather than the actual amount of $[quin\ 2]_i$ is given.) All the experiments performed in "calcium medium" ($[Ca^{2+}]_0 = 10^{-3}$ M) were done in the above described medium, whereas those performed in "calcium-free medium" were performed in the same medium without calcium with the addition of 1 mM EGTA ($[Ca^{2+}]_0 = 10^{-9}$ M).

Exocytosis Experiments: 1.25×10^6 neutrohils were suspended in 500 μl of calcium medium or calcium-free medium containing 2.5 μg cytochalasin and warmed at 37°C for 5 min before the addition of stimuli at the indicated concentration and time. Incubation was terminated by rapid cooling in ice and centrifugation (800 g for 10 min). Enzymes and vitamin B_{12}-binding protein were assayed in the supernatants and calculated as a percentage of total cell protein released from an aliquot of the same cell suspension treated with 0.025% Triton X-100 for 5 min at 37°C. For control, in several experiments, recoveries were calculated by comparing the amounts in the supernatant, plus pellet, with those of the original cell suspension and gave similar results (14).

Biochemical Assays: Lactic acid dehydrogenase was measured as previously described in fresh supernatant from the same day (11); the values never exceeded 7% of the total lactic acid dehydrogenase content. β-glucuronidase and *N*-acetyl-β-glucosaminidase were measured fluorimetrically with 4-methylumbelliferyl substrates (14). For enzyme assay, the incubation mixture consisted of 0.05 M sodium acetate buffer, pH 4.0, 0.05% Triton X-100, 5 mM substrate, and sample in a total volume of 0.2 ml. The reaction was carried out at 37°C for 30 min and was stopped by addition of 3 ml of a glycine-NaOH buffer, pH 10.4, containing 5 mM EDTA. The unsaturated vitamin B_{12}-binding protein was assayed by a slight modification of the method of Kane et al. (14). In brief, 0.1 ml of sample was mixed with 0.4 ml 0.025% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.5, before addition of 1 ml of a solution containing 1.33 ng [^{57}Co]cyanocobalamin in 0.1 M potassium phosphate, pH 7.5 followed by incubation and separation of free and bound [^{57}Co]cyanocobalamin.

Superoxide Production: O_2^- production by neutrophils was monitored continuously in a double-beam spectrophotometer, thermostated at 37°C as previously described (15). In each assay, neutrophils (2.5×10^6) were added to each of two 1-ml cuvettes containing cytochrome c (50 nmol), cytochalasin B (5 μg), and after 5 min either fMLP or PMA (at indicated concentrations) were added. Superoxide dismutase (10 μg) was also present in the reference cuvette, and the rate of O_2^--dependent cytochrome-c reduction was monitored at 550 nm. The molar extinction coefficient for this change in absorption at 550 nm is 21,000. The initial rates of O_2^- production were calculated from the first derivative of the curve for O_2^- release as previously described (15).

Statistical Analysis: Values are expressed as mean ± SD. Statistical analysis was performed by the two-tailed unpaired Student's t-test.

RESULTS

Effect of Intracellular Quin 2, $[Quin\ 2]_i$, on $[Ca^{2+}]_i$-Transients Induced by the Chemotactic Peptide fMLP

To compare changes in $[Ca^{2+}]_i$ with exocytosis, in particular the release of β-glucuronidase (primary granules), all experiments were performed in the presence of cytochalasin B (16). In Fig. 1, *A–D*, the $[Ca^{2+}]_i$ changes induced by fMLP in the same batch of neutrophils containing low or high quin 2 intracellular concentrations (quin 2) are shown. In calcium-containing medium, at low $[quin\ 2]_i$ there is a rapid rise of $[Ca^{2+}]_i$ that reaches micromolar levels, followed by a progressive decrease to basal levels. At high $[quin\ 2]_i$ the amplitude of the $[Ca^{2+}]_i$ rise is markedly reduced reaching at its maximum ~300 nM. The kinetics of the $[Ca^{2+}]_i$ transients are very different at high or low quin 2 loadings. At low loading upon addition of fMLP, there is a rapid rise that is completed in <10 s followed by a slow return to the basal level. At high loading the rapid phase is drastically reduced in amplitude and a slow phase of $[Ca^{2+}]_i$ rise is now observed that lasts for >4 min. The fast component has to be attributed to release of Ca^{2+} from intracellular stores, since it is observed also in the presence of EGTA, while the slow component is probably due to an increased influx from the extracellular medium since it is abolished when external Ca^{2+} is removed.

In calcium-free medium, while at low $[quin\ 2]_i$ loadings, an important $[Ca^{2+}]_i$ rise can still be measured (up to 850 nM): at high loadings the $[Ca^{2+}]_i$ rise is barely significant, from 90 to 115 nM. The differences of $[Ca^{2+}]_i$ rise at the high and low loading in Ca^{2+}-free medium should be attributed to the different calcium-buffering capacity provided by quin 2, since the amount of Ca^{2+} released in the two cases is rather similar.

[1] *Abbreviations used in this paper:* $[Ca^2]_i$, cytosolic-free calcium; fMLP, formylmethyonyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; $[quin\ 2]_i$, intracellular quin 2 concentration; quin 2/AM, quin 2 acetoxy-methylester.

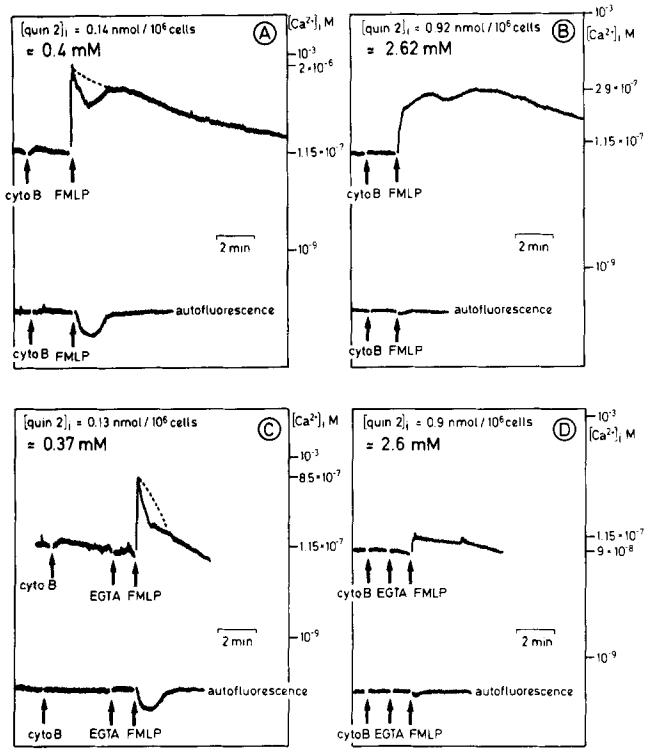

FIGURE 1 Effect of intracellular quin 2 in $[Ca^{2+}]_i$ transients. $[Ca^{2+}]_i$ changes in neutrophils containing low or high quin 2 concentrations induced by fMLP in calcium medium or calcium-free medium. In each panel the changes in autofluorescence in control cells are indicated. The dotted lines represent the graphical correction of $[Ca^{2+}]_i$ transients taking into account the changes in autofluorescence. This correction factor is significant at low quin 2 loading but not at high loading because of the difference in signal intensity. Intracellular quin 2 concentration was calculated taking into account a volume of 0.35 μl free water/per 10^6 neutrophils (23). We noticed that cytochalasin B frequently induces the transient small rise of $[Ca^{2+}]_i$ reported in this figure. However, this effect was not routinely reproducible and was extremely variable in extent in the various batches of cells.

The amount of calcium released from intracellular pools can be calculated (9): quin 2 binds calcium ions with a stoichiometry of 1:1; thus the initial amount of calcium liberated from stores is the product of the percent changes in quin 2 saturation times its intracellular concentration. For example, in Fig. 1, the amount of calcium released at low loading in Ca^{2+}-free medium was 53 pmol $Ca^{2+}/10^6$ cells (Δ 41% × 130 pmol quin $2/10^6$ cells) whereas at high loading it was 49 pmol $Ca^{2+}/10^6$ cells (Δ 5.5% × 900 pmol quin $2/10^6$ cells). In this calculation the assumption is made (9) that the endogenous Ca^{2+} buffering is negligible compared with that provided by quin 2. Thus only the lower limit of the amount of Ca^{2+} released from stores can be inferred from this approach, the error being larger than the lower $[quin\ 2]_i$. As discussed in detail by Tsien and Rink (10) from a series of experiments of this kind, the endogenous Ca^{2+}-buffering capacity can be calculated (Pozzan, T., and D. Lew, manuscript in preparation).

Effect of $[quin\ 2]_i$ on Granule Content Release and O_2^- Generation Induced by fMLP

In Fig. 2, *A* and *B*, a time course of release of β-glucuronidase (a marker of primary granules) and vitamin B_{12}-binding protein (a marker of secondary granules) in the same batch of neutrophils loaded with various concentrations of quin 2 and stimulated by 10^{-7} fMLP is shown. Increasing quin 2 concentrations decreases the amount of primary and secondary granule exocytosis in calcium-containing medium. The rate of granule release, on the other hand, appears less decreased; however measurements <15 s were not performed. The inhibitory effect on granule content release is much more pronounced in calcium-free medium even in cells loaded with the lowest quin 2 concentrations. Fig. 2*C* indicates the maximal free cytosolic calcium reached in the same batch in calcium or calcium-free medium of neutrophils at various quin 2 loadings. The maximal $[Ca^{2+}]_i$ rise reached correlated well with the maximal extent of granule content released under these various conditions in the same batch of cells as shown in Fig. 2, *D* and *E*.

Table I summarizes the results of three such similar experiments. Included also are results of *N*-acetyl-β-glucosaminidase, an enzyme present in primary granules and C-particles. Statistically significant inhibitions of granule release were observed in calcium medium at 60 μM quin 2/AM (loading conditions) at early times whereas in calcium-free medium they were observed at 30 μM quin 2/AM at all times tested.

In all the above experiments, we have used 10^{-7} M fMLP, a concentration that is almost maximal both for granule-enzyme release and superoxide production in unloaded cells. We tried also higher fMLP concentrations to test whether one could overcome this inhibition by increasing the fMLP concentrations: this was not the case and the fMLP dose response curve was very similar in quin 2–loaded cells and control cells (not shown).

Table II indicates the effect of quin 2 on the initial rates of O_2^- production induced by the chemotactic peptide, fMLP. In contrast to granule release, where the initial rate of release was less affected, the initial rates of superoxide production upon stimulation with fMLP are markedly inhibited by increasing quin 2 concentrations. This is not, however, a perfect comparison, since the enzyme released is a function of the amount released while the O_2^- measured is a function of both the amount of active oxidase produced and the time it has been allowed to generate its product (O_2^-). The inhibition by quin 2 was not significantly different in the presence or absence of extracellular Ca^{2+}. Similar qualitative results on the extent of O_2^- production were obtained in these same experiments (Table II).

Effect of Intracellular quin 2 on $[Ca^{2+}]_i$ Transients and Granule Content Release Induced by the Calcium Ionophore, Ionomycin

Fig. 3 shows that quin 2 affects $[Ca^{2+}]_i$ rise induced by the calcium ionophore, ionomycin, in a predictable way: in calcium medium increasing quin 2 loadings decreases the rate of $[Ca^{2+}]_i$ rise without affecting its final extent (Fig. 3, *A* and *B*). At a loading of 0.22 nmol/quin $2/10^6$ cells, $[Ca^{2+}]_i$ reaches maximal detectable levels at ~10 s whereas at 0.9 nmol/10^6 cells $[Ca^{2+}]_i$ reaches maximal detectable levels at 2 min. On the contrary, in calcium-free medium, the rate of calcium increase is practically unaffected by the extent of intracellular quin 2 while the magnitude of the $[Ca^{2+}]_i$ rise is dramatically decreased from 1,500 to 230 nM in these two conditions. Again the difference has to be ascribed to the extra cytosolic calcium-buffering capacity provided by quin 2, since the

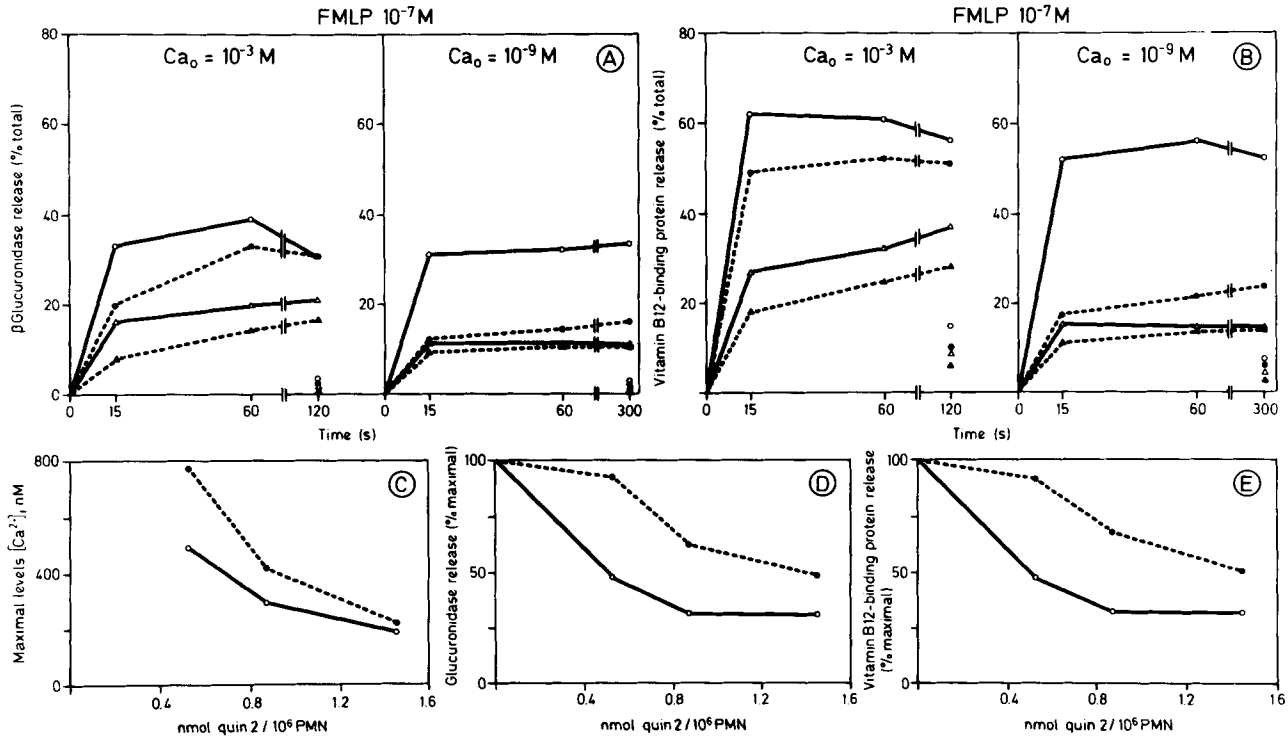

FIGURE 2 Effect of intracellular quin 2 on granule exocytosis and $[Ca^{2+}]_i$ changes induced by fMLP. Neutrophils were incubated as described in Materials and Methods in the presence of 0, 30, 60, and 100 μM quin 2/AM and then tested in parallel. (*A* and *B*) Time course of β-glucuronidase (*A*) and vitamin B_{12}-binding protein release (*B*) in calcium or calcium-free medium. In this experiment, ○, no quin 2; ●, 0.4 nmol quin 2/10^6 PMN; △, 0.8 nmol quin 2/10^6 PMN; ▲, 1.2 nmol quin 2/10^6 PMN. (*C–E*) Maximal $[Ca^{2+}]_i$ levels, maximal β-glucuronidase release, maximal vitamin B_{12}-binding protein release as a function of intracellular quin 2 in calcium (●), or calcium-free medium (○). The values in *D* and *E* represent the maximal release values from *A* and *B* and are shown for comparison with *C*. All these experiments were performed on the same batch of neutrophils.

amount of calcium released is similar both at high and low loading, i.e., 108 and 95 pmol $Ca^{2+}/10^6$ cells, respectively. Fig. 4 (and Table III) shows the effect of $[quin\ 2]_i$ on the rate and extent of β-glucuronidase, *N*-acetyl-β-glucosaminidase, and vitamin B_{12}-binding protein released from neutrophils treated with ionomycin. Paralleling the changes in Ca^{2+}, increasing $[quin\ 2]_i$ decreases the rate of protein release, while the final extent of secretion eventually approaches the same level, although at different rates for various granule populations. No attempt was made to study the effect of $[quin\ 2]_i$ on secretion in calcium-free medium, since even in unloaded cells, secretion with ionomycin is very small. Furthermore, since the extent and the rate of O_2^- production induced by ionomycin, both in Ca^{2+} and Ca^{2+}-free medium are also very small, no systematic investigation of the effect of $[quin\ 2]_i$ on this parameter was undertaken.

Effect of Depletion of Intracellular Mobilizable Calcium on Exocytosis and O_2^- Production

In a previous report we have provided strong evidence suggesting that another signal in addition to $[Ca^{2+}]_i$ rise must be generated intracellularly to explain the stimulatory effect of fMLP (11). The main evidence for this is that in calcium medium, after ionomycin has raised $[Ca^{2+}]_i$, fMLP, though not inducing any further $[Ca^{2+}]_i$ rise is able to stimulate O_2^- generation and myeloperoxidase release.

Fig. 5, *B* and *C* show that such a stimulation by fMLP can be observed provided that $[Ca^{2+}]_i$ is elevated above resting levels. Addition of fMLP, after ionomycin, when $[Ca^{2+}]_i$ is returning to the resting levels, and while the cells are being depleted leads to a progressive decrease in exocytosis and O_2^- production. Interestingly, loaded cells remain susceptible to fMLP stimulation longer than unloaded controls. This correlates with the observation that although the $[Ca^{2+}]_i$ rise induced by ionomycin in calcium-free medium is inversely proportional to $[quin\ 2]_i$, the time required to reach basal levels increases as the loading increases (not shown, see also Fig. 3*D*).

Evidence That the Inhibitory Effect of $[quin\ 2]_i$ on Neutrophil Functions Is Mainly Attributable to Buffering Calcium Transients

It may be argued that the inhibitory effect of $[quin\ 2]_i$ on cell responses are not due to buffering $[Ca^{2+}]_i$ transients but rather to nonspecific inhibition of cell functions. Hesketh et al. (4) reported in lymphocytes two toxic side-effects that might complicate the interpretation of our results: (*a*) loading the cells with quin 2 stimulates reactions that are believed in lymphocytes to be causally related to a rise of $[Ca^{2+}]_i$; (*b*) as a consequence of intracellular generation of formaldehyde, a byproduct of enzymatic hydrolysis of acetoxy-methylester, a reduction of ATP levels was observed in lymphocytes loaded with quin 2. The first problem does not seem to be relevant in neutrophils since we have previously observed that there is no secretion or superoxide production during and after loading with quin 2/AM up to concentrations of 100 μM (11). Fig. 6*A* shows that the ATP level is maintained at around 80–100% in the whole range of quin 2/AM concentrations

TABLE I

Effect of Intracellular Quin 2 on Granule Content Release Induced by FMLP (10^{-7} M)

Loading concentration	Time	Total cellular protein					
		β-Glucuronidase		Glucosaminidase		Vitamin B_{12}-binding protein	
μM quin 2/AM	*s*						
$Ca_0^{2+} = 10^{-3}$ M							
0		38 ± 11‡		44 ± 6		58 ± 9	
30*	15	32 ± 9	n.s.	46 ± 4	n.s.	49 ± 1	n.s.
60		21 ± 11	$P < 0.05$	28 ± 9	$P < 0.015$	27 ± 2	$P < 0.05$
100		13 ± 6	$P < 0.01$	21 ± 1	$P < 0.01$	20 ± 4	$P < 0.01$
0		36 ± 14		45 ± 4		56 ± 7	
30	60	36 ± 16	n.s.	47 ± 7	n.s.	50 ± 2	n.s.
60		21 ± 10	$P < 0.05$	30 ± 9	$P < 0.05$	32 ± 2	$P < 0.05$
100		18 ± 8	$P < 0.05$	23 ± 6	$P < 0.01$	27 ± 5	$P < 0.02$
0		38 ± 7		42 ± 1		52 ± 6	
30	120	35 ± 16	n.s.	46 ± 8	n.s.	49 ± 4	n.s.
60		23 ± 12	n.s.	32 ± 8	n.s.	36 ± 2	$P < 0.05$
100		19 ± 9	$P < 0.05$	26 ± 4	$P < 0.01$	29 ± 2	$P < 0.05$
$Ca_0^{2+} = 10^{-9}$ M							
0		32 ± 5		34 ± 9		46 ± 10	
30	15	14 ± 9	$P < 0.01$	18 ± 4	$P < 0.05$	18 ± 2	$P < 0.01$
60		8 ± 2	$P < 0.01$	12 ± 1	$P < 0.01$	14 ± 2	$P < 0.01$
100		8 ± 2	$P < 0.01$	11 ± 1	$P < 0.01$	13 ± 2	$P < 0.01$
0		33 ± 5		34 ± 9		47 ± 14	
30	60	15 ± 10	$P < 0.01$	19 ± 4	$P < 0.05$	21 ± 2	$P < 0.01$
60		10 ± 1	$P < 0.01$	12 ± 2	$P < 0.01$	15 ± 2	$P < 0.01$
100		9 ± 3	$P < 0.01$	13 ± 0.05	$P < 0.01$	15 ± 2	$P < 0.01$
0		34 ± 4		34 ± 10		44 ± 12	
30	300	15 ± 8	$P < 0.01$	19 ± 5	$P < 0.05$	23 ± 3	$P < 0.01$
60		10 ± 2	$P < 0.01$	13 ± 2	$P < 0.01$	15 ± 2	$P < 0.01$
100		9 ± 3	$P < 0.01$	12 ± 0.5	$P < 0.01$	15 ± 2	$P < 0.01$

Results are mean ± SD of three determinations performed on three different neutrophil preparations. Statistical analysis was performed by comparing values at various loadings at each time point to the values in unloaded cells at the same time point. *n.s.*, not significant.

* Intracellular quin 2 (nanomoles quin 2/10^6 PMN) under various loading conditions were as follows in the three experiments: 0.44 ± 0.09 (30 μM); 0.78 ± 0.08 (60 μM); 1.2 ± 0.26 (100 μM).

‡ Granule release in the absence of stimulus under various loading conditions, respectively for vitamin B_{12}-binding protein glucosaminidase and β-glucuronidase were as follows: at time 120 s in calcium-containing medium: 16 ± 1; 9 ± 3; 3 ± 2 (0 μM quin 2); 12 ± 2; 9 ± 2; 3 ± 2 (30 μM); 8 ± 0.5; 8 ± 2; 3 ± 2 (60 μM); 5 ± 1; 6 ± 1; 3 ± 3 (100 μM) and time 300 s in calcium-free medium (10^{-9} M Ca^{++}): 10 ± 1; 9 ± 2; 5 ± 1 (0 μM quin 2) 8 ± 1; 8 ± 1; 4 ± 2 (30 μM); 8 ± 2; 7 ± 0.5; 4 ± 1 (60 μM); 5 ± 2; 7 ± 3; 2 ± 1 (100 μM).

used in this study. The effect on neutrophil functions of generating intracellularly formaldehyde was tested by loading the cells with Anis/AM: Anis/AM is a compound that results from the splitting of the quinidine ring into halves. Anis/AM has an affinity for calcium of 10^{-3} M and generates half of the amount per mol of formaldehyde, compared to quin 2/AM.

In Fig. 6*B* it is shown that the secretion of β-glucuronidase induced by fMLP is not affected by Anis/AM up to 200 μm. Though Anis is not itself fluorescent and therefore the efficiency of trapping cannot be checked directly, we have observed in lymphocytes that Anis/AM affects ATP levels similarly to quin 2/AM. Further evidence indicating that all the effects of $[quin\ 2]_i$ on fMLP-induced secretion and O_2^- production, are not due to unspecific side-effects is provided in the experiments of Fig. 6*C*.

In previous experiments in our laboratory, and by others, it was demonstrated that exocytosis and O_2^- production by PMA occurs without changes in cytosolic-free calcium. The effect of $[quin\ 2]_i$ on PMA-induced stimulation thus constitutes an ideal control to distinguish effects of $[quin\ 2]_i$ on calcium transients from nonspecific interactions with cell functions. Fig. 6*C* shows that neither the release of vitamin B_{12}-binding protein, nor O_2^- production stimulated by PMA are affected by quin 2 cellular content, whether tested in calcium or calcium-free medium.

DISCUSSION

In a series of recent studies using the fluorescent calcium indicator, quin 2, or obelin-loaded neutrophils, it was possible to demonstrate that a variety of stimuli, including chemotactic peptides, raise cytosolic-free calcium, indicating that calcium could function as an intracellular messenger (5, 11, 17). However, these same experiments pointed out that chemotactic peptides generate other intracellular signal(s) and raised the question as to what role calcium ions play in neutrophil activation (11). In addition, PMA, an extremely potent stimulator of the oxidative metabolism and secondary granule release stimulates neutrophils without raising $[Ca^{2+}]_i$ (18, 19).

We have addressed the question of whether calcium plays a role in neutrophil activation by trapping into the cytoplasm of neutrophils various concentrations of the calcium indicator, quin 2, which binds calcium with high affinity. This allowed us experimentally to progressively increase the cytosolic calcium-buffering capacity and subsequently monitor the kinetics of calcium rises and functional response induced by fMLP or ionomycin.

In the case of exocytosis there was a clear-cut correlation

between the final extent of $[Ca^{2+}]_i$ rise and exocytosis of the contents of both primary (β-glucuronidase) and secondary (vitamin B_{12}-binding protein) granules. Increasing $[quin\ 2]_i$ decreased the maximal calcium rises and exocytosis in response to fMLP and these decreases were even more pronounced in calcium-free buffer where calcium influx from the extracellular medium is negligible and calcium originates only from intracellular pools. The calcium ionophore ionomycin that bypasses membrane surface receptors and tends to dissipate calcium gradients (20) causes rapid rises in $[Ca^{2+}]_i$ and degranulation at low quin 2-loadings. Increasing quin 2 loadings progressively decreased the rates of $[Ca^{2+}]_i$ rises and exocytosis induced by the ionophore, but since in contrast to fMLP the effects of the ionophore are sustained, eventually both parameters tended to reach maximal levels. A further argument for an important role of calcium released from intracellular stores by fMLP is indicated by the fact that depleting these stores with ionomycin in calcium-free medium will lead to time-dependent decreases of fMLP-induced responses. It is noteworthy that under the same experimental conditions, PMA can still fully activate the exocytotic process and O_2^- generation (not shown). The present experimental findings (using fMLP and ionomycin as stimulants) indicate that the rate of elevation of cytosolic-free calcium levels and the final maximal extent of the calcium rise, control the kinetics and extent of secretion induced by these agents in human neutrophils.

TABLE II

Effect of Intracellular Quin 2 on Superoxide Production (O_2^-) Induced by FMLP (2.10^{-7} M)

Loading concentration	Maximal initial rates* of O_2^- production ($n = 3$)	Maximal extent‡ of O_2^- production ($n = 3$)
μM quin 2/AM	%	%
$Ca^{2+} = 10^{-3}$ M		
0	100 ± 10	100 ± 30
30§	50 ± 14 $P < 0.01$	73 ± 29 n.s.
60	39 ± 9 $P < 0.01$	75 ± 15 n.s.
100	25 ± 0 $P < 0.01$	48 ± 5 $P < 0.05$
$Ca_0^{2+} = 10^{-9}$ M		
0	100 ± 4	100 ± 13
30	53 ± 5 $P < 0.01$	87 ± 2 n.s.
60	32 ± 13 $P < 0.01$	74 ± 14 $P < 0.05$
100	24 ± 6 $P < 0.01$	74 ± 24 $P < 0.05$

Results are mean ± SD of three determinations. Statistical analysis was performed as in Table I.

* Maximal initial rates of O_2^- production were 8.6 ± 2.6 nM $O_2^-/10^6$ PMN/min in calcium medium and 6.8 ± 2.6 nM $O_2^-/10^6$ PMN/min in Ca^{++}-free medium by unloaded cells in this experiment.

‡ Maximal extent of O_2^- production was 18 ± 13 nM $O_2^-/10^6$ PMN in calcium medium and 16.8 ± 8.7 nM $O_2^-/10^6$ PMN in calcium-free medium by unloaded cells in this experiment. For these experiments O_2^- production was continuously monitored in the spectrophotometer for 10 min at 37°C.

§ Intracellular quin 2 (nmol quin $2/10^6$ cells) under various loadings were as follows: 0.39 ± 0.12 (30 μM); 0.75 ± 0.15 (60 μM); 1.2 ± 0.2 (100 μM).

The relationship between $[Ca^{2+}]_i$ rises and O_2^- production is not as clear-cut as the one observed in exocytosis. We have previously observed that rises in calcium above micromolar levels induced by the calcium ionophore ionomycin results in negligible O_2^- production, in contrast to stimulation of exocytosis (11). In the present report we show that depletion of intracellular calcium stores by ionomycin leads to total abolishment of fMLP-induced O_2^- production. Furthermore, if

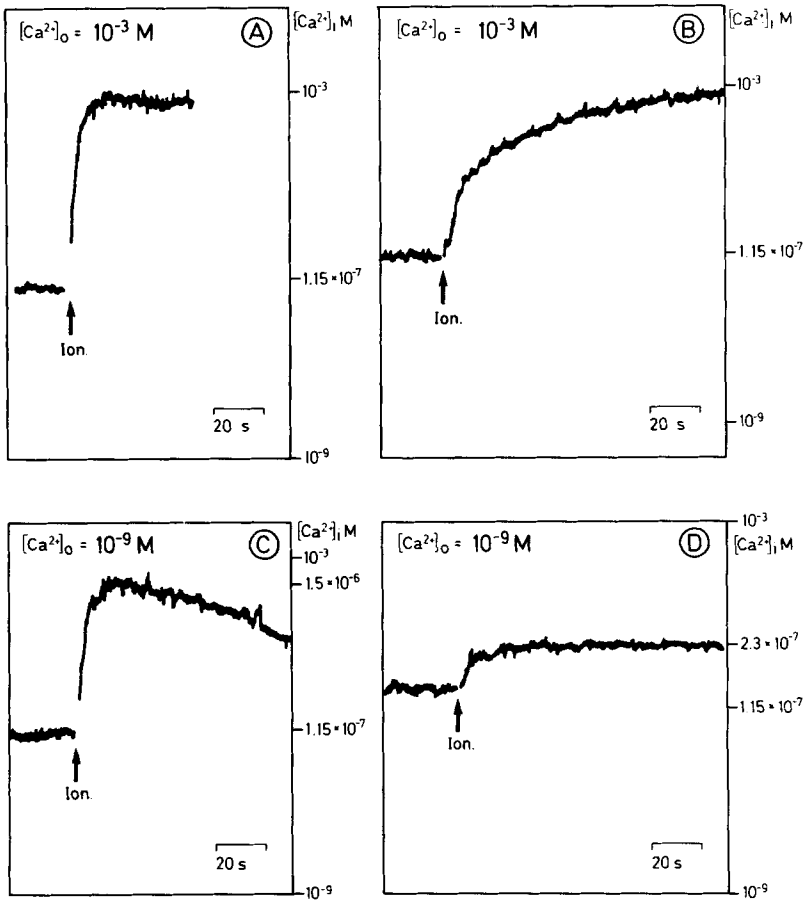

FIGURE 3 Effect of intracellular quin 2 on $[Ca^{2+}]_i$ changes in neutrophils exposed to ionomycin (ion) in calcium or calcium-free medium. (*A* and *C*) $[quin\ 2]_i = 0.22$ nmol quin $2/10^6$ PMN (0.6 mM); (*B* and *D*) $[quin\ 2]_i = 0.9$ nmol quin $2/10^6$ PMN (2.6 mM).

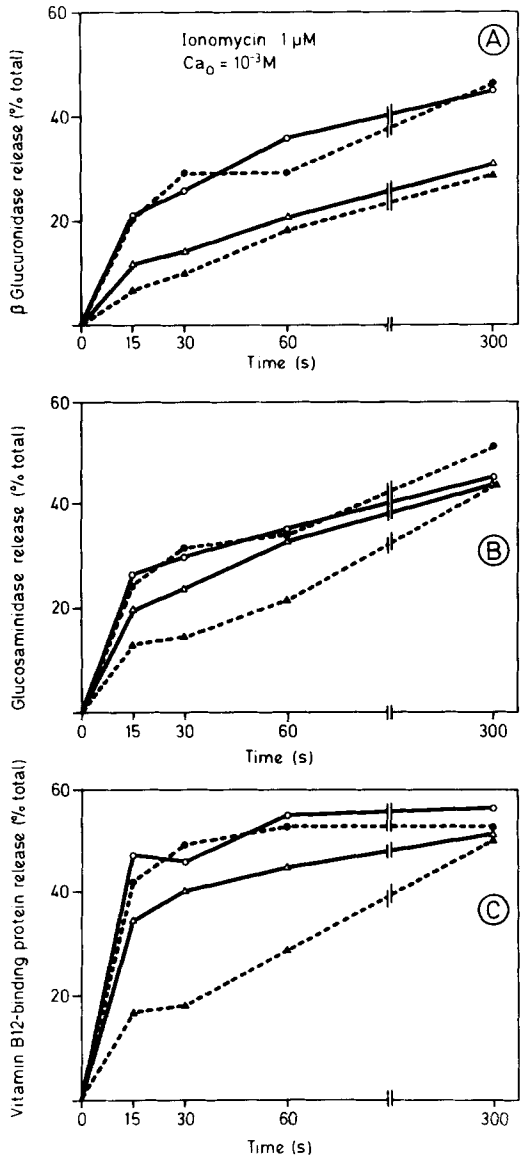

FIGURE 4 Effect of intracellular quin 2 on granule exocytosis induced by ionomycin as a function of time. Extracelluar calcium was 10^{-3} M throughout. (*A*) β-glucuronidase release; (*B*) glucosaminidase release; (C) vitamin B_{12}-binding-protein release. O, 0 nmol quin 2/10^6 PMN; ●, 0.37 nmol quin 2/10^6 PMN; Δ, 0.72 nmol quin 2/10^6 PMN; ▲, 1.1 nmol quin 2/10^6 PMN.

cells are loaded with increasingly higher concentrations of quin 2 there is a pronounced decrease in the initial rates and extent of superoxide production induced by fMLP. However, in contrast to exocytosis, no major differences were observed in calcium or calcium-free medium. The initial calcium rise that occurs in the first seconds, is the same in the presence or absence of extracellular calcium and the initial $[Ca^{2+}]_i$ rise is decreased to a similar extent by quin 2 loading under both conditions. Following this initial calcium rise, $[Ca^{2+}]_i$ is maintained at high levels or continues to rise secondary to calcium influx from the extracellular medium, only in the presence of normal extracellular calcium. These results suggest that the important calcium pool for activation of the superoxide generating system is the one that is rapidly mobilized from intracellular stores upon interaction of fMLP with its receptor, possibly by an intermediate of phosphoinositol metabolism (21). However, this role of calcium does not seem to apply to the sustained activity of the O_2^- generating system. Thus under most conditions, where both parameters were measured simultaneously, $[Ca^{2+}]_i$ remains elevated longer than the superoxide production detected extracellularly. Another observation that favors the view that maintenance of O_2^- production is modulated by $[Ca^{2+}]_i$ differently than initiation of O_2^- production, stems from a report showing reversible inhibition by submicromolar calcium of NADPH-dependent O_2^- generation by activated inside-out plasma membrane vesicles from macrophages (15).

Four lines of evidence indicate that quin 2 loading does not alter cell function in a nonspecific manner, but rather is related to its calcium-buffering properties. Firstly, PMA, a compound that activates neutrophils without raising $[Ca^{2+}]$ is able to elicit granule release independently of quin 2 loading or of the presence of calcium (Fig. 6*C* and references 18 and 19). Secondly, Anis/AM used to control the effect of trapping intracellularly acetoxymethylester derivatives did not change cell function. Thirdly, even in high quin 2 loading, ionomycin causes full stimulation of enzyme release, albeit at lower rates. Fourthly, the reduction of fMLP-induced exocytosis by quin 2 loading is particularly sensitive to the omission of external calcium.

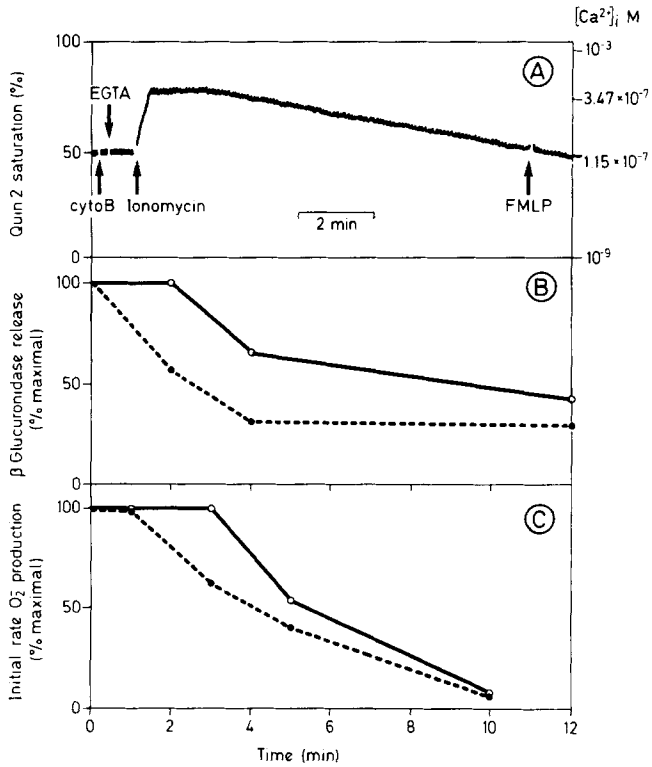

FIGURE 5 Effect of depletion of intracellular calcium on $[Ca^{2+}]_i$ transients, exocytosis and O_2^- production induced by fMLP (10^{-7} M). Cells were incubated in calcium-free medium. Cytochalasin B (5 μg/ml), and EGTA (1 mM) were added, followed by ionomycin (1 μM). fMLP was then added at various times after ionomycin as indicated in *B* and C, and β-glucuronidase release or O_2^- production were measured. (*A*) Kinetics of $[Ca^{2+}]_i$; (*B*) β-glucuronidase release measured 5 min after addition of fMLP and expressed as percent of maximal values (fMLP and ionomycin added simultaneously) after subtraction of basal levels; (C) Initial rate of O_2^- production. All experiments were performed on the same batch of neutrophils. ●, unloaded cells; O, loaded cells (0.6 nmol 10^6 quin 2 PMN).

TABLE III

Effect of Intracellular Quin 2 on Granule Content Release Induced by Ionomycin (1 μM)

Loading concentration	Time	Total cellular protein					
		β-Glucuronidase		Glucosaminidase		Vitamin B_{12}-binding protein	
μM	s	%		%		%	
0		20 ± 3		27 ± 6		41 ± 16	
30	15	16 ± 4	n.s.	20 ± 3	n.s.	31 ± 13	n.s.
60		11 ± 3	$P < 0.01$	17 ± 7	$P < 0.05$	25 ± 9	n.s.
100		7 ± 1	$P < 0.01$	12 ± 4	$P < 0.01$	15 ± 6	$P < 0.05$
0		28 ± 4		35 ± 7		44 ± 9	
30	30	24 ± 6	n.s.	28 ± 5	n.s.	39 ± 11	n.s.
60		12 ± 3	$P < 0.05$	20 ± 8	n.s.	26 ± 13	n.s.
100		10 ± 5	$P < 0.01$	15 ± 8	$P < 0.01$	18 ± 3	$P < 0.01$
0		36 ± 11		33++		54*	
30	60	27 ± 7	n.s.	31		52	
60		22 ± 9	$P < 0.01$	24		41	
100		14 ± 7	$P < 0.05$	18		29	
0		37 ± 6		43 ± 3		54 ± 7	
30	300	33 ± 13	n.s.	37 ± 13	n.s.	48 ± 12	n.s.
60		25 ± 8	n.s.	32 ± 13	n.s.	46 ± 12	n.s.
100		23 ± 7	n.s.	33 ± 12	n.s.	41 ± 19	n.s.

Results are mean ± SD of three determinations performed on three different neutrophil preparations. Statistical analysis was performed as in Table I. Intracellular quin 2 (nanomoles quin 2/10^6 cells) under various loadings were as follows: 0.38 ± 0.10 (30 μM; 0.8 ± 0.1 (60 μM); 1.3 ± 0.2 (100 μM).

* Average of duplicates.

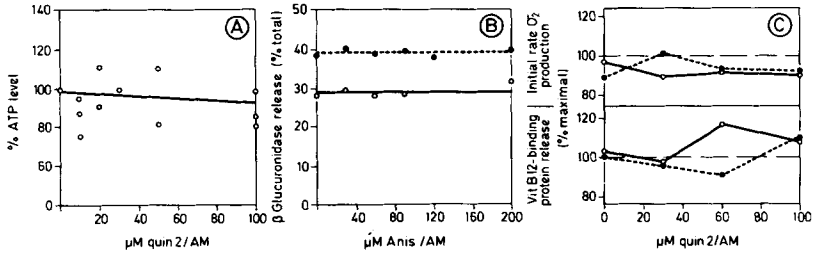

Figure 2 Evidence for the specificity of quin 2. (*A*) The effect of quin 2/AM loading on the levels of intracellular ATP. ATP was measured on an aliquot of the quin 2 loaded cells as described by Pozzan et al. (31) and expressed as percent ATP of untreated cells. Intracellular quin 2 concentrations ranged in this experiment from 0 to 1.3 nmol quin 2/10^6 cells. (*B*) The effect of Anis/AM loading on β-glucuronidase release in Ca^{2+} (●) or Ca^{2+}-free medium (○) induced by fMLP (10^{-7} M). Anis/AM loading was performed as for quin 2/AM. Anis/AM is a nonfluorescent quinolein derivative of quin 2 (see text). (*C*) The effect of quin 2/AM loading on phorbol myristate acetate (30 nmol) stimulated vitamin B_{12}-binding protein release and O_2^- production in Ca^{2+} (●) or Ca^{2+}-free medium (○). Intracellular quin 2 concentrations ranged in this experiment from 0 to 1.2 nmol quin 2/10^6 cells.

In the present investigation, the effect of increasing intracellular calcium buffering capacity has been studied in detail only with fMLP and ionomycin and demonstrates a role of calcium in the control of granule content release by exocytosis and O_2^- activation. Preliminary observations indicate that the same conclusion may be drawn for most physiological stimulators of neutrophils, capable of eliciting rises in $[Ca^{2+}]_i$ such as leukotriene B4, or C5a (22, 23).

The effects of quin 2 loading on Ca^{2+} transients in human neutrophils is similar to that observed in B lymphocytes stimulated with antisurface immunoglobulin (9). However, while the process of capping in B lymphocytes appears to be independent of intracellular calcium, the effect of buffering intracellular calcium on calcium-dependent functions of these cells was not assessed (9). It is possible that increased calcium buffering will affect various cell types differently; thus in Ca^{2+} medium there was a slight decrease of insulin release from rat insulinoma cells (7), whereas no changes in steroidogenesis were found in adrenal glomerulosa cells (24).

We would thus like to conclude that an elevation of $[Ca^{2+}]_i$ is not only important but also a necessary event for the activation of metabolic responses by physiological agonists in human neutrophils. Despite this, there are other factors that are critical for full expression of fMLP stimulation of metabolic responses in these cells. Thus, an increase in $[Ca^{2+}]_i$ induced by calcium ionophores is not sufficient to induce secretion in calcium-free medium, or O_2^- production, even in Ca^{2+} medium (11). Likewise, in calcium-free medium in the presence of ionomycin, fMLP causes metabolic activation without a further increase in $[Ca^{2+}]_i$ (11). Preliminary results by our group (32, 33) suggest that the lacking signal that synergizes with $[Ca^{2+}]_i$ to stimulate the cells, is the activation of the ubiquitous phospholipid-dependent protein, kinase c, by a receptor mediated generation of phosphatidylinositol metabolites, and its polyphosphate derivatives (25–27). Thus while the relationship of the rise in $[Ca^{2+}]_i$ and protein kinase c activation by chemotactic peptides is not clear (28–30), both pathways appear necessary for neutrophil stimulation.

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