Evidence that phorbol ester interferes with stimulated Ca^{2+} redistribution by activating Ca^{2+} efflux in neutrophil leucocytes

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The free calcium ion concentration, $[\text{Ca}^{2+}]_i$, in the cytoplasmic matrix of quin2-loaded neutrophil leucocytes increases rapidly after addition of concanavalin A. This increase is effectively abolished by a short (3 min) preincubation with 10 nM-TPA (12-*O*-tetradecanoylphorbol 13-acetate). TPA also inhibits a $[\text{Ca}^{2+}]_i$ rise of similar magnitude induced by low concentrations (10 nM) of calcium ionophore A23187, suggesting that phorbol ester does not interfere with a physiological influx mechanism. To investigate the effects of TPA further, cells were depleted of Ca^{2+} during quin2 loading and then re-equilibrated with normal extracellular $[\text{Ca}^{2+}]$. The return to a stable $[\text{Ca}^{2+}]_i$ value was preceded by a transient overshoot in $[\text{Ca}^{2+}]_i$, implying delayed activation of an efflux mechanism by rising $[\text{Ca}^{2+}]_i$. TPA abolished the transient, suggesting preactivation by TPA of the efflux mechanism before Ca^{2+} influx. TPA also stimulates net Ca^{2+} efflux from neutrophils and neutrophil cytoplasts. These observations are consistent with the thesis that TPA stimulates a Ca^{2+} -efflux mechanism in these cells.

INTRODUCTION

Unstimulated neutrophil leucocytes maintain a stable low intracellular free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, in the range 10–100 nM (Schell-Frederick, 1984; Gennaro *et al.*, 1984). This stability presumably reflects a steady state in which the rate of influx of Ca^{2+} into the cytoplasmic matrix is balanced by efflux from the cytoplasm out of the cell or into intracellular stores. Thus changes in $[\text{Ca}^{2+}]_i$ can occur by modulation of either influx or efflux mechanisms. Efflux of Ca^{2+} from the cytoplasmic matrix of neutrophils against an electrochemical gradient is mediated by ATP-dependent Ca^{2+} transporters located in the plasma membrane (Volpi *et al.*, 1982; Lagast *et al.*, 1984a) and a non-mitochondrial intracellular membrane pool (Prentki *et al.*, 1984). A $\text{Na}^+/\text{Ca}^{2+}$ exchanger is also present in plasma membranes, but is considered to have a minor role in calcium homeostasis in resting cells (Carafoli, 1984). Increases in $[\text{Ca}^{2+}]_i$ during receptor-mediated stimulation are assumed to be the result of increased Ca^{2+} influx into the cytoplasmic matrix, either from the extracellular medium or from an intracellular pool (for review see Rasmussen & Barrett, 1984).

The phorbol ester TPA has been reported to stimulate a wide variety of cell types, including neutrophils, with effects similar to those produced by physiological stimuli (Lehmeyer *et al.*, 1979; Castagna *et al.*, 1982; Tsien *et al.*, 1982a; Rosoff *et al.*, 1984; Sagi-Eisenberg *et al.*, 1985), but associated with no change (Sagi-Eisenberg *et al.*, 1985) or a slow decrease (Tsien *et al.*, 1982a; Sha'afi *et al.*, 1983; Lagast *et al.*, 1984b; Schell-Frederick, 1984) in $[\text{Ca}^{2+}]_i$ as determined by using the fluorescent calcium indicator quin2. Activation of protein kinase C has been proposed as the primary mechanism of action of TPA (Nishizuka, 1984).

Although TPA appears to induce a cellular response by mechanisms which do not involve an increase in $[\text{Ca}^{2+}]_i$, effects on $^{45}\text{Ca}^{2+}$ fluxes (Malaisse *et al.*, 1980; Mottola &

Romeo, 1982; Korchak *et al.*, 1984) and Ca^{2+} extrusion from neutrophils (Mottola & Romeo, 1982) have been observed. Inhibition by TPA of agonist-induced increases in $[\text{Ca}^{2+}]_i$ has also been reported (Tsien *et al.*, 1982a; Sha'afi *et al.*, 1983; Lagast *et al.*, 1984b; Gennaro *et al.*, 1984; Sagi-Eisenberg *et al.*, 1985). Various hypotheses have been proposed to account for these observations. For example, TPA may prevent Ca^{2+} influx (Sagi-Eisenberg *et al.*, 1985), it may deplete or immobilize intracellular Ca^{2+} stores (Sha'afi *et al.*, 1983), or it may activate a plasma-membrane Ca^{2+} transporter (Tsien *et al.*, 1982a). This last possibility is consistent with the observation that the ATP-dependent Ca^{2+} transporter in a plasma-membrane preparation from guinea-pig neutrophils appears to have a higher activity when membranes are isolated from TPA-treated cells (Lagast *et al.*, 1984b).

Using the fluorescent Ca^{2+} chelator quin2 (Tsien *et al.*, 1982b), we have demonstrated an inhibition by TPA of passive, ionophore-mediated and ConA-stimulated rises in $[\text{Ca}^{2+}]_i$ in neutrophil leucocytes, and a stimulation by TPA of net Ca^{2+} extrusion by neutrophils and cytoplasts. These results are interpreted as a TPA-induced activation of a plasma-membrane Ca^{2+} -efflux mechanism.

EXPERIMENTAL

Preparation of cells and cytoplasts

Neutrophil leucocytes were prepared from freshly collected pig blood as described previously (Sheterline *et al.*, 1984a). The cytoplast preparation was essentially as outlined by Sheterline *et al.* (1984b), except that the Ficoll gradient steps were 23%, 18% and 15%. A suspension of 1.5×10^8 cells in modified Hanks salt solution (Sigma Chemical Co., Poole, Dorset, U.K.) containing 20 μM -cytochalasin B (Sigma) was layered on each gradient (about 13.5 ml total) and centrifuged in a SW40 rotor at 26000 rev./min (90000 g_{av}) in a Beckman L-8

Abbreviations used: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; ConA, concanavalin A; $[\text{Ca}^{2+}]_i$, intracellular free calcium concentration; DMSO, dimethyl sulphoxide.

centrifuge for 50 min at 37 °C. Cytoplasts were collected from the boundary between the 18% and 15% Ficoll layers and washed five times in Hepes-buffered Hanks salt solution to remove cytochalasin (Sheterline *et al.*, 1984a).

Quin2 loading and measurement of $[Ca^{2+}]_i$

The method used for loading cells with quin2 was essentially that described by Tsien *et al.* (1982b). The buffer used throughout was Hepes-buffered Hanks, but Phenol Red was omitted and bovine serum albumin (1 mg/ml; Sigma, globulin-free) was included. Where low extracellular Ca^{2+} is specified for incubations, $CaCl_2$ was omitted and 1 mM-EGTA included. Ca^{2+} -depleted cells were prepared by loading with quin2 in the EGTA buffer. Cells at 2×10^7 /ml were incubated for 15 min at 37 °C with 10 μ M-quin2 acetoxymethyl ester (Amersham International, Amersham, Bucks., U.K.) added from a 20 mM stock solution in DMSO. Parallel cell preparations were incubated with an equivalent volume of DMSO for autofluorescence measurements. After loading, cells were centrifuged for 3 min at 200 $g_{av.}$, resuspended at 10^7 /ml and stored at room temperature. Quin2 content was 0.23–0.33 nmol/ 10^6 cells. Fluorescence was monitored in an LS-5 luminescence spectrometer (Perkin-Elmer, Beaconsfield, Bucks., U.K.) by using a water-jacketed cuvette holder maintained at 37 °C, fitted with a magnetic stirring device (Rank Bros., Cambridge, U.K.). Excitation and emission wavelengths were 333 nm and 494 nm, with 5 nm and 10 nm slit widths respectively. For each measurement, cells were washed once by a brief (1–2 s) centrifugation in a Beckman Microfuge, resuspended at 10^7 cells/3 ml and then placed in the cuvette. Cells were stirred throughout each experiment and additions were made during continuous recording. A23187 and TPA (Sigma) were added, at concentrations indicated in the Results and discussion section, as solutions in DMSO. The final DMSO concentration never exceeded 0.1% (v/v) and the effect of DMSO alone was measured on parallel cell preparations. ConA (BDH Chemicals, Poole, Dorset, U.K.) was added from a 10 mg/ml stock solution in Hepes-buffered Hanks. In experiments where Ca^{2+} was added back to the external medium, Ca^{2+} was added (final concn. 2 mM) from a 0.5 M- $CaCl_2$ stock, the pH of the medium being maintained by addition of an appropriate volume of 0.5 M-NaOH.

At the end of each experiment, the quin2 signal was calibrated, to allow calculation of $[Ca^{2+}]_i$ and to estimate the quin2 loading. To correct for quin2 which may have leaked out from cells, 0.4 mM- $MnCl_2$, which quenches extracellular quin2 fluorescence, was added from a 0.1 M stock. To lyse the cells and measure $L_{min.}$, 0.1% (v/v) Triton X-100 was added from a 3% stock. $L_{max.}$ was measured by addition of 1.7 mM-diethylenetriaminepentaacetic acid, a Mn^{2+} chelator, from a 0.1 M stock in equimolar $CaCl_2$. $[Ca^{2+}]_i$ was then calculated by using the equation $[Ca^{2+}]_i = K_d(I - 0.16 I_{tot.}/I_{tot.} - I)$ (Hesketh *et al.*, 1983), where I was the fluorescence signal measured from quin2-loaded cells, corrected for autofluorescence measured during parallel treatments of unloaded cells, and $I_{tot.}$ was the total quin2 signal, equal to $L_{max.} - L_{min.}$. The value used for the K_d of Ca-quin2 was 115 nM, as determined by Tsien *et al.* (1982b). $I_{tot.}$ was also used to estimate the quin2 content of the cells, by using a standard curve generated with quin2 free acid (Lancaster Synthesis, Morecambe, Lancs., U.K.).

Measurement of Ca^{2+} extrusion

Cells (10^7 /3 ml) and cytoplasts (1.2×10^6 – 1.2×10^7 /ml) were incubated at 37 °C in Hepes-buffered Hanks containing 1 mg of albumin/ml but omitting $CaCl_2$ and EGTA. They were stored in Ca^{2+} -containing buffer before use, then washed once in Ca^{2+} -free Hanks just before addition to the cuvette and equilibrated at 37 °C for 5 min before additions were made. In some experiments, contaminating Ca^{2+} was removed from the medium by pretreatment with Chelex-100 (Bio-Rad Laboratories, Watford, Herts., U.K.). Quin2 free acid was added from a 20 mM stock in DMSO to a final concentration of 10 or 16.7 μ M, a concentration sufficient to set the free $[Ca^{2+}]$ of the medium at approx. 100 nM. Ca^{2+} extrusion was measured as increasing quin2 fluorescence, indicating changing free $[Ca^{2+}]$, for 20 min after additions. At the end of each experiment, $L_{max.}$ was measured after addition of $CaCl_2$ to 4 mM, and $L_{min.}$ after addition of $MnCl_2$ to 0.8 mM. Total Ca^{2+} content of the medium at various times was calculated as the sum of free $[Ca^{2+}]$ plus [Ca-quin2] and converted into nmol of Ca^{2+} extruded/ 10^7 cells or cytoplasts. The K_d of Ca-quin2 was assumed to be 115 nM; this K_d was measured at pH 7.05, but is reported to be insensitive to the increased pH (7.4) in the extracellular medium (Tsien *et al.*, 1982b).

RESULTS AND DISCUSSION

The fluorescent Ca^{2+} chelator quin2 can be trapped inside the cytoplasmic matrix of cells, where it acts as a real-time indicator of $[Ca^{2+}]_i$ changes (Tsien *et al.*, 1982b). Neutrophil leucocytes were loaded with quin2, and in Ca^{2+} -containing medium the fluorescence signal reported a $[Ca^{2+}]_i$ of 42.5 ± 6.2 nM (mean $\pm$ S.E.M., $n = 10$).

The lectin ConA binds to and cross-links surface glycoproteins of neutrophils, leading to capping of lectin and the membrane proteins to which it is bound (Sheterline & Hopkins, 1981). ConA stimulates an increase in $[Ca^{2+}]_i$, which may be involved in regulation of myosin light-chain phosphorylation during capping (Bourguignon & Bourguignon, 1984) and is shown in Fig. 1(a) as a fluorescence increase. $[Ca^{2+}]_i$ rose steadily with no detectable lag after addition of lectin. In several experiments, ConA always caused a similar pattern of $[Ca^{2+}]_i$ change, but the $t_{\frac{1}{2}}$ for the rise and the $[Ca^{2+}]_i$ reached were linearly dependent on the quin2 content of the cells, demonstrating increased intracellular Ca^{2+} buffering by quin2. For example, cells loaded with 0.063 nmol of quin2/ 10^6 cells showed a ConA-stimulated increase in $[Ca^{2+}]_i$ to 400 nM, with a $t_{\frac{1}{2}}$ for the rise of 2.3 min, whereas for cells containing 0.381 nmol of quin2/ 10^6 cells the $[Ca^{2+}]_i$ reached was 165 nM and the $t_{\frac{1}{2}}$ was 6.4 min. For the experiment shown in Fig. 1, the quin2 content was 0.32 nmol/ 10^6 cells; $[Ca^{2+}]_i$ rose to 287 nM and the $t_{\frac{1}{2}}$ for the rise was 4.5 min.

The Ca^{2+} influx induced by ConA is largely dependent on extracellular Ca^{2+} . Fig. 1(b) demonstrates the effect of ConA on cells in low- Ca^{2+} medium. The residual increase in $[Ca^{2+}]_i$ is possibly due to release of Ca^{2+} from intracellular stores. The free $[Ca^{2+}]$ of the low-extracellular- Ca^{2+} medium was estimated as approx. 4 nM (by using quin2 free acid), which suggests that significant influx of Ca^{2+} across the plasma membrane is unlikely. This result therefore implies that ConA induces net influx of Ca^{2+} across the plasma membrane.

Fig. 1. Effect of TPA on the changes in $[\text{Ca}^{2+}]_i$ induced by ConA and A23187 in quin2-loaded neutrophils

Neutrophils were loaded with quin2 in Ca^{2+} medium, and measurements of fluorescence changes were made in Ca^{2+} or EGTA medium as indicated. Additions of TPA (10 nM), ConA (20 $\mu\text{g}/\text{ml}$) or A23187 (10 nM) were made where shown by the arrows. The scale at the right of each trace calibrates the fluorescence signal for $[\text{Ca}^{2+}]_i$ (nm).

The effect of TPA on the ConA-stimulated $[\text{Ca}^{2+}]_i$ change is shown in Fig. 1(c); 10 nM-TPA was added to the cells 3 min before ConA, but the conditions were otherwise the same as for Fig. 1(a). Conditions for optimum effect of TPA were determined in experiments on Ca^{2+} -depleted cells, described below. Fig. 1(c) demonstrates that TPA almost abolishes the $[\text{Ca}^{2+}]_i$ rise. Since the rise is mainly due to influx at the plasma membrane, it seems likely that TPA exerts its effect either here, or by a stimulation of an efflux mechanism, which could nullify the $[\text{Ca}^{2+}]_i$ rise caused by stimulated influx. Reports of an inhibition by TPA of $[\text{Ca}^{2+}]_i$ rises in other cell types also used receptor-directed stimuli to induce the Ca^{2+} influx (Lagast *et al.*, 1984b; Sagi-Eisenberg *et al.*, 1985; MacIntyre *et al.*, 1985). These data can therefore be interpreted in a similar way.

The ionophore A23187 can also induce $[\text{Ca}^{2+}]_i$ rises, but by-passes any physiological influx mechanisms. This lipid-soluble compound promotes electroneutral exchange of Ca^{2+} and H^+ across cell membranes, driven by concentration gradients (Pressman, 1976). Fig. 1(d) demonstrates that there is a rapid rise in $[\text{Ca}^{2+}]_i$ on addition of A23187 to quin2-loaded neutrophils. Comparison with Fig. 1(e), measured in low extracellular Ca^{2+} , shows that the rise is largely dependent on Ca^{2+} influx at the plasma membrane. Fig. 1(f) shows that, after TPA preincubation, there is a dramatic inhibition of the $[\text{Ca}^{2+}]_i$ change, although there is a small but steady rise

over 10 min. The $[\text{Ca}^{2+}]_i$ changes demonstrated in Fig. 1(a) and 1(d) both represent primarily an increase in net Ca^{2+} influx at the plasma membrane, but induced by different mechanisms. The dramatic inhibition of both these stimulated increases in $[\text{Ca}^{2+}]_i$ by TPA is more readily explained, therefore, by stimulated Ca^{2+} efflux rather than by blocking of Ca^{2+} influx.

The effect of TPA on a third mechanism of Ca^{2+} influx was also examined. For these experiments, neutrophils were incubated with quin2 acetoxymethyl ester in the absence of extracellular Ca^{2+} . Under these conditions, the cells accumulate quin2 as usual, but are unable to take up extra Ca^{2+} to compensate for the increased intracellular Ca^{2+} buffering. The result is a lowered $[\text{Ca}^{2+}]_i$, and also a depletion of intracellular Ca^{2+} stores as Ca^{2+} is sequestered by quin2, which has a higher affinity for Ca^{2+} than do these stores (Tsien *et al.*, 1982b). The absence of releasable Ca^{2+} stores in such cells was demonstrated by ConA stimulation in low extracellular Ca^{2+} , which had no effect on $[\text{Ca}^{2+}]_i$ (results not shown). The quin2 fluorescence of Ca^{2+} -depleted neutrophils prepared in this way indicated a $[\text{Ca}^{2+}]_i$ of 20.7 ± 2.4 nM ($n = 5$). When Ca^{2+} is added back to the extracellular medium of these cells, there is a rapid influx of Ca^{2+} . The changes in quin2 fluorescence under various conditions are shown in Fig. 2. The upper trace in both Figs. 2(a) and 2(b) shows typical $[\text{Ca}^{2+}]_i$ changes during Ca^{2+} uptake by Ca^{2+} -depleted cells. There is a rapid increase

Fig. 2. Effect of TPA on the $[Ca^{2+}]_i$ transient induced in quin2-loaded Ca^{2+} -depleted neutrophils by addition of extracellular Ca^{2+} .

Neutrophils were depleted of Ca^{2+} by loading with quin2 in medium containing 1 mM-EGTA, and measurements were made in the same. (a) TPA (large arrow) was added at the concentrations shown, 3 min before 2 mM- Ca^{2+} (small arrow). (b) 1 nM-TPA (large arrow) was added at the times shown before addition of 2 mM- Ca^{2+} (small arrow). The left-hand scales calibrate each control trace for $[Ca^{2+}]_i$ (nM), but similar scales can be drawn for each trace. The broken lines join gaps in the traces during additions.

in $[Ca^{2+}]_i$, which must be due to Ca^{2+} entering across the plasma membrane, as all freely exchangeable cell Ca^{2+} has been chelated by quin2. The $[Ca^{2+}]_i$ reaches a sharp maximum, then recovers to a lower value, 160.8 ± 35.8 nM ($n = 3$), after 7 min.

The transient overshoot in $[Ca^{2+}]_i$ is consistent with a delay in activation of an efflux mechanism by rising $[Ca^{2+}]_i$, which overcomes influx and allows the subsequent achievement of steady state. This result is in contrast with the effect of Ca^{2+} addition to Ca^{2+} -depleted lymphocytes reported by Tsien *et al.* (1982b). In these cells, the $[Ca^{2+}]_i$ rose to a new value without a transient overshoot. Possibly this represents differences in Ca^{2+} homeostatic mechanisms and sensitivity to Ca^{2+} depletion between the two cell types. The plasma-membrane Ca^{2+} transporter of neutrophils is likely to play a major role in the recovery that occurs in these experiments (Borle, 1981), but there is evidence for a role of intracellular membranes in recovery from rapid changes in $[Ca^{2+}]_i$ in neutrophils (Prentki *et al.*, 1984). A transient rise in $^{45}Ca^{2+}$ content of red blood cells induced by A23187 was shown by Scharff *et al.* (1983), who used a model involving hysteretic activation of the plasma-membrane Ca^{2+} -

ATPase to explain the transient. The red-blood-cell Ca^{2+} -ATPase is known to be activated by Ca^{2+} -calmodulin, but the activation is not instantaneous, presumably because of slow binding (Vincenzi *et al.*, 1980). The Ca^{2+} pump in neutrophil plasma membrane is also sensitive to calmodulin *in vitro* (Lagast *et al.*, 1984a), and may therefore show slow activation by Ca^{2+} -calmodulin by a similar mechanism. It is noteworthy in these experiments that $[Ca^{2+}]_i$ recovers to 160.8 nM, which is considerably higher than the $[Ca^{2+}]_i$ found in cells loaded with quin2 in the presence of extracellular Ca^{2+} (42.5 nM). The reason for this is not clear, but it suggests a change in relative affinities of the various cellular mechanisms for Ca^{2+} homeostasis.

The model of Scharff *et al.* (1983) also predicts that a transient overshoot in $[Ca^{2+}]_i$ should not occur if the exit pump is in an activated state at the start of Ca^{2+} influx. The experiment shown in Fig. 2(a) demonstrates the effect of TPA on the Ca^{2+} influx, abolishing the transient at low concentrations, and decreasing the equilibrium $[Ca^{2+}]_i$ at higher concentrations; 10 nM-TPA was sufficient to give a maximum effect. The equilibrium value of $[Ca^{2+}]_i$ after 7 min was 39.6 ± 4.0 nM ($n = 3$) at 10 nM-TPA. Fig. 2(b) demonstrates that the effect of 1 nM-TPA is not immediate, but that a 3 min preincubation is necessary to produce maximum effect. The delay in effect of TPA would be consistent with a mechanism involving activation of protein kinase C, followed by a rate-limiting, possibly phosphorylation, event.

The effect of TPA on $[Ca^{2+}]_i$ increases can be explained in terms of activation by TPA of a Ca^{2+} -efflux mechanism at the plasma membrane. This is consistent with the demonstration by Lagast *et al.* (1984b) of a TPA-induced increase in the activity of an ATP-dependent Ca^{2+} transporter in a plasma-membrane preparation from guinea-pig neutrophils. They reported an inhibition by TPA of the rise in $[Ca^{2+}]_i$ induced by chemotactic peptide, which they attributed to the increased activity of the Ca^{2+} transporter. This inference predicts that TPA would also cause an increase in net Ca^{2+} efflux from cells. Quin2 free acid was therefore added to the extracellular medium to monitor changes in total $[Ca^{2+}]$ in the medium. Neutrophils incubated in a low- Ca^{2+} medium (free $[Ca^{2+}]$ approx. 100 nM) showed a steady loss of Ca^{2+} over 20 min (Fig. 3a). On addition of TPA, after a lag of approx. 30 s, the rate of Ca^{2+} extrusion increases and is maintained for at least 20 min. This effect is concentration-dependent. The curves show slight non-linearity, which may be due to a decreasing rate of efflux with time. In these experiments, however, the calculation of absolute Ca^{2+} values depends on the assumption that the K_d for Ca-quin2 is the same in our extracellular medium as in an 'intracellular buffer'; a slight inaccuracy in the K_d used would affect the shape of the calculated curve. The fluorescence changes measured, however, demonstrate an obvious effect of TPA on Ca^{2+} extrusion from these cells. The control cells, after 20 min, had lost 0.317 ± 0.087 ($n = 4$) nmol/ 10^7 cells; cells incubated with 10 nM-TPA for 20 min had extruded 1.907 ± 0.230 ($n = 4$) nmol/ 10^7 cells.

An increased relative rate of Ca^{2+} efflux from TPA-treated cells might be expected to cause a fall in $[Ca^{2+}]_i$; therefore the effect of TPA on $[Ca^{2+}]_i$ of quin2-loaded neutrophils was examined in more detail. At 10 nM, TPA had very little effect on $[Ca^{2+}]_i$ of cells in normal or low extracellular Ca^{2+} . During a 15 min

Fig. 3. Effect of TPA on Ca^{2+} extrusion by neutrophils (a) and cytoplasts (b)

Ca^{2+} extrusion was measured from the fluorescence change of quin2 free acid in the extracellular medium. Additions made at zero time were: $\circ$, DMSO; $\triangle$, 2 nM-TPA; $\square$, 10 nM-TPA. All curves represent mean data from four cell and cytoplast preparations.

incubation in TPA, $[\text{Ca}^{2+}]_i$ changed from 30.0 ± 4.5 to 28.7 ± 3.7 nM ($n = 4$) in 1.3 mM- Ca^{2+} medium and from 28.6 ± 1.1 to 22.6 ± 2.2 nM ($n = 4$) in 1 mM-EGTA medium. The small decrease in $[\text{Ca}^{2+}]_i$ in low extracellular Ca^{2+} was not statistically significant for the small number of samples. Incubation in low extracellular Ca^{2+} alone also had no effect; the $[\text{Ca}^{2+}]_i$ of quin2-loaded neutrophils changed from 32.9 ± 1.7 to 31.2 ± 2.8 nM ($n = 4$) during a 15 min incubation in low- Ca^{2+} medium. This observation implies buffering of $[\text{Ca}^{2+}]_i$ by intracellular stores, since unloaded cells show net loss of Ca^{2+} under similar conditions (Fig. 3a).

TPA is known to induce secretion of specific granules by neutrophils (Lehmeyer *et al.*, 1979), so the possibility that stimulated Ca^{2+} extrusion was due to loss of

Ca^{2+} -containing granules was considered. We therefore prepared granule-free cytoplasts to minimize this problem. These plasma-membrane-bounded cytoplasmic fragments contain very few secretory granules (Sheterline *et al.*, 1984b; P. Sheterline & J. E. Rickard, unpublished work), but Ca^{2+} extrusion was stimulated more than 2-fold by 10 nM-TPA (Fig. 3b). The mean value for Ca^{2+} extrusion was 0.209 ± 0.100 ($n = 4$) nmol/ 10^7 cytoplasts after 20 min for control preparations, compared with 0.446 ± 0.117 ($n = 4$) nmol/ 10^7 cytoplasts after 20 min in 10 nM-TPA. The large s.e.m. values reflect differences in absolute values for Ca^{2+} extrusion between cytoplast preparations, but each preparation showed a similar pattern and extent of stimulation in the presence of TPA.

These data taken together indicate an effect of TPA in activating a Ca^{2+} -efflux mechanism at the plasma membrane. The known effect of TPA on protein kinase C suggests a mechanism involving protein phosphorylation, perhaps involving direct phosphorylation of the Ca^{2+} -ATPase or of an (unknown) regulatory protein. It should be noted that these results cannot exclude the possibility that TPA is acting via some other mechanism than activation of protein kinase C. However, the concentration (10 nM) at which maximum effects occur is close to the measured K_d (8 nM) for binding of a related phorbol ester to protein kinase C (Nishizuka, 1984), whereas effects other than on protein kinase C, for example on bilayer properties, take place at micromolar concentrations (Jacobson *et al.*, 1975). TPA has structural similarities with diacylglycerol, the presumed physiological activator of protein kinase C, and this protein is the only cellular binding site for phorbol esters so far described. This and other evidence support the view that the many cellular effects of TPA can be accounted for by activation of protein kinase C (for review, see Nishizuka, 1984). Activation of Na^+/H^+ exchange at the plasma membrane, leading to cytoplasmic alkalinization, has also been shown to be an early event of TPA activation in some cells (Rosoff *et al.*, 1984; Moolenaar *et al.*, 1984; Besterman & Cuatrecasas, 1984), which suggests that activated protein kinase C may regulate the behaviour of more than one plasma-membrane transporter.

TPA is a non-physiological stimulus, but activation of protein kinase C occurs during cell stimulation by certain physiological ligands via the generation of diacylglycerol from the action of a phospholipase C on phosphatidylinositol 4,5-bisphosphate (Nishizuka, 1984). Raised intracellular Ca^{2+} concentrations are also observed during cell activation, and the two effects appear to act synergistically in stimulation of cell function (Berridge & Irvine, 1984). Protein kinase C-mediated activation of Ca^{2+} efflux, as demonstrated here, is therefore an apparent paradox. However, a delayed activation of efflux may be involved as a negative-feedback mechanism in modulation of stimulated influx, and in recovery from raised cell $[\text{Ca}^{2+}]_i$ (Sagi-Eisenberg *et al.*, 1985; MacIntyre *et al.*, 1985). This effect has been observed in several cell types, suggesting that it may be a general result of the activation of protein kinase C.

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