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Use of fluo-3 to measure cytosolic Ca^{2+} in platelets and neutrophils

Loading cells with the dye, calibration of traces, measurements in the presence of plasma, and buffering of cytosolic Ca^{2+}

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A description is given of the methodology, and problems encountered, for the use of a new fluorescent Ca^{2+}-indicator dye, fluo-3, in neutrophils and platelets. The higher K_d and longer excitation wavelength of fluo-3 can have significant advantages over fura-2. Although neutrophils and platelets are used as examples, these observations will be applicable to other cell types. The K_d of fluo-3 for binding Ca^{2+} at 37 °C was measured and found to be 864 nM; the previously published value was 400 nM at 22 °C. The K_d of fluo-3, like that of fura-2, is therefore very temperature-dependent. Protocols for loading cells, and preventing leakage of fluo-3, are described; probenecid, known to inhibit fura-2 leakage from cells, was found to be essential to get good fluo-3 signals from platelets. Calibration of fluo-3 fluorescence signals to $[Ca^{2+}]$ and methods for obtaining maximum and minimum fluorescence signals are described; these methods differ from those used with fura-2. Agonist-stimulated responses of fluo-3-loaded neutrophils and platelets are shown, and the calculated cytosolic $[Ca^{2+}]$ is comparable with that previously obtained with fura-2. Responses of cells in the presence of plasma are also shown; such measurements, unobtainable with quin2, fura-2 or indo-1, are possible with fluo-3, owing to its longer excitation wavelengths. Co-loading of cells with bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid and fluo-3 is included as an example of how cytosolic $[Ca^{2+}]$ can be buffered and manipulated. Many of these observations will be of value when using fluo-3 (or other Ca^{2+}-indicator dyes) in most cell types.

INTRODUCTION

The use of fluorescent indicator dyes for the measurement of $[Ca^{2+}]_i$ has enabled considerable advances in our knowledge of cellular Ca^{2+} regulation (for review, see Cobbold & Rink, 1987). Quin2 was the first fluorescent $[Ca^{2+}]_i$-indicator dye to be described (Tsien *et al.*, 1982), and has provided much important information on many different cell types, including platelets (Rink *et al.*, 1982; Hallam *et al.*, 1984) and neutrophils (Pozzan *et al.*, 1983; Andersson *et al.*, 1986). However, quin2 has limitations: the K_d is low (115 nM at 37 °C), making it difficult to measure accurately peak $[Ca^{2+}]_i$ in stimulated cells; the quantum yield is relatively low, which means that high cytosolic concentrations are required for measureable signals (these high concentrations of dye will buffer changes in $[Ca^{2+}]_i$); quin2 requires excitation at u.v. wavelengths near the cut-off for transmission through glass, and these wavelengths tend to excite cellular autofluorescence (e.g. nicotinamide nucleotides), as well as fluorescence of proteins and drugs. The second generation of fluorescent indicators includes fura-2 (Grynkiewicz *et al.*, 1985). Fura-2 overcame many of the problems associated with quin2: the K_d is higher (224 nM at 37 °C) and the quantum yield is higher (up to 30-fold) than for quin2. The higher quantum yield of fura-2 allows measurement of $[Ca^{2+}]_i$ at lower cytosolic concentrations of dye, where buffering of $[Ca^{2+}]_i$ is less of a problem. The spectral shift of fura-2 fluorescence on binding Ca^{2+} is a major advantage, permitting ratio-measurements of $[Ca^{2+}]_i$; however, the excitation wavelengths of fura-2 are still shorter than would be desirable (for the same reasons as described above for quin2). Since the introduction of fura-2, it has been used to measure $[Ca^{2+}]_i$ in many cell types (e.g. Tsien & Poenie, 1986), including platelets (Pollock *et al.*, 1986; Merritt & Hallam, 1988; Sage *et al.*, 1989) and neutrophils (Merritt, 1989; Merritt *et al.*, 1989).

A new family of Ca^{2+}-indicator dyes, including fluo-3, has recently been described (Minta *et al.*, 1989), and results using fluo-3 with lymphocytes and fibroblasts have been reported (Kao *et al.*, 1989). Although fluo-3 does not exhibit a spectral shift on binding Ca^{2+}, it has several advantages over quin2 and fura-2. The K_d of fluo-3 is higher (400 nM at 22 °C), which allows more sensitive measurement at higher $[Ca^{2+}]_i$. Fluo-3 is highly fluorescent: binding of Ca^{2+} increases dye fluorescence by 40-fold. The visible excitation wavelength of fluo-3 is more convenient for fluorescence microscopy and flow cytometry than are the u.v. wavelengths of quin2 and fura-2. In addition, the higher excitation wavelengths of fluo-3 allow measurements in the presence of protein, as well as other compounds that are fluorescent at the wavelengths required for fura-2 and quin2. The higher K_d and fluorescence yield of fluo-3 permit measurement of $[Ca^{2+}]_i$ under conditions where there is less buffering of Ca^{2+}; however, cells can be co-loaded with fluo-3 and BAPTA if buffering of $[Ca^{2+}]_i$ is required. Use of BAPTA to buffer $[Ca^{2+}]_i$ in neutrophils has previously been documented (Nasmith & Grinstein, 1987).

Here, we describe methodology (and the problems encountered) for the use of fluo-3 in neutrophils and platelets. (Although neutrophils and platelets are used as examples, these results will be applicable to other cell types.) Since the original description of fluo-3 only gave a K_d value for binding to Ca^{2+} at 22 °C (Minta *et al.*, 1989), and binding of Ca^{2+} to the other related dyes (fura-2 and quin2) is known to be temperature-dependent (Grynkiewicz *et al.*, 1985), the first stage was to

Abbreviations used: BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'N'*-tetra-acetic acid; $[Ca^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; FMLP, *N*-formylmethionyl-leucyl-phenylalanine; U46619, dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$; AM, acetoxymethyl ester; LTB_4, leukotriene B_4.

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calculate the K_d of fluo-3 at 37 °C and to validate this K_d in an intact-cell system. Methods for loading the dye into cells (problems encountered and their solutions) are then described. The next problem was calibration to the cellular fluo-3 fluorescence to $[Ca^{2+}]_i$: owing to the high K_d of fluo-3 and the inability of Mn^{2+} to quench its fluorescence fully, the method used for fura-2 and quin2 could not be extended to use with fluo-3. $[Ca^{2+}]_i$-calibrated fluorescence traces of fluo-3-loaded platelets and neutrophils stimulated with various agonists are then shown. Responses of cells suspended in 50% plasma are compared with responses of cells in buffer. (It is not possible to make measurements in the presence of plasma with quin2 or fura-2.) Finally, the use of BAPTA plus fluo-3 to manipulate $[Ca^{2+}]_i$ is described, with a discussion of buffering of $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Fluo-3 acetoxymethyl ester (AM) and fluo-3 free acid were initially gifts from Dr. Roger Y. Tsien (Department of Pharmacology, School of Medicine, University of California San Diego, CA, U.S.A.), and were then purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). Fura-2 AM and fura-2 free acid were also from Molecular Probes. Probenecid, BSA (fraction V), thrombin (bovine), FMLP and digitonin were from Sigma; BAPTA AM and Hepes (ultrapure) were from Calbiochem; ATP and ADP were from Boehringer; U46619 was from the Upjohn Co. (Kalamazoo, MI, U.S.A.), LTB_4 was from Smith, Kline & French Laboratories Ltd. (Philadelphia, PA, U.S.A.). The buffer for these experiments contained 145 mM-NaCl, 5 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Hepes (pH 7.4 at 37 °C) and 10 mM-glucose.

Neutrophils were prepared from the blood of normal healthy volunteers. Blood was collected into anticoagulant (20 units of heparin/ml), then centrifuged for 15 min at 800 *g*. The upper layer of platelet-poor plasma was removed, and was used to add back to the neutrophils for measurements of fluo-3 fluorescence in the presence of plasma. An equivalent volume of 0.9% NaCl was added back to the blood to reconstitute the volume. Neutrophils were then prepared by dextran sedimentation of erythrocytes, concentration of the resulting leucocyte-rich plasma, followed by separation of white cells on a Percoll density gradient. This method was described in detail by Merritt *et al.* (1989). The washed neutrophils were then resuspended at approx. 6×10^6 cells/ml in buffer supplemented with 1 mM-$CaCl_2$ and 1% BSA.

For loading with fluo-3, the neutrophil suspension was incubated for 30 min at 37 °C with 2 μM-fluo-3 AM. For co-loading with fluo-3 and BAPTA, the neutrophil suspension was incubated for 30 min at 37 °C with 2 μM-fluo-3 AM and 3.5 μM-BAPTA AM. For co-loading with fluo-3 and fura-2, the neutrophil suspension was incubated for 30 min at 37 °C with 1 μM-fluo-3 AM and 0.25 μM-fura-2 AM. In all experiments, a control sample of cell suspension was not incubated with dye; these cells were used to measure autofluorescence. The cells were then centrifuged for 5 min at 300 *g*, and resuspended at approx. 2×10^6 cells/ml in buffer supplemented with 1 mM-$CaCl_2$ and 1% BSA. The resulting cell suspensions were kept at room temperature until use. For fluorescence measurements, portions (0.7 ml) of cell suspension were centrifuged (30 s at 6500 rev./min in a Microfuge) and resuspended in medium with no added $CaCl_2$ or BSA (or in a 1:1 mixture of this medium and plasma). Fluo-3 fluorescence was measured at 37 °C in a Perkin–Elmer LS-5 fluorimeter at 505 nm excitation, 530 nm emission. Fluo-3 (505 nm excitation) and fura-2 (340 nm excitation) fluorescence (530 nm emission) were measured simultaneously in a dual-excitation-wavelength fluorimeter (Spex). All additions are shown in the Figures, which are typical of at least three others obtained from different cell preparations. Autofluorescence measurements were obtained from cells that had been treated similarly but not loaded with dye.

Platelets were prepared from the blood of normal healthy volunteers, as previously described (Hallam *et al.*, 1984). For preparation of platelet-poor plasma, blood was collected into neutral citrate anticoagulant (7 mM-citric acid, 139 mM-trisodium citrate, 93 mM-glucose) in blood/anticoagulant ratio of 9:1 (v/v), and was centrifuged for 15 min at 800 *g*. The upper layer of platelet-poor plasma was removed, and was used to add back to the platelets for measurements of fluo-3 fluorescence in the presence of plasma. For preparation of platelets, blood was collected into acidic citrate anticoagulant (65 mM-citric acid, 85 mM-trisodium citrate, 110 mM-glucose) in a blood/anticoagulant ratio of 6:1 (v/v), and was centrifuged for 5 min at 500 *g*. The upper layer of platelet-rich plasma was removed. Aspirin (100 μM) was added to the platelet-rich plasma.

In order to prevent leakage of dye (see the Results and discussion section), probenecid (2.5 mM) was added to the platelet-rich plasma and to all the buffers throughout the experiments. For loading with fluo-3, the platelet-rich plasma was incubated for 30 min at 37 °C with 2 μM-fluo-3 AM. For co-loading with fluo-3 and BAPTA, the platelet-rich plasma was incubated for 30 min at 37 °C with 2 μM-fluo-3 AM and 20 μM-BAPTA AM. In all experiments, a portion of cell suspension was not incubated with dye; these cells were used to measure autofluorescence. The cells were then centrifuged for 15 min at 200 *g*, and resuspended at approx. 3×10^8 cells/ml (twice the final density) in buffer supplemented with 2.5 mM-probenecid and 0.2 mM-$CaCl_2$. The resulting cell suspensions were kept at room temperature until use. For fluorescence measurements, portions (0.35 ml) of cell suspension were added to 0.35 ml of buffer or plasma. Fluo-3 fluorescence was measured at 37 °C in a Perkin–Elmer LS-5 fluorimeter at 505 nm excitation, 530 nm emission. All additions are shown in the Figures, which are typical of at least three others obtained from different cell preparations. Autofluorescence measurements were obtained from cells that had been treated similarly but not loaded with dye.

RESULTS AND DISCUSSION

Determination of the K_d of fluo-3 for binding Ca^{2+} at 37 °C

The original description of fluo-3 quotes a K_d of 400 nM for binding Ca^{2+} at 22 °C (Minta *et al.*, 1989). However, it is known that binding of Ca^{2+} to quin2 and fura-2, as well as EGTA, is highly temperature-dependent; for example, the K_d of fura-2 is 135 nM at 20 °C and 224 nM at 37 °C (Grynkiewicz *et al.*, 1985). Most cellular responses are measured at 37 °C, so it was important to obtain a K_d value for fluo-3 at 37 °C. Since the K_d of fura-2 is known at 37 °C, fura-2 was used to measure the $[Ca^{2+}]$, and from the $[Ca^{2+}]$, $F_{max.}$ and $F_{min.}$ the K_d of fluo-3 was calculated by using the equation: $[Ca^{2+}] = K_d (F - F_{min.})/(F_{max.} - F)$. The excitation spectra of fluo-3 and fura-2 are well separated, so it was possible to use both dyes together in the same solution and switch excitation wavelengths to measure the fluorescence of each dye. Fig. 1 shows excitation spectra (emission at 530 nm) for Ca^{2+}-bound and Ca^{2+}-free fluo-3 (0.5 μM) plus fura-2 (0.5 μM) in a solution containing 135 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Mops/KOH (pH 7.05 at 37 °C). This also shows that fluo-3 is virtually non-fluorescent in the absence of Ca^{2+}. The K_d of fluo-3 was therefore calculated by measuring the fura-2 (340 nm excitation) and fluo-3 (505 nm excitation) fluorescence of the above solution containing various amounts of $CaCl_2$ and EGTA to cover a range of $[Ca^{2+}]$. $F_{max.}$ was obtained

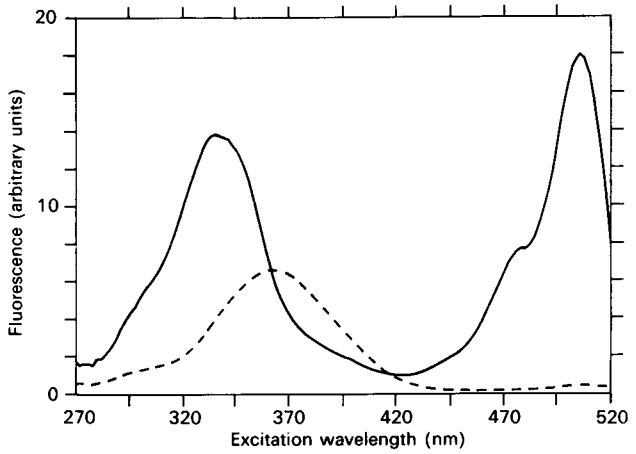

Fig. 1. Excitation spectra of fluo-3 plus fura-2 in the presence (——) and absence (----) of Ca^{2+}

Excitation spectra were recorded at an emission wavelength of 530 nm. The solution contained 135 mM-KCl, 1 mM-$MgCl_2$, 10 mM-Mops/KOH (pH 7.05 at 37 °C), 0.5 μM-fluo-3 (free acid), 0.5 μM-fura-2 (free acid), and saturating $CaCl_2$ or excess EGTA.

by adding $CaCl_2$ (plus diethylenetriaminepenta-acetic acid to chelate heavy metals that might cause quenching) until a maximum signal was obtained with both dyes (more $CaCl_2$ was needed with fluo-3, owing to its higher K_d). $F_{min.}$ was obtained by adding EGTA (plus Tris to maintain the pH) until a minimum signal was obtained. Based on a large number of determinations (approx. 100 separate determinations in six independent experiments), the K_d of fluo-3 at 37 °C was found to be 864 nM (all measured values were within 20 % of this mean value). This shift in K_d from 400 nM at 22 °C to 864 nM at 37 °C is of a similar order to the change in K_d of fura-2 from 135 nM at 20 °C to 224 nM at 37 °C, and obviously has a major effect on calibration of fluorescence to $[Ca^{2+}]_i$.

Loading of neutrophils and platelets with fluo-3

When neutrophils were loaded with fluo-3 by incubation for 30 min at 37 °C with 2 μM-fluo-3 AM, the fluorescence trace shown in Fig. 2(*a*) was obtained. The cells, in the presence of 1 mM-$CaCl_2$, responded rapidly to FMLP (10 nM) with a substantial increase in fluorescence, which then declined to a level above baseline. This response to FMLP is essentially similar to that previously reported for fura-2-loaded human neutrophils (Merritt *et al.*, 1989). Addition of digitonin (30 μM) to lyse the cells then caused a further large increase in fluorescence, which (in the presence of extracellular Ca^{2+}) is a measure of $F_{max.}$. The autofluorescence is the fluorescence of a parallel batch of cells that have not been exposed to fluo-3, and gives a measure of $F_{min.}$ (fluo-3 is virtually non-fluorescent in the absence of Ca^{2+}; $F_{min.}$ is < 3 % of $F_{max.}$). Fig. 2(*a*) shows that a good fluorescence signal was obtained relative to the autofluorescence background. These loading conditions, which resulted in a cytosolic fluo-3 concentration of approx. 0.5 mM, were therefore routinely used for neutrophils. The Materials and methods section describes the protocol for maintaining the fluo-3-loaded neutrophils at room temperature in buffer containing $CaCl_2$ and BSA until use; these conditions were found to minimize dye leakage over time ($F_{max.}$ was constant over several hours). Just before use, each batch of neutrophils was resuspended in fresh buffer; as well as allowing changes in the buffer, this protocol removed any extracellular dye that had accumulated over time. Dye leakage was minimal during the experiments (as shown in Fig. 4).

When platelets were loaded in a similar way to neutrophils (2 μM-fluo-3 for 30 min at 37 °C), the fluorescence trace shown in Fig. 2(*b*) (trace on the left, labelled −probenecid) was obtained. The fluorescence scale is the same as in Fig. 2(*a*) (for neutrophils). Stimulation of these platelets with thrombin (1 unit/ml) resulted in only a very small increase in fluorescence, and addition of digitonin (30 μM) to obtain $F_{max.}$ caused little further increase in fluorescence. The total fluorescence signal of these platelets was very small compared with the autofluorescence of the cells, and compared with the fluorescence signal obtained from neutrophils

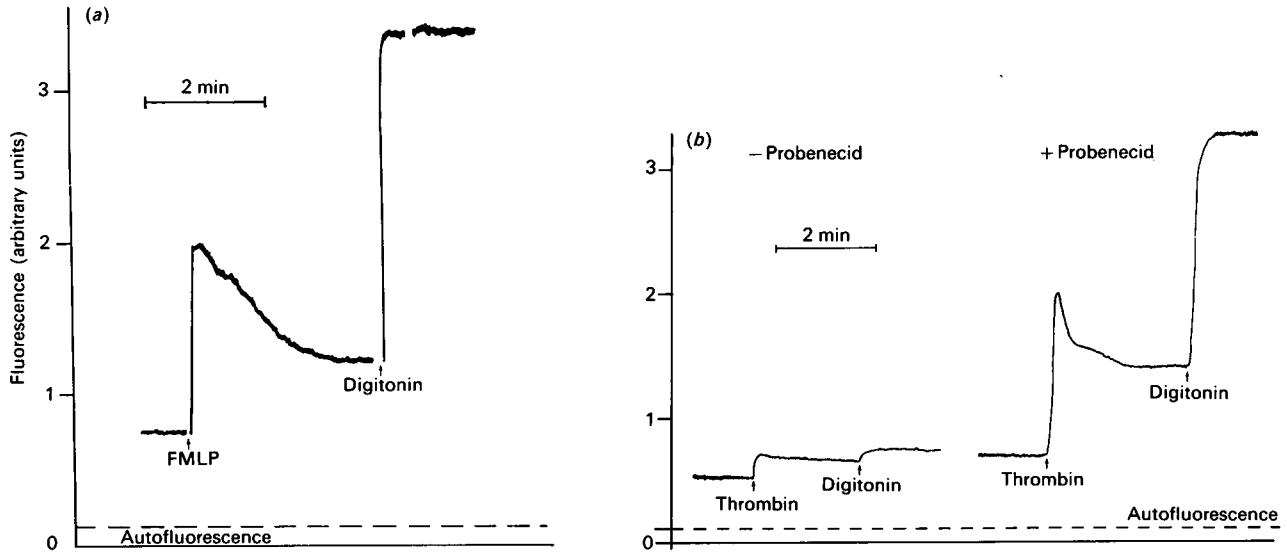

Fig. 2. Fluorescence traces of fluo-3-loaded neutrophils (*a*) and platelets (*b*)

Fluorescence was recorded at 505 nm excitation, 530 nm emission. Neutrophils (*a*) or platelets (*b*) were suspended in buffer containing 1 mM-$CaCl_2$, and the following additions were made at the times shown: FMLP (10 nM), thrombin (1 unit/ml), digitonin (30 μM). Autofluorescence is a measure of cells, treated similarly, that have not been exposed to dye. In (*b*) probenecid (2.5 mM) was present throughout for the experiment labelled '+probenecid', and was not included in that labelled '−probenecid'.

loaded under similar conditions. It was therefore apparent that these platelets contained very little fluo-3. The organic-anion-transport inhibitor, probenecid, has been reported to inhibit loss of fura-2 (free acid) from the cytoplasm of macrophages (Steinberg *et al.*, 1987; DiVirgilio *et al.*, 1988). Fig. 2(*b*) (trace on right, labelled +probenecid) shows the effect of probenecid (2.5 mM) on the incorporation of fluo-3 into platelets. Probenecid (2.5 mM) was present while the platelets were loaded with fluo-3, and throughout the experiment. Under these conditions, in the presence of 1 mM-$CaCl_2$, thrombin (1 unit/ml) evoked a large and rapid increase in $[Ca^{2+}]_i$ to a transient peak, which then declined to a maintained elevated level; this response is similar to that previously described for fura-2-loaded platelets stimulated with thrombin (Merritt & Hallam, 1988). Addition of digitonin (30 μM) then caused a further large increase in fluorescence to $F_{max.}$. The fluorescence signal relative to autofluorescence was now substantial, and comparable with that seen with neutrophils (Fig. 2*a*). Under these conditions, the cytosolic fluo-3 concentration was approx. 0.5 mM. All subsequent experiments with platelets therefore included probenecid (2.5 mM) throughout.

Calibration of fluo-3 fluorescence

Fig. 3 shows fluorescence traces, calibrated to $[Ca^{2+}]_i$, for neutrophils co-loaded with fluo-3 and fura-2. The fluorescence of the two dyes was measured simultaneously at 530 nm emission in a dual-excitation-wavelength fluorimeter at excitation wavelengths of 340 nm (fura-2) and 505 nm (fluo-3). The autofluorescence is the fluorescence of cells that have not been exposed to any dye. For fluo-3, autofluorescence equals $F_{min.}$. For fura-2, autofluorescence equals the fluorescence of the Mn^{2+}-quenched dye (F_{Mn}), and for fura-2 it has been determined that, under these experimental conditions, $F_{min.} - F_{Mn} = 0.33\,(F_{max.} - F_{Mn})$. Fig. 3 shows that Mn^{2+} does not totally quench fluo-3 fluorescence, so a more satisfactory way of obtaining $F_{min.}$ for fluo-3 is to measure the autofluorescence of cells that have not been exposed to dye. An alternative method to obtain $F_{min.}$ is lysis of the cells (can only be used with cells in suspension) with digitonin, followed by addition to enough EGTA to obtain $F_{min.}$. High concentrations of EGTA are required to obtain a true $F_{min.}$, and care must be taken to ensure that the pH is maintained. When working with uniform suspensions of cells, we consider use of a parallel portion of 'control' cells to be a more satisfactory method of obtaining $F_{min.}$. With fura-2, ionomycin is generally used to obtain $F_{max.}$, and Fig. 3 shows that addition of ionomycin (2 μM) results in a virtually maximum increase in fluorescence; subsequent addition of digitonin (30 μM) had little further effect. Fig. 3 shows that it is not possible to obtain $F_{max.}$ for fluo-3 with ionomycin; subsequent addition of digitonin (30 μM) results in a further large increase in fluorescence. This result may be a reflection of the higher K_d of fluo-3 compared with fura-2. Fig. 3 clearly shows that, in order to obtain a true $F_{max.}$ for fluo-3, digitonin must be used. Having established values of $F_{max.}$ and $F_{min.}$, $[Ca^{2+}]_i$ is calculated from the equation: $[Ca^{2+}]_i = K_d\,(F - F_{min.})/(F_{max.} - F)$. The calculated values for $[Ca^{2+}]_i$ are shown in Fig. 3, which also illustrates the difference in scales owing to the different K_d values of the two dyes. Fig. 3 shows that calibration of the traces is similar with the two dyes, which validates the estimation of the K_d for fluo-3 described above.

$[Ca^{2+}]_i$ traces of fluo-3-loaded platelets and neutrophils in buffer or 50% plasma

Figs. 4 and 5 show calibrated fluorescence traces of neutrophils (Fig. 4) and platelets (Fig. 5) suspended in either buffer (Figs. 4*a* and 5*a*) or 50% plasma (Figs. 4*b* and 5*b*). All additions are shown on the traces. Neutrophils were stimulated with FMLP

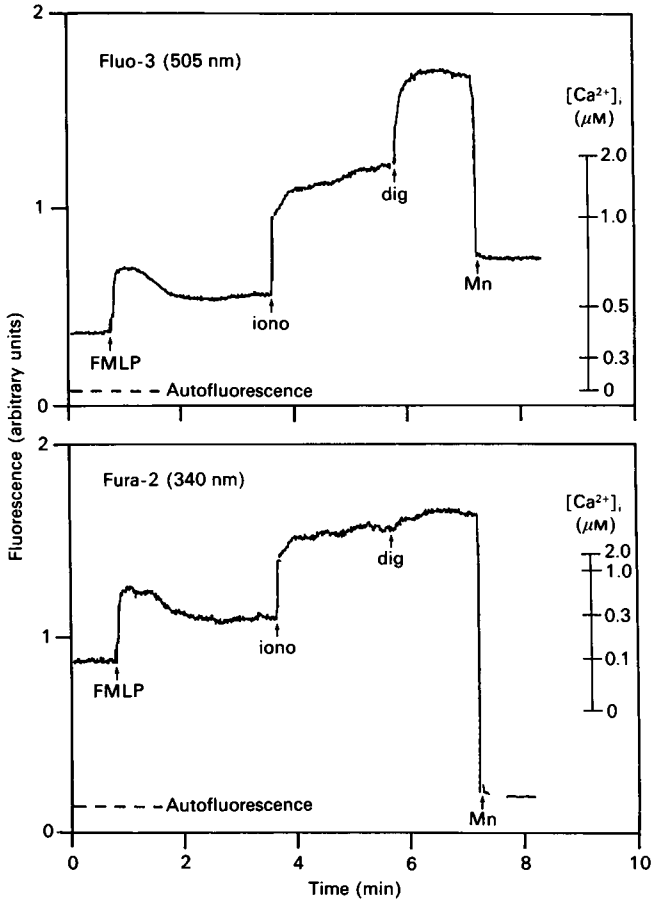

Fig. 3. Fluorescence traces of neutrophils co-loaded with fluo-3 and fura-2

Fluorescence (530 nm emission) was recorded in a dual-excitation-wavelength fluorimeter at excitation wavelengths of 505 nm (fluo-3) and 340 nm (fura-2). The fluo-3 and fura-2 fluorescences were recorded simultaneously. Neutrophils were suspended in medium containing 1 mM-$CaCl_2$, and the following additions were made at the times shown: FMLP (10 nM), ionomycin (iono, 2 μM), digitonin (dig, 30 μM), $MnCl_2$ (Mn, 5 mM). Autofluorescence is a measure of cells, treated similarly, that have not been exposed to dye. These traces are calibrated to $[Ca^{2+}]_i$ as described in the text.

(10 nM), LTB_4 (10 nM) or ATP (10 μM). Platelets were stimulated with thrombin (1 unit/ml), ADP (20 μM) or U46619 (1 μM).

For cells in buffer (Figs. 4*a* and 5*a*), $CaCl_2$ (1 mM) or EGTA (1 mM) was added where shown. With each agonist, the responses of both platelets and neutrophils were larger and more maintained in the presence of extracellular Ca^{2+}, which illustrates the contributions of Ca^{2+} influx and release from intracellular stores. In each case the resting $[Ca^{2+}]_i$ of around 100 nM was elevated to a transient peak of around 1 μM. These responses of fluo-3-loaded cells are similar to those previously reported for fura-2-loaded neutrophils (Merritt, 1989; Merritt *et al.*, 1989) and platelets (Pollock *et al.*, 1986; Merritt & Hallam, 1988).

Figs. 4(*b*) and 5(*b*) show that neutrophils and platelets respond similarly to agonist stimulation in the presence of plasma or buffer. This is the first demonstration of measurement of $[Ca^{2+}]_i$ in cells in the presence of plasma. (As mentioned in the Introduction, this method is also applicable for other compounds that are fluorescent at fura-2/quin2 wavelengths.) Heparinized plasma was used for the experiments with neutrophils, so there was sufficient Ca^{2+} present in the plasma to support Ca^{2+} influx, and EGTA (5 mM) was added to remove extracellular Ca^{2+}. A high concentration of digitonin (300 μM) was required to obtain

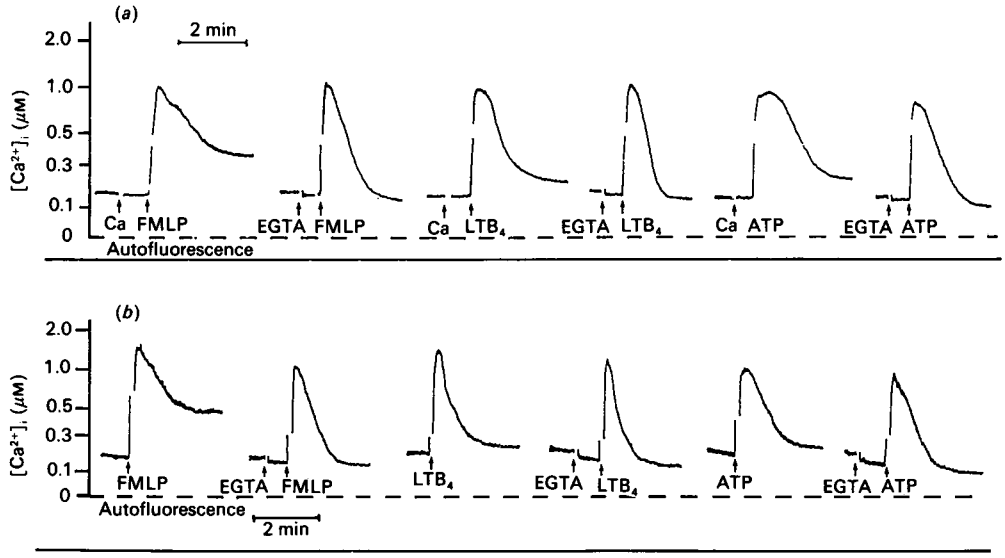

Fig. 4. Calibrated fluorescence traces of fluo-3-loaded neutrophils suspended in buffer (*a*) or 50% plasma (*b*)

Fluorescence was measured at 505 nm excitation, 530 nm emission, and calibrated to $[Ca^{2+}]_i$ as described in the text. Autofluorescence is the fluorescence of cell suspensions in buffer (*a*) or plasma (*b*) that have not been loaded with fluo-3. The following additions were made at the times shown: $CaCl_2$ (Ca, 1 mM, added to buffer only), EGTA (1 mM added to buffer; 5 mM added to plasma), FMLP (10 nM), LTB_4 (10 nM), ATP (10 μM).

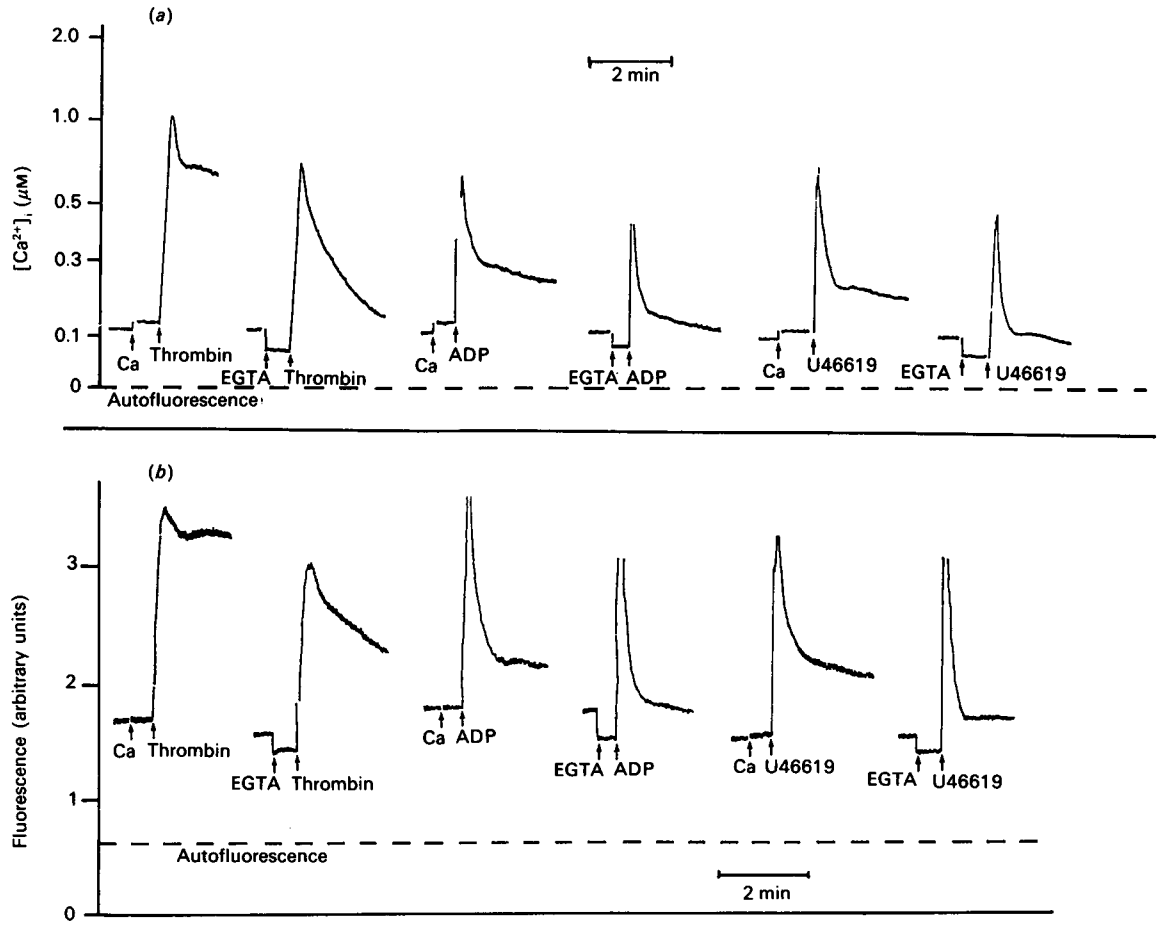

Fig. 5. Fluorescence traces of fluo-3-loaded platelets suspended in buffer (*a*) or 50% plasma (*b*)

Fluorescence was measured at 505 nm excitation, 530 nm emission, and calibrated to $[Ca^{2+}]_i$ as described in the text. [As discussed in the text, it was not possible to calibrate the responses in plasma (Fig. 5*b*).] Autofluorescence is the fluorescence of cell suspensions in buffer (*a*) or plasma (*b*) that have not been loaded with fluo-3. The following additions were made at the times shown: $CaCl_2$ (Ca, 1 mM), EGTA (1 mM added to buffer; 5 mM added to plasma), thrombin (1 unit/ml), ADP (20 μM), U46619 (1 μM).

a value for $F_{max.}$ in the presence of plasma. Calibration of the traces for neutrophils resulted in values of $[Ca^{2+}]_i$ very similar to those obtained in the presence of buffer with either fluo-3 (Fig. 4*a*) or with fura-2 (Merritt, 1989; Merritt *et al.*, 1989). It was not possible to use heparinized plasma for the experiments with platelets, because heparin inhibits platelet responses; citrated plasma therefore had to be used. This presented problems, because $CaCl_2$ had to be added to support Ca^{2+} influx, and this caused the plasma to clot. Addition of 1 mM-$CaCl_2$ to the citrated plasma was found to be sufficient to support Ca^{2+} influx with minimal clotting; however, the free $[Ca^{2+}]$ was too low to obtain a value for $F_{max.}$, and addition of further Ca^{2+} caused clotting. It was therefore not possible to calibrate the responses of platelets in plasma.

Co-loading of cells with BAPTA and fluo-3 to buffer changes in $[Ca^{2+}]_i$

Fig. 6 shows fluo-3 fluorescence traces of cells co-loaded with fluo-3 and BAPTA (a non-fluorescent Ca^{2+} chelator). Inclusion of BAPTA into the cytosol considerably increases the Ca^{2+}-binding capacity of the cytosol, such that changes in $[Ca^{2+}]_i$ are buffered. In the absence of extracellular Ca^{2+} (1 mM-EGTA added), only a finite amount of Ca^{2+} is available for release from intracellular stores. When the cytosolic Ca^{2+} buffering is minimal (i.e. fluo-3 only), as in Figs. 4(*a*) and 5(*a*), this finite amount of Ca^{2+} released from intracellular stores is sufficient to cause a large increase in $[Ca^{2+}]_i$; however, when there is increased cytosolic Ca^{2+} buffering (i.e. fluo-3 plus BAPTA), as in Fig. 6, the finite amount of Ca^{2+} released from intracellular stores can evoke only a small increase in $[Ca^{2+}]_i$. In the presence of extracellular Ca^{2+}, when an infinite amount of Ca^{2+} is available, there was still a large rise in $[Ca^{2+}]_i$ in the buffered cells. The response of the neutrophils was, however, very slow. Use of Ca^{2+} chelators to buffer and manipulate changes in $[Ca^{2+}]_i$ can be very useful in experiments designed to study the role of $[Ca^{2+}]_i$ in cellular function.

Summary and conclusions

Fluo-3 has certain advantages over fura-2, in terms of its higher K_d, permitting greater sensitivity of measurement at peak

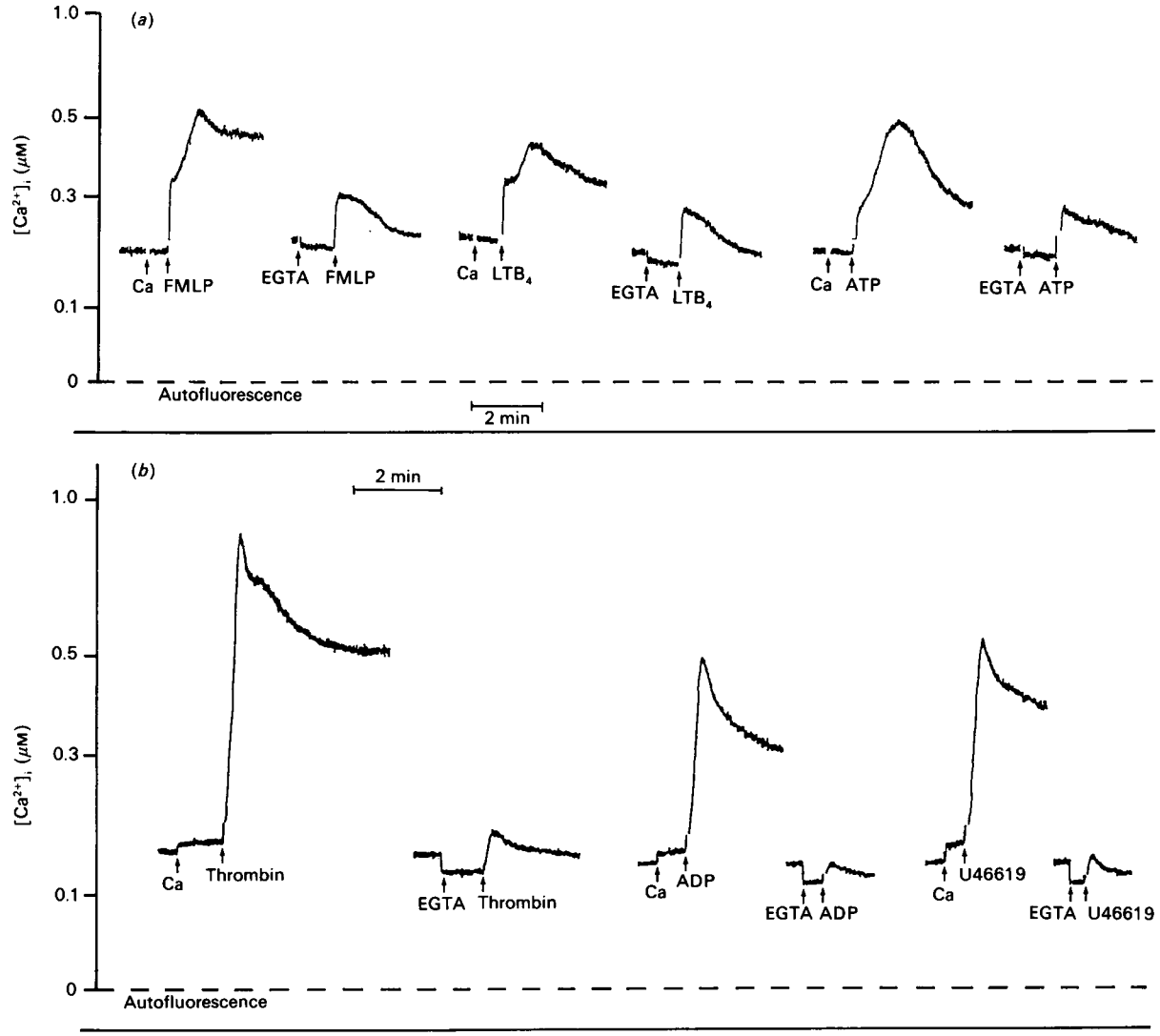

Fig. 6. Fluorescence traces of neutrophils (*a*) and platelets (*b*) co-loaded with fluo-3 and BAPTA

Fluorescence was measured at 505 nm excitation, 530 nm emission, and calibrated to $[Ca^{2+}]_i$ as described in the text. Autofluorescence is the fluorescence of cell suspensions that have not been loaded with dye. The following additions were made at the times shown: $CaCl_2$ (Ca, 1 mM), EGTA (1 mM), FMLP (10 nM), LTB_4 (10 nM), ATP (10 μM), thrombin (1 unit/ml), ADP (20 μM), U46619 (1 μM).

$[Ca^{2+}]_i$, and its longer excitation wavelength, which allows measurements in the presence of plasma. However, fluo-3 is not without its problems, many of which can be overcome as described here. The K_d of fluo-3 at 37 °C has now been estimated, and the resultant calibrations of cellular responses are in good agreement with those obtained with fura-2. Calibration of the dye, including measurement of $F_{max.}$ and $F_{min.}$ has been described. In some cells (platelets), fluo-3 leaks out very quickly; this can be prevented by using probenecid. Once these problems had been overcome, it was possible to obtain calibrated $[Ca^{2+}]_i$ responses of fluo-3-loaded platelets and neutrophils in the presence of both buffer and plasma, and also to co-load the cells with both BAPTA and fluo-3 in order to manipulate agonist-stimulated changes in $[Ca^{2+}]_i$. Many of these observations should be of value when using fluo-3 with suspensions of other cell types.

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