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Biochemical Pharmacology 68 (2004) 1721–1728
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Investigation into the relationship between calyculin A-mediated potentiation of NADPH oxidase activity and inhibition of store-operated uptake of calcium by human neutrophils

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Received 30 April 2004; accepted 13 July 2004

Abstract

The primary objective of the current study was to investigate possible relationships between calyculin A (CA)-mediated potentiation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and inhibition of store-operated uptake of Ca^{2+} by chemoattractant-activated human neutrophils. Treatment of neutrophils with 100 nM CA, but not at lower concentrations (12.5–50 nM), prior to the addition of the *N*-formylated chemotactic tripeptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 μM), both potentiated and prolonged the activity of NADPH oxidase which was accompanied by exaggerated membrane depolarisation, delayed and attenuated membrane repolarisation, and inhibition of store-operated Ca^{2+} influx. Inclusion of diphenylene iodonium chloride (DPI, 10 μM), an inhibitor of NADPH oxidase, antagonised the effects of CA on NADPH oxidase activity and the membrane repolarisation responses of FMLP-activated neutrophils, but failed to restore store-operated influx of Ca^{2+}. Similarly, CA also inhibited store-operated influx of Ca^{2+} into FMLP-activated neutrophils from a patient with chronic granulomatous disease, a primary immunodeficiency disorder characterised by the absence of a functional NADPH oxidase. CA also inhibited the store-operated influx of Ca^{2+} into control neutrophils treated with 1 μM thapsigargin, a selective inhibitor of the endomembrane Ca^{2+}-ATPase, which does not activate NADPH oxidase. Taken together, these observations demonstrate that augmentation of NADPH oxidase activity is not primarily involved in CA-mediated inhibition of the store-operated influx of Ca^{2+} into activated human neutrophils.

Keywords: Calyculin A; NADPH oxidase; Membrane potential; Store-operated calcium influx

1. Introduction

In addition to generating antimicrobial oxidants, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the membrane-associated, superoxide-generating electron transporter of neutrophils, is believed to fulfil an important, physiological anti-inflammatory function by regulating the store-operated influx of Ca^{2+} [1–4]. This is a consequence of the electrogenic, membrane-depolarising activity of the oxidase, which limits the influx of Ca^{2+}. When the cells are depolarised, the driving force for entry of Ca^{2+} is markedly reduced because the electrical component of the electrochemical gradient for Ca^{2+} is abolished [5–7]. NADPH oxidase-mediated exclusion of extracellular cations enables Ca^{2+} mobilised from intracellular stores, following neutrophil activation to be cleared efficiently by the plasma membrane and endomembrane Ca^{2+}-ATPases, unencumbered by incoming cation [8,9]. Following stimulation of neutrophils with the NADPH oxidase-activating chemotactic tripeptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), store-operated influx of Ca^{2+} proceeds gradually over several minutes coincident with membrane repolarisation [10,11].

Abbreviations: CA, calyculin A; CGD, chronic granulomatous disease; di-O-C_5(3), dipentyloxacarbocyanine; DMSO, dimethylsulphoxide; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks balanced salt solution; DPI, diphenylene iodonium chloride; LECL, lucigenin-enhanced chemiluminscence; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline.

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0006-2952/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2004.07.004

Calyculin A (CA), an inhibitor of protein phosphatases 1 and 2A [12], is a well-recognised antagonist of store-operated influx of Ca^{2+} in a variety of different eukaryotic cell types [13–18], including human neutrophils [19,20]. These inhibitory effects of CA on restoration of cellular Ca^{2+} homeostasis have generally been attributed to alterations in the cytoskeleton, with consequent uncoupling of cellular storage vesicles from Ca^{2+}-uptake channels in the plasma membrane [21–23]. However, CA has also been reported to potentiate the activity of NADPH oxidase by preventing dephosphorylative inactivation of the electron transporter in neutrophils [24,25], suggesting that this mechanism may also contribute to CA-mediated inhibition of store-operated influx of Ca^{2+} in these cells. This issue needs to be clarified to enable accurate interpretation of data when using CA as a probe of Ca^{2+} handling by neutrophils, as well as by other cell types, which possess an active NADPH oxidase.

In the current study, we have investigated the relationship between CA-mediated augmentation of neutrophil NADPH oxidase activity and inhibition of store-operated influx of Ca^{2+}.

2. Materials and methods

Calyculin A and all other chemicals and reagents (unless indicated) were purchased from Sigma and dissolved in dimethylsulphoxide (DMSO) to give a stock concentration of 100 μM. Dilutions were made in the same solvent and the final concentration of DMSO ± CA in the various assay systems described below was 0.1%.

2.1. Neutrophils

Purified neutrophils were prepared from heparinised blood (5 U of preservative-free heparin/ml) of healthy adult human volunteers as previously described [10,11], and resuspended to 1×10^7/ml in phosphate-buffered saline (PBS, 0.15 M, pH 7.4). Neutrophils were also isolated from the blood of a single patient with the autosomal recessive form of chronic granulomatous disease (CGD, deficiency of $p47^{phox}$, GT deletion in exon 2).

2.2. Superoxide production, assembly of NADPH oxidase and oxygen consumption

The effects of CA (12.5–100 nM) on superoxide generation by unstimulated neutrophils and cells activated with the synthetic chemotactic tripeptide FMLP (1 μM final) were measured using lucigenin (bis-*N*-methylacridinium nitrate)-enhanced chemiluminescence (LECL). Neutrophils (2×10^6) were preincubated with CA for 10 min at 37 °C in indicator-free Hank's balanced salt solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$) containing 0.2 mM lucigenin after which they were activated with FMLP, and the LECL responses measured with an LKB Wallac 1251 chemiluminometer. LECL readings were integrated for 11 s intervals and recorded as mV s.

The effects of the NADPH oxidase inhibitor, diphenylene iodonium chloride (DPI, 10 μM final), on CA (100 nM)-mediated augmentation of neutrophil superoxide production were also investigated using this system. DPI, together with CA, was present with the cells throughout the 10 min preincubation period at 37 °C.

To investigate the effects of CA (100 nM) on the assembly of NADPH oxidase, neutrophils (2×10^6) were preincubated with this agent for 10 min at 37 °C, followed by addition of FMLP (1 μM) in a final volume of 5 ml HBSS. After 10 min of incubation at 37 °C, the reactions were terminated by transferring the tubes to an ice-bath. The cells were then pelleted by centrifugation at 4 °C and the pellets resuspended in 0.34 M sucrose supplemented with 0.5 mM phenylmethylsulphonyl fluoride and disrupted by sonication. Cellular debris was removed by centrifugation and the membrane fractions in the supernatants were harvested, following centrifugation at 70,000 × *g* for 30 min. The resultant membrane pellets were dispersed in sucrose and assayed for NADPH oxidase activity using LECL. Reaction mixtures (1 ml) contained lucigenin, membrane fractions (300 μl), and NADPH (2 mM, final), which were added last to initiate superoxide generation.

Oxygen consumption by FMLP (1 μM)-activated neutrophils was measured using a three-channel oxygen electrode (model CW1, Hansatech Ltd.). The cells (2×10^6) were preincubated for 10 min at 37 °C in HBSS in the presence and absence of CA (100 nM) followed by the addition of FMLP, and PO_2 monitored further for 15 min.

2.3. Spectrofluorimetric measurements of Ca^{2+} fluxes

Fura-2 was used as the fluorescent Ca^{2+}-sensitive indicator for these experiments [26]. Neutrophils (1×10^7/ml) were loaded with fura-2 by incubation with fura-2/AM (2 μM) for 30 min at 37 °C in PBS, washed twice and resuspended in indicator-free HBSS, pH 7.4, containing 1.25 mM $CaCl_2$. The fura-2 loaded cells (2×10^6/ml) were then preincubated for 10 min at 37 °C followed by transfer to disposable reaction cuvettes which were maintained at 37 °C in a Hitachi 65010S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline was obtained (1 min), CA (12.5–100 nM) or an equal volume of DMSO (3 μl to control systems) was added to the neutrophils followed, after 1 min, by FMLP (1 μM). The subsequent increase in fluorescence intensity was monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6×10^6 neutrophils.

In an additional series of experiments, the effects of DPI (10 μM), added before FMLP or 1 min after the chemoattractant (at the time of maximum membrane depolarisa-

tion) on the fura-2 responses of neutrophils were investigated. Delayed addition of DPI (1 min after FMLP) was undertaken to monitor the involvement of NADPH oxidase in membrane repolarisation, uncomplicated by the well-recognised inhibitory effects of DPI on membrane depolarisation in FMLP-activated neutrophils [3].

A modification of the fura-2 fluorescence procedure was used to investigate the effects of CA on the store-operated influx of Ca^{2+}, uncomplicated by receptor-mediated activation of phospholipase C and NADPH oxidase [17,21]. Cells which had been resuspended in nominally Ca^{2+}-free HBSS, immediately followed by loading with fura-2, were preincubated for 3 min at 37 °C followed by addition of thapsigargin (1 μM, final), a highly selective inhibitor of the endomembrane Ca^{2+}-ATPase, which depletes intracellular Ca^{2+} stores [17,20,27]. This was followed, 6 min later, by addition of CA (100 nM), DPI (10 μM), CA + DPI, or DMSO and a further preincubation for 1 min (10 min preincubation at 37 °C in total) after which the cells were transferred to a cuvette in the fluorescence spectrophotometer. Store-operated influx of Ca^{2+} was initiated by the addition of $CaCl_2$ (250 μM, final) and the increase in fluorescence intensity monitored for 5 min.

2.4. Mn^{2+} quenching of fura-2 fluorescence

Cells loaded with fura-2, as described above, were activated with FMLP (1 μM) in HBSS containing 300 μM $MnCl_2$ (added 5 min prior to FMLP) and fluorescence quenching as a surrogate measure of Ca^{2+} influx was monitored at an excitation wavelength of 360 nm, which is an isobestic wavelength, and at an emission wavelength of 500 nm [1]. This procedure was used to investigate the effects of CA (100 nM) on the influx of Mn^{2+} into—(i) FMLP (1 μM) activated neutrophils with or without DPI (10 μM) added before and after 1 min of the chemoattractant; and (ii) FMLP-activated CGD neutrophils.

2.5. Radiometric assessment of Ca^{2+} fluxes

Calcium-45 chloride ($^{45}Ca^{2+}$, 370 GBq) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in unstimulated and FMLP (1 μM)-activated neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$ for experiments designed to measure net efflux of the cation, as well as their application in the measurement of net influx of Ca^{2+} following activation of the cells with FMLP, have previously been described in detail [8,10,11]. For these experiments CA was used at a fixed, final concentration of 100 nM added either 1 min before, or 30 s after FMLP when mobilisation of Ca^{2+} from neutrophil intracellular stores is complete. Delayed addition of CA (30 s after FMLP), was undertaken to exclude possible inhibitory effects of CA on release of the cation from stores as a mechanism of decreased store-operated uptake of Ca^{2+}. The effects of DPI (10 μM), added to neutrophils before or 1 min after FMLP when membrane depolarisation was maximal on the CA-mediated inhibition of net influx of Ca^{2+} were also investigated using this system.

2.6. Membrane potential

The potential sensitive fluorescent dye, dipentyloxacarbocyanine (di-O-C_5(3)), was used to measure changes in membrane potential in activated neutrophils [28]. The cells (1×10^6/ml) were preincubated for 10 min at 37 °C with and without CA (100 nM) in HBSS containing 80 nM (final) di-O-C_5(3), after which they were transferred to disposable reaction cuvettes and held at 37 °C in a Hitachi 65010S fluorescence spectrophotometer with excitation and emission wavelengths set at 460 nm and 510 nm, respectively. The neutrophils were then activated with FMLP (1 μM) and the subsequent alterations in fluorescence intensity monitored over a 5–10 min period. The final volume in each cuvette was 3 ml containing a total of 3×10^6 neutrophils. This procedure was also used to determine the effects of DPI (10 μM) on CA-mediated augmentation of FMLP-activated membrane depolarisation and attenuation of repolarisation, with DPI being added before FMLP, or 1 min after the chemoattractant at the time of maximum depolarisation (to eliminate the inhibitory effects of DPI on depolarisation).

2.7. Expression and statistical analysis of results

The results of each series of experiments are expressed as the mean values ± S.E.M., with the exception of the fura-2 experiments for which the traces are shown. Statistical analysis was performed using the paired Student's *t*-test when comparing two groups or by analysis of variance with subsequent Tukey–Kramer multiple comparisons test for multiple comparisons.

3. Results

3.1. Superoxide production, NADPH oxidase assembly and oxygen consumption

CA at 100 nM as shown in Fig. 1, but not at the lower concentrations tested (12.5–50 nm, not shown), both potentiated and prolonged FMLP-activated superoxide production by the cells, with only trivial effects on basal responses. Inclusion of DPI (10 μM) completely eliminated the FMLP-activated generation of superoxide by neutrophils in both the absence and presence of CA (100 nM). The LECL responses of unstimulated cells were 140 ± 11 mV s, while the corresponding responses of FMLP-activated control cells and for those treated with

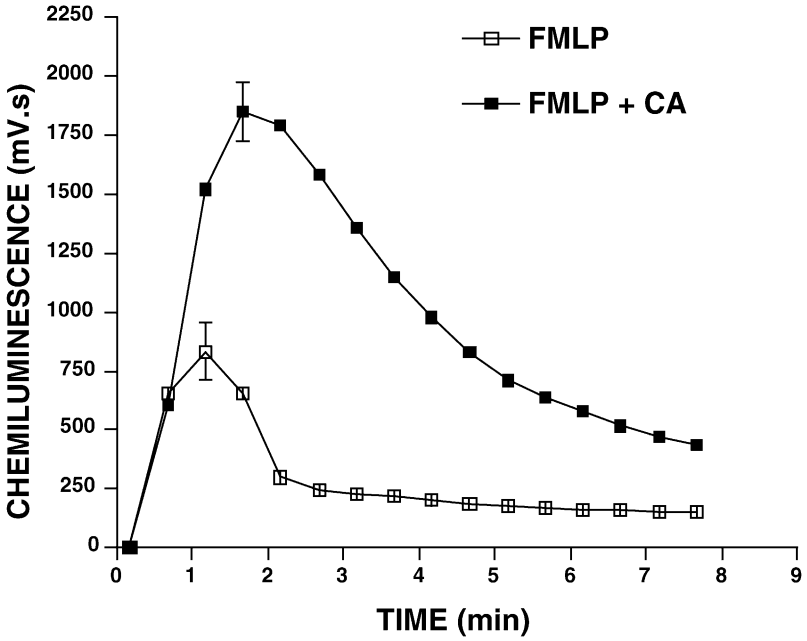

Fig. 1. Time course of superoxide production by FMLP (1 μM)-activated neutrophils in the absence and presence of CA (100 nM). The peak lucigenin-enhanced chemiluminescence values are expressed as the mean ± S.E.M.

CA only, DPI only or CA + DPI were 889 ± 78, 1561 ± 187, 102 ± 7, and 132 ± 8 mV s, respectively.

Treatment of neutrophils with CA (100 nM) caused significant ($P < 0.05$) augmentation of the activity of NADPH oxidase in membrane fractions isolated from FMLP-activated neutrophils. The peak LECL responses, measured at around 4 min after the addition of NADPH to membrane fractions were 204 ± 44, 299 ± 35, 320 ± 124 and 9946 ± 2094 mV s for unstimulated neutrophils, unstimulated neutrophils + CA, FMLP-activated neutrophils, and FMLP-activated neutrophils + CA, respectively. In agreement with these results, O_2 consumption by FMLP-activated neutrophils was dramatically increased by CA (not shown).

3.2. Spectrofluorimetric measurement of Ca^{2+} fluxes

The results shown in Fig. 2 are typical traces of the FMLP-activated fura-2 fluorescence responses of neutrophils from three different donors in the absence or presence of 100 nM CA, the concentration of the phosphatase inhibitor at which the observed effects were maximal. Treatment of neutrophils with CA caused a slight reduction in the abruptly occurring, peak fura-2 fluorescence responses of the cells (mean reduction of 18.6%, $P < 0.05$; data from 11 experiments). A striking acceleration in the rate of decline in peak fluorescence intensity was also observed in CA-treated, FMLP-activated neutrophils, compatible with an inability of the cells to sustain the che-

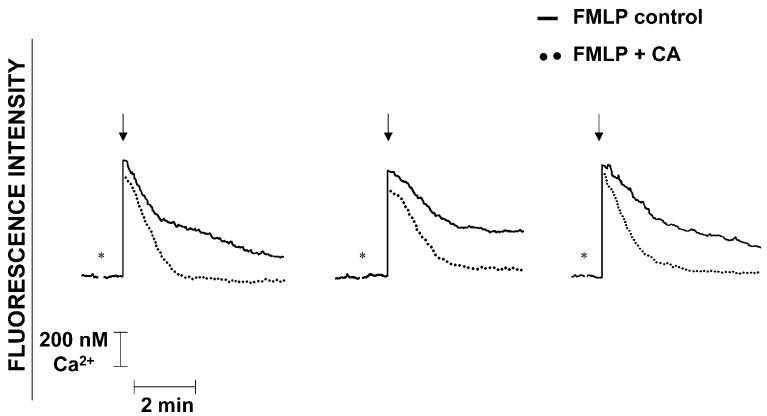

Fig. 2. FMLP (1 μM)-activated fura-2 fluorescence responses of control and CA (100 nM)-treated neutrophils. CA, or appropriate solvent control, was added as indicated (*), followed by the addition of FMLP, indicated by (↓) after a stable baseline was obtained (±1 min). These are three typical traces from 11 different experiments.

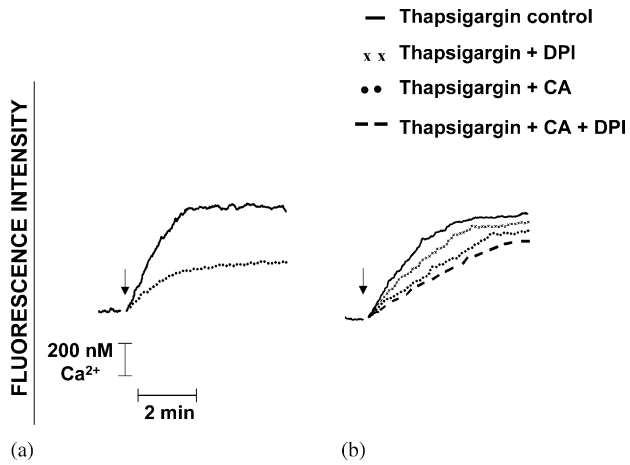

Fig. 3. Traces showing the effect of CA (100 nM) on the influx of Ca^{2+} (measured by fura-2 fluorescence) into thapsigargin (1 μM)-treated neutrophils without DPI (10 μM; trace a) and with DPI (10 μM; trace b). Thapsigargin, CA ± DPI and Ca^{2+} (250 μM $CaCl_2$; added as denoted by ↓) were added sequentially after 3, 9 and 10 min of preincubation, respectively.

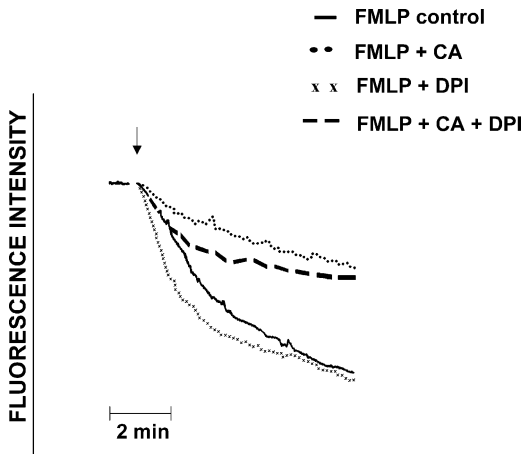

Fig. 4. Effects of DPI (10 μM) on CA (100 nM)-mediated modulation of FMLP (1 μM)-activated Mn^{2+} quenching of the fura-2 fluorescence responses. These traces are representative of two sets of results obtained for each. The arrow (↓) denotes the addition of FMLP.

moattractant-mediated elevation in cytosolic Ca^{2+}. This was unaffected by addition of DPI, either before or after 1 min of the FMLP to the neutrophils (not shown).

The effects of CA (100 nM) on the store-operated influx of Ca^{2+} into thapsigargin (1 μM)-treated neutrophils with and without DPI are shown in Fig. 3. Treatment of neutrophils with CA attenuated the thapsigargin-mediated, store-operated influx of Ca^{2+}, which was unaffected by DPI. These observations support the contention that CA-mediated inhibition of store-operated uptake of Ca^{2+} is independent of potentiation of NADPH oxidase.

3.3. Mn^{2+} quenching of fura-2 fluorescence

The effects of a 2 min pre-treatment with CA (100 nM) and DPI (10 μM), individually and in combination, on FMLP-activated influx of Mn^{2+} into control neutrophils are shown in Fig. 4. Addition of FMLP (1 μM) to the fura-2-loaded neutrophils was accompanied, after a short lag of 30–60 s, by a decline in fluorescence intensity due to influx of Mn^{2+}. The rate and magnitude of the decline in fura-2 fluorescence were decreased by CA. DPI per se accelerated the decline in fluorescence intensity, compatible with accelerated influx of Mn^{2+} into FMLP-activated neutrophils, but failed to eliminate the effects of CA.

The effects of CA (100 nM) on the influx of Mn^{2+} into FMLP-activated neutrophils from the CGD patient are shown in Fig. 5. As with neutrophils from control subjects, treatment of CGD neutrophils with CA strikingly attenuated the influx of Mn^{2+} following addition of FMLP.

3.4. Radiometric assessment of Ca^{2+} fluxes

Treatment of neutrophils with FMLP resulted in an abrupt efflux of Ca^{2+} from the cells, which was significantly ($P < 0.05$) decreased by treatment with CA (100 nM). At 60 s after the addition of FMLP, the amounts of Ca^{2+} extruded from control cells and those treated with CA were 179.6 ± 10.2 and 90.1 ± 13.2 pmoles $Ca^{2+}/10^7$ cells, respectively.

Treatment of neutrophils with CA (100 nM) also resulted in striking attenuation ($P < 0.05$) of the FMLP-activated, store-operated influx of Ca^{2+}, which was insensitive to DPI (10 μM) added either prior to, or 1 min after FMLP. The magnitudes of the net influx of Ca^{2+} into neutrophils, measured 5 min after addition of the chemoattractant, were 122 ± 9, 33 ± 3 and 33 ± 12 pmoles $Ca^{2+}/10^7$ cells for the control cells and for cells treated with CA only or CA + DPI (added prior to FMLP), respectively.

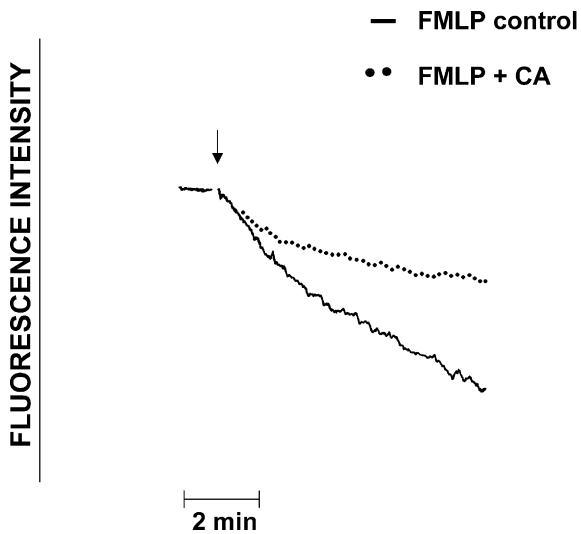

Fig. 5. Effect of CA (100 nM) on the influx of Mn^{2+} into FMLP-activated neutrophils from a patient with chronic granulomatous disease. The arrow (↓) denotes the addition of FMLP.

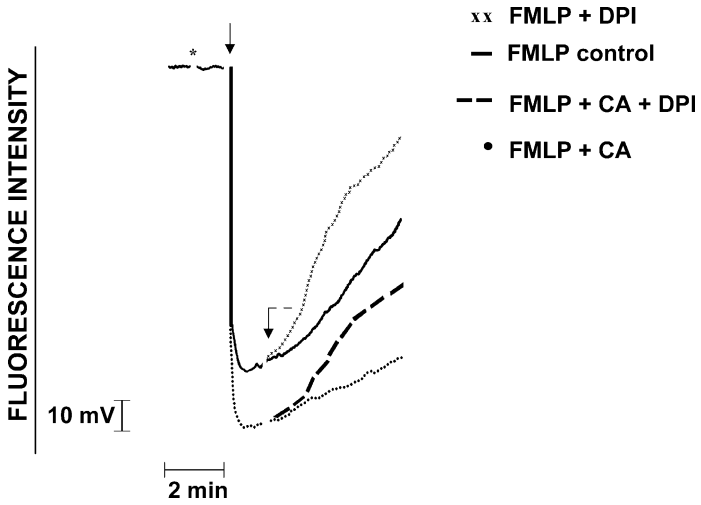

Fig. 6. Effect of DPI (10 μM) on CA (100 nM)-mediated alterations in neutrophil membrane potential following activation of the cells with FMLP (1 μM), which was added 1 min after CA (indicated by *). DPI, or appropriate solvent control, was added (as indicated by ⌐) approximately 1 min after FMLP, at a time when membrane depolarisation would have been maximal. This trace is representative of seven sets of results obtained. The arrow (⇓) denotes the addition of FMLP.

In an additional series of experiments, delayed addition of CA (30 s after FMLP) was also associated with inhibition of influx of Ca^{2+} (mean percentage inhibition 66 ± 3%; $P < 0.05$; data from six determinations), albeit to a lesser extent than that observed when CA was added to the cells prior to FMLP. These observations demonstrate that CA-mediated inhibition of store-operated uptake of the cation is not secondary to reduced mobilisation from intracellular stores.

3.5. Membrane potential

The effects of CA (100 nM) on the FMLP-activated membrane depolarisation and repolarisation responses of neutrophils in the absence and presence of DPI (10 μM), added 1 min after the chemoattractant when depolarisation was maximal, are shown in Fig. 6 (data shown from a single representative experiment). Treatment of neutrophils with CA potentiated FMLP-activated membrane depolarisation, which was associated with delayed onset and reduced magnitude of repolarisation, while addition of DPI at the time of maximum depolarisation increased the rate of repolarisation in control cells and restored this response to CA-treated cells (Fig. 6).

Because maximal membrane depolarisation was greater in CA-treated cells, we also calculated the data from all six experiments in the series as the rates of membrane repolarisation over the 5 min period commencing 1 min after activation of the cells with FMLP. The rates of membrane repolarisation for FMLP-activated control cells (without CA and/or DPI), and for cells treated with CA only, DPI only, or CA + DPI were 7.2 ± 1.6, 3.3 ± 0.5, 15.2 ± 3.3 and 8.7 ± 1.9 mV min, respectively, ($P < 0.05$ for comparison of the CA-treated and DPI-treated cells with the control cells, as well as for comparison of the CA-treated and CA + DPI-treated cells; the rates of repolarisation in the control and CA ± DPI-treated cells were not significantly different), demonstrating that DPI attenuates the inhibitory effects of CA on membrane depolarisation in FMLP-activated neutrophils.

4. Discussion

In agreement with previous reports [13–18], the results of the current study clearly demonstrate that CA inhibits the store-operated influx of Ca^{2+} into neutrophils activated with the chemoattractant, FMLP. Although CA also reduced the magnitude of the abruptly occurring peak increments in cytosolic Ca^{2+}, as well as the efflux of Ca^{2+} from FMLP-activated neutrophils, these events are probably secondary to attenuation of store-operated influx of the cation. This contention is supported by observations that CA, at the maximal concentration used in the current study, does not interfere with inositol triphosphate-mediated release of Ca^{2+} from the intracellular stores of eukaryotic cells [14], while decreased influx of Ca^{2+} would presumably result in increased utilisation of Ca^{2+} mobilised from stores for store-refilling, with a consequent reduction in efflux [8]. Moreover, using a radiometric system, we observed that addition of CA to FMLP-activated neutrophils at a time when the mobilisation of Ca^{2+} from intracellular stores is complete (30 s after the chemoattractant), also resulted in significant inhibition of store-operated influx of Ca^{2+}. The inhibitory effects of CA on store-operated influx of Ca^{2+} were also observed with thapsigargin-treated cells, a process which is independent of the generation of inositol triphosphate [29].

Calyculin A-mediated inhibition of the store-operated influx of Ca^{2+} has been attributed to the effects of this agent

on the cytoskeleton, resulting in cortical actin rearrangement and uncoupling of storage vesicles from Ca^{2+} entry channels [18,21–23]. However, CA as demonstrated in the present and previous studies [24,25], also potentiates the activity of NADPH oxidase by preventing the dephosphorylative inactivation of this electron transporter. This represents a potential alternative mechanism of CA-mediated inhibition of Ca^{2+} influx, since in cells such as neutrophils, which up-regulate NADPH oxidase following receptor-mediated mobilisation of intracellular Ca^{2+}, the electrogenic activity of the oxidase results in abrupt membrane depolarisation and inhibition of Ca^{2+} entry.

However, several lines of evidence appeared to exclude a possible contribution of CA-mediated potentiation of NADPH oxidase to inhibition of the store-operated influx of Ca^{2+} into FMLP-activated neutrophils. Firstly, using both the radiometric and Mn^{2+} quenching of fura-2 fluorescence procedures to measure influx of Ca^{2+}, we detected only trivial effects of the NADPH oxidase inhibitor, DPI, on CA-mediated attenuation of store-operated influx of Ca^{2+} in FMLP-activated neutrophils. Importantly, at the concentration of 10 μM used for these experiments, DPI almost completely neutralised the potentiating actions of CA on FMLP-activated NADPH oxidase activity and membrane depolarisation, and restored membrane repolarisation. Because of the exaggerated membrane depolarisation response of CA-treated, FMLP-activated neutrophils, the attenuating effects of DPI on CA-mediated inhibition of membrane repolarisation were most evident when comparing the rates, as opposed to the magnitudes, of membrane repolarisation. Secondly, using neutrophils from a patient with CGD, which fail to activate NADPH oxidase or undergo significant membrane depolarisation on exposure to FMLP [2], the inhibitory effects of CA on store-operated influx of Ca^{2+} were essentially similar to those of FMLP-activated cells from control subjects. We consider this to be particularly good evidence in support of the lack of involvement of NADPH oxidase in CA-mediated inhibition of store-operated uptake of Ca^{2+} by neutrophils because this condition (CGD) is associated with the absence of a functional oxidase. Thirdly, as alluded to above, CA was found to be a potent inhibitor of store-operated influx of Ca^{2+} into thapsigargin-treated neutrophils, irrespective of the presence or absence of DPI, which like CGD cells represents a system relatively uncomplicated by the activation of NADPH oxidase.

Interestingly, Itagaki et al. [30] have recently reported that CA abolishes the store-operated influx of Ca^{2+} into human neutrophils activated with the chemoattractant, platelet-activating factor (PAF), which does not activate NADPH oxidase [31], and in a limited series of experiments, cells activated with FMLP. These effects of CA were attributed to cytoskeletal reorganisation resulting in internalisation of transient receptor potential channels [30]. However, the study of Itagaki and colleagues was not designed to investigate the possible involvement of potentiation of NADPH oxidase activity and consequent interference with membrane repolarisation as a possible potential additional mechanism of CA-mediated inhibition of store-operated uptake of Ca^{2+}, which is the central theme of the current study.

Although common targets, such as MAP kinases, may underlie CA-mediated potentiation of NADPH oxidase activity [32] and inhibition of store-operated influx of Ca^{2+}, differential mechanisms must also be involved in these events. In the case of NADPH oxidase, these are likely to include cytosolic phospholipase A_2 and the translocatable oxidase component $p67^{phox}$, both of which are activated by MAP kinases [33,34]. However, in the case of cytoskeletal reorganisation, the precise phosphorylative mechanisms and targets of CA, which result in internalisation of putative store-operated Ca^{2+} channels [30] remain to be established.

In conclusion, the lack of significant involvement of NADPH oxidase in CA-mediated inhibition of influx of Ca^{2+} into neutrophils is an important finding because it means that CA can be used to probe involvement of the cytoskeleton in store-operated Ca^{2+} influx into these cells uncomplicated by NADPH oxidase-mediated effects on membrane potential.

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