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Counteracting effects of NADPH oxidase and the Na^+/Ca^{2+} exchanger on membrane repolarisation and store-operated uptake of Ca^{2+} by chemoattractant-activated human neutrophils

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Abstract

This study was designed to investigate the possible involvement of NADPH oxidase and the Na^+/Ca^{2+} exchanger in regulating membrane repolarisation and store-operated uptake of Ca^{2+} by FMLP (1 μM)-activated human neutrophils. Diphenyleneiodonium chloride (DPI, 5–10 μM) and KB-R7943 (2.5–10 μM), inhibitors of NADPH oxidase and the reverse mode of the Na^+/Ca^{2+} exchanger respectively, were used as pharmacological probes. Transmembrane fluxes of Ca^{2+}, K^+ and Na^+ were determined radiometrically, while alterations in membrane potential and cytosolic Ca^{2+} were evaluated using spectrofluorimetric procedures. DPI, added to the cells at the time of maximum FMLP-activated membrane depolarisation, accelerated the rates of both membrane repolarisation and influx of Ca^{2+}, while KB-R7943 effectively antagonised these processes. SKF 96365 (10 μM), an antagonist of store-operated Ca^{2+} channels, abolished the influx of Ca^{2+} into FMLP-activated neutrophils, but had no effects on membrane repolarisation, suggesting that the Na^+/Ca^{2+} exchanger is primarily involved in mediating membrane repolarisation, thereby facilitating uptake of Ca^{2+} via store-operated channels. These observations are compatible with prominent negative and positive regulatory roles for NADPH oxidase and the Na^+/Ca^{2+} exchanger respectively in regulating the rates of membrane repolarisation and store-operated uptake of Ca^{2+} by chemoattractant-activated neutrophils.

Keywords: Calcium; Chemoattractants; Membrane depolarisation/repolarisation; Na^+/Ca^{2+} exchanger; NADPH oxidase; Neutrophils; Store-operated calcium channels

1. Introduction

Receptor-mediated activation of human neutrophils results in an abrupt, transient increase in cytosolic Ca^{2+} which is a prerequisite for activation of the pro-inflammatory activities of these cells. The relative contributions of Ca^{2+} mobilised from intracellular stores and from extracellular reservoirs to neutrophil activation appear to vary according to both the type of activator [1,2] and the Ca^{2+}-dependent cell function [3,4]. In the case of neutrophils activated with the *N*-formylated chemotactic tripeptide, FMLP, Ca^{2+} released from intracellular stores appears to be primarily involved in activating superoxide generation, because net influx of the cation is evident only after peak responses have subsided [1,5–8].

Early attenuation of uptake of extracellular Ca^{2+} by FMLP-activated neutrophils is the consequence of an abrupt membrane depolarisation due primarily to activation of the electrogenic NADPH oxidase [6,7,9,10], thereby decreasing the driving force for Ca^{2+} influx due to elimination of the electrical component of the electrochemical gradient for Ca^{2+} [11–13]. Interestingly, membrane repolarisation becomes evident at around 1–2 min after activation of the cells with the chemoattractant and proceeds gradually over a 5–10-min time course, coincident

Abbreviations: Benzamil, (*N*-[benzylamidino]-3,5-diamino-6-chloropyrazinecarboxamide; di-O-C_5(3), dipentyloxacarbocyanine; CGD, chronic granulomatous disease; DPI, diphenyleneiodonium; EGTA, ethylene glycol-bis(beta-amino-ethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; KB-R7943, 2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl] isothiourea methanesulphonate; HBSS, Hanks' balanced salt solution; RMP, resting membrane potential; SKF 96365, (1-[β-(3-[4-methoxyphenethyl]-1*H*-imidazole hydrochloride

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with Ca^{2+} influx, supportive of a mechanistic relationship between these two events [1,5,8].

Nevertheless, the exact molecular/biochemical mechanisms, which regulate membrane repolarisation and store-operated influx of Ca^{2+} in human neutrophils, have not been conclusively established. In the current study, we have investigated the possible involvement of NADPH oxidase and the Na^+/Ca^{2+} exchanger as modulators of the rates of membrane repolarisation and Ca^{2+} influx in chemoattractant-activated neutrophils. Our results are compatible with counteracting roles for these two membrane transporters, which may explain the prolonged time course of membrane repolarisation and refilling of Ca^{2+} stores in activated neutrophils.

2. Materials and methods

2.1. Materials

KB-R7943, a selective inhibitor of the reverse mode (Ca^{2+} influx, Na^+ efflux) of the Na^+/Ca^{2+} exchanger [14] was kindly provided by Dr. K. Yokata, Nippon Organon K.K. Importantly, during preliminary studies with KB-R7943 we determined that this agent at a fixed concentration of 5 μM had no inhibitory effects on any of the following neutrophil functions: ATP production; NADPH oxidase activity; Na^+, K^+-ATPase activity; plasma membrane Ca^{2+}-ATPase activity and store-operated Ca^{2+} channels. For those experiments in which KB-R7943 was used at a higher concentration (10 μM), the agent was added 1 min after FMLP when Ca^{2+} release from stores, Ca^{2+}-ATPase mediated efflux of the cation, and membrane depolarisation are complete.

Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co, while all radiochemicals were purchased from Perkin-Elmer Life Sciences.

2.2. Neutrophils

Purified human neutrophils were prepared from heparinised venous blood (five units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 × *g* for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) before sedimentation with 3% gelatin in order to remove most of the erythrocytes. Following centrifugation (280 × *g* at 10 °C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4 °C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1×10^7/ml in PBS and held on ice until used.

A limited number of experiments were performed using neutrophils from two individuals, brother and sister aged 25 and 29 years respectively, with the autosomal recessive form of chronic granulomatous disease (CGD) (deficiency of $p47^{phox}$, GT deletion in exon 2).

2.3. Membrane potential

The potential sensitive fluorescent dye, dipentyloxacarbocyanine (di-O-C_5(3)), was used to measure changes in membrane potential in activated neutrophils [15]. The cells (1×10^6/ml) were pre-incubated for 10 min at 37 °C in indicator-free Hanks balanced salt solution (HBSS, pH 7.4) 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 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 460 and 510 nm, respectively. The neutrophils were then activated with the synthetic chemotactic tripeptide FMLP (1 μM final), 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 used to determine the effects of each of the following on resting membrane potential (RMP) and on alterations in membrane potential following activation of the cells with FMLP: (i) diphenyleneiodonium chloride (DPI, 5–10 μM), a selective inhibitor of NADPH oxidase [16]; (ii) KB-R7943 (5–10 μM) and benzamil (200 μM), inhibitors of the Na^+/Ca^{2+} exchanger; and (iii) SKF 96365 (10 μM), an antagonist of store-operated Ca^{2+} channels [17]. These agents were added to the cells 1 min prior to FMLP, with the exception of DPI, which was added after FMLP at the time of maximum depolarisation (30–60 s). This was done to monitor the possible involvement of NADPH oxidase on membrane repolarisation, uncomplicated by the well-recognised inhibitory effects of DPI on membrane depolarisation in FMLP-activated neutrophils [9].

Two approaches were used in an additional series of experiments designed to investigate the requirement for extracellular Ca^{2+} in the membrane depolarisation/repolarisation responses of FMLP-activated neutrophils. In the first of these, the Ca^{2+}-chelating agent EGTA (10 mM) was added to cells suspended in Ca^{2+}-replete (1.25 mM $CaCl_2$) HBSS 1 min prior to FMLP. In the second, neutrophils were suspended in nominally Ca^{2+}-free HBSS containing 100 μM EGTA to remove residual Ca^{2+} and activated with FMLP followed 5 min later by addition of 1 mM $CaCl_2$.

To calibrate alterations in membrane potential, neutrophils were bathed in HBSS containing Na^+, K^+ and Cl^- ions at fixed concentrations of 141, 5.7 and 145 mM, respectively. The intracellular concentrations of these ions were assumed to be 20, 130 and 80 mM, respectively for Na^+, K^+ and Cl^- taking into consideration reported estimations of intracellular concentrations in neutrophils varying from 13.5–26 mM for Na^+, 120–138 mM for K^+, and

80 mM for Cl^- [18–22]. The neutrophil permeability coefficients for sodium (P_{Na}), potassium (P_k) and chloride (P_{Cl}) ions are approximately 5×10^{-9}, 5×10^{-8} and 5×10^{-9} cm/s, respectively [22]. This information allows the resting membrane potential (RMP) of neutrophils in HBSS to be calculated according to the constant-field equation of Goldmann [22]. The RMP was manipulated by altering the extracellular concentration of K^+ by addition of KCl to give final extracellular potassium concentrations of 31, 56, 80 and 106 mM. The constant-field equation was used to calculate the estimated new RMP for each extracellular concentration of K^+ and this was correlated with the measured decrease (cm) in RMP which allowed estimation of the magnitude of alteration in fluorescence intensity in units of mV/cm. The results of three separate experiments for each concentration of extracellular K^+ were used to calculate the mean value for changes in fluorescence intensity and membrane potential as about 10 mV/cm.

2.4. Superoxide anion generation by neutrophils

The time course of superoxide production by FMLP (1 μM)-activated neutrophils, as well as the effects of DPI and KB-R7943 (both at 5 μM), were measured using a lucigenin (bis-*N*-methylacridinium nitrate)-enhanced chemiluminescence procedure [7]. Neutrophils (10^6) were preincubated for 15 min at 37 °C in HBSS with 0.2 mM lucigenin with and without DPI or KB-R7943, followed by transfer of the vials to a Lumac Biocounter (Lumac Biosystems). Following measurement of basal chemiluminescence, FMLP (1 μM) was added to the vials and the chemiluminescence responses of the cells monitored over a 5-min time course. The final volume in each vial was 1 ml, and the results are expressed in relative light units.

2.5. 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 measure of Ca^{2+} influx was monitored at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm [6]. This procedure was used to investigate the effects of DPI (10 μM) and KB-R7943 (10 μM), added 1 min following activation with FMLP, on the rates of Ca^{2+} influx. In an additional series of experiments, the effect of KB-R7943 (5 μM) on Mn^{2+} quenching of fura-2 fluorescence in CGD neutrophils was investigated.

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

$^{45}Ca^{2+}$ (calcium-45 chloride, specific activity 28.81 mCi/mg) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and FMLP-activated neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as their application in the measurement of the net influx of Ca^{2+} following the activation of neutrophils with FMLP have been described in detail elsewhere [1]. This procedure was used to monitor the effects of DPI (5 μM), KB-R7943 (2.5–10 μM) and SKF 96385 (10 μM) on the influx of Ca^{2+} into FMLP (1 μM)-activated neutrophils from control and CGD (only KB-R7943 at 5 μM) subjects.

2.7. Efflux of $^{22}Na^+$ from FMLP-activated neutrophils

To measure net efflux of $^{22}Na^+$ from FMLP-activated neutrophils, uncomplicated by concomitant uptake of the radiolabelled cation, the cells (2×10^6/ml) were washed with and resuspended in 50 mM Hepes–Tris buffer (pH 7.4) supplemented with 135 mM choline chloride, 1.1 mM glucose, 1.25 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM KCl, and 1 mM KH_2PO_4 containing 5 μCi/ml $^{22}Na^+$ (sodium-22, specific activity 399 mCi/mg). The cell suspension was then incubated for 20 min at 37 °C to allow uptake of $^{22}Na^+$ after which the cells were washed once with and resuspended in HBSS to 1×10^7/ml. The $^{22}Na^+$-loaded neutrophils were then preincubated for 10 min at 37 °C with and without KB-R7943 (2.5–10 μM) or SKF 96365 (10 μM) and the extent of efflux of $^{22}Na^+$ monitored over a 5-min time course following the addition of FMLP (1 μM, final) or an equal volume of HBSS to unstimulated systems. The final volume in each tube was 5 ml containing a total of 10^7 neutrophils. Reactions were terminated by the addition of ice-cold PBS and the amount of cell-associated $^{22}Na^+$ determined using an LKB Wallac 1261 Multigamma counter following lysis of the cells with 0.5 ml of 0.5% Triton X-100/0.05 M NaOH. Appropriate background systems consisting of identically processed, $^{22}Na^+$-exposed cells, which had been maintained at 4 °C throughout the entire time course of the experiments, were included and the values for these were subtracted from the corresponding experimental systems.

Importantly, the FMLP-activated membrane depolarisation/repolarisation responses of neutrophils which had been subjected to the $^{22}Na^+$-loading procedure followed by transfer to HBSS did not differ from those of cells which were maintained in HBSS throughout, but were otherwise identically processed (data not included).

The validity of the neutrophil $^{22}Na^+$-loading procedure was evaluated by measurement of the extent of uptake of the radiolabelled cation in the presence and absence of the Na^+, K^+-ATPase inhibitor, ouabain (50 μM, final) and KB-R7943 (5 μM) individually and in combination.

2.8. Influx of $^{22}Na^+$ and $^{86}Rb^+$ into FMLP-activated neutrophils

To measure the net influx of $^{22}Na^+$ into FMLP-activated neutrophils the cells (2×10^6/ml) were washed with and

resuspended in Hepes–Tris/choline chloride buffer and preincubated for 10 min at 37 °C followed by simultaneous addition of FMLP (1 μM) and $^{22}Na^{+}$ (5 μCi/ml) or $^{22}Na^{+}$ only to control, unstimulated systems. The amount of cell-associated $^{22}Na^{+}$ was then measured as described above at 10, 20, 30 and 60 s after the addition of FMLP to the neutrophils, this being the time course of efflux of Ca^{2+} from FMLP-activated neutrophils [1,21].

$^{86}Rb^{+}$ was used as a tracer for measuring K^{+} uptake and efflux [22]. For uptake studies neutrophils (2×10^{6}/ml) were suspended in isotonic Tris buffer (20 mM Tris, 122 mM NaCl, 5 mM KCl, 1 mM $MgSo_4$, 1 mM KH_2PO_4, 5 mM glucose, pH 7.4) containing 2 μCi/ml of $^{86}Rb^{+}$ (Rubidium-86 chloride, specific activity 1.48 mCi/mg) for 15 min at 37 °C followed by addition of FMLP (1 μM) or an equal volume of buffer to unstimulated systems (final volume in each tube of 5 ml). The time courses of uptake of $^{86}Rb^{+}$ by control and FMLP-activated neutrophils were then monitored over a 10 min period (10, 20 and 30 s, 1, 2, 3, 5 and 10 min). Reactions were terminated by the addition of ice-cold Tris buffer and the cells washed twice, followed by lysis of the cell pellets with 0.5 ml of 0.5% Triton X-100/0.05 M NaOH and measurement of radioactivity in the lysates by liquid scintillation spectrometry. Na^{+}, K^{+}-ATPase activity was taken as the difference in $^{86}Rb^{+}$ uptake in the presence and absence of 50 μM ouabain.

For efflux experiments, neutrophils were preloaded with $^{86}Rb^{+}$ by incubating the cells (10^{7} cells/ml) with $^{86}Rb^{+}$ (5 μCi/ml) for 20 min in isotonic Tris buffer. The cells were then washed twice with and resuspended in Tris buffer at 2×10^{6}/ml. The rates of efflux of the radiolabelled cation from control and FMLP-activated neutrophils were then monitored over a 60 s time course (10, 20, 30 and 60 s).

2.9. Statistical analysis

The results of each series of experiments are expressed as the mean values ± the standard error of the mean (S.E.M.). Levels of statistical significance were calculated using the Student's *t*-test when two groups were compared, or by analysis of variance with a subsequent Tukey–Kramer multiple comparisons test for multiple groups. Correlations between parameters were calculated using Pearson's correlation coefficient.

3. Results

3.1. FMLP-activated neutrophil depolarisation/repolarisation, superoxide production and transmembrane Ca^{2+} fluxes

A comparison of these responses was performed using neutrophils from six different individuals and a typical set of results from a single individual is shown in Fig. 1. Exposure of the cells to FMLP was accompanied by the characteristic abrupt decrease in membrane potential, efflux of $^{45}Ca^{2+}$ from the cells and activation of superoxide production, all of which levelled off at around 30 s after the addition of FMLP. Repolarisation was evident at 1–2 min after addition of FMLP and was associated with net influx of extracellular Ca^{2+} and a decline in superoxide production by the cells. These results confirm the relationship between NADPH oxidase-mediated alterations in membrane potential and Ca^{2+} fluxes in FMLP-activated neutrophils. The results shown from this point onward are devoted to the novel aspects of the study.

The effects of DPI (5 μM) and KB-R7943 (10 μM) added to the cells 10 and 1 min, respectively prior to

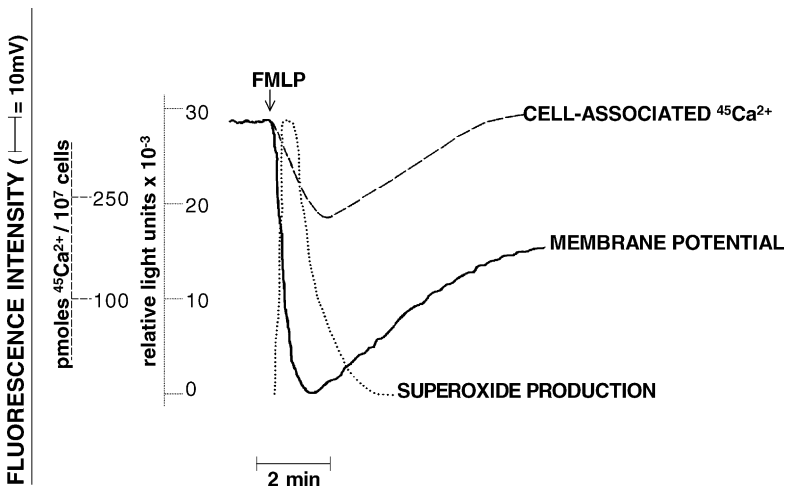

Fig. 1. Comparison of the alterations in membrane potential (depolarisation/repolarisation), Ca^{2+} efflux (represented by a fall in cell-associated $^{45}Ca^{2+}$) and influx of Ca^{2+} (rise in cell-associated $^{45}Ca^{2+}$), as well as superoxide generation, which accompany activation of human neutrophils with the *N*-formylated chemotactic tripeptide, FMLP. The data shown are those for neutrophils from a single individual and representative of the data obtained using cells from six different individuals. Addition of FMLP (1 μM, final) is denoted by the arrow (↓).

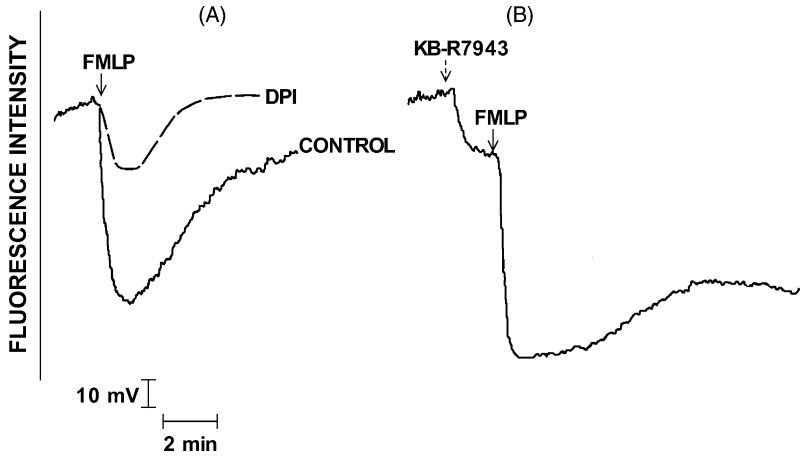

Fig. 2. Effects of DPI (5 μM; Trace A) and KB-R7943 (10 μM; Trace B) added 10 and 1 min (↓), respectively prior to 1 μM FMLP (↓), on the membrane potential of matched neutrophils. The traces shown are those from a single representative experiment (eight in the series).

FMLP on RMP and FMLP-activated membrane depolarisation/repolarisation are shown in Fig. 2. Although treatment of neutrophils with DPI did not affect RMP, this agent markedly attenuated FMLP-mediated depolarisation with a consequent reduction in the time taken to recovery of pre-activation membrane potential. KB-R7943 per se caused membrane depolarisation, and had opposite effects to those of DPI on the FMLP-mediated depolarisation and repolarisation responses of neutrophils, with a more pronounced depolarisation, and a longer time taken to onset of repolarisation, as well as a slower rate taken to onset of repolarisation and a slower rate of recovery. Similar results (not shown) to those observed with KB-R7943 were obtained with the non-selective Na^+/Ca^{2+} exchange inhibitor, benzamil (200 μM final, $n = 4$), while SKF 96365 had no effects on either the membrane depolarisation or repolarisation responses of FMLP-activated neutrophils ($n = 6$; results not shown).

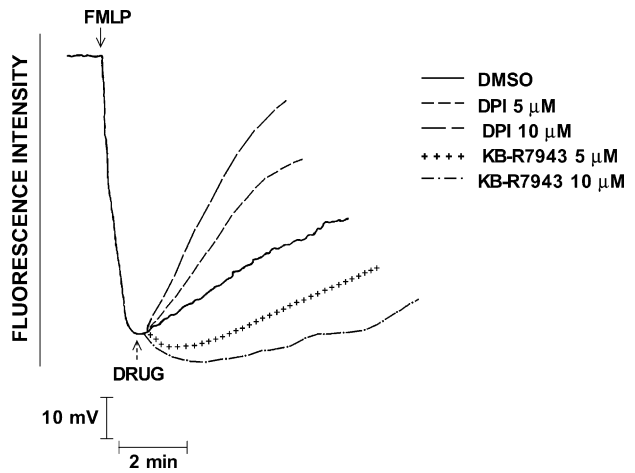

Fig. 3. The effects of DPI and KB-R7943 (both at 5 and 10 μM), as well as those of the DMSO solvent, added 1 min (↑) after 1 μM FMLP (↓) at the time of maximal membrane depolarisation, on the time course of the subsequent membrane repolarisation response. The traces shown are those from a single experiment (six in the series).

To determine the effects of DPI and KB-R7943 (both at 5 and 10 μM) on FMLP-activated membrane repolarisation, uncomplicated by their effects on depolarisation, these agents were added to neutrophils 1 min after FMLP at the time of maximum depolarisation. These results are shown in Fig. 3. DPI markedly accelerated the rate of membrane repolarisation, while with KB-R7943 the time taken to onset of repolarisation and rate of repolarisation were markedly protracted and decreased, respectively.

The effects of addition of EGTA (10 mM) to the cell-suspending medium on FMLP-activated alterations in neutrophil membrane potential are shown in Fig. 4A. The addition of EGTA had no effect on the resting potential over a 10-min test period (not shown) and did not alter the magnitude of the depolarisation response after addition of FMLP. However, inclusion of EGTA almost completely abolished the membrane repolarisation response. The corresponding responses of neutrophils suspended in minimally Ca^{2+}-free HBSS containing 100 μM EGTA to chelate residual Ca^{2+} are shown in Fig. 4B. The results were similar to those observed in Ca^{2+}-replete HBSS ± EGTA at 10 mM. Replenishment of extracellular Ca^{2+} during the phase of EGTA-mediated suppression of membrane repolarisation, resulted in a recovery of membrane potential at a rate and of a magnitude similar to that observed with control cells (Fig. 4B).

3.2. Effects of DPI and KB-R7943 on superoxide generation

Pretreatment of neutrophils with DPI (5 μM) almost completely inhibited the lucigenin-enhanced chemiluminescence responses of FMLP-activated neutrophils, while KB-R7943 (5 μM) was without effect. The peak responses, detected at 30 s after addition of FMLP, were 27336 ± 2029, 1165 ± 476, ($P < 0.001$) and 30581 ± 2297 relative light units for the control system and those containing DPI and KB-R7943 respectively, relative to a value of 4093 ± 337

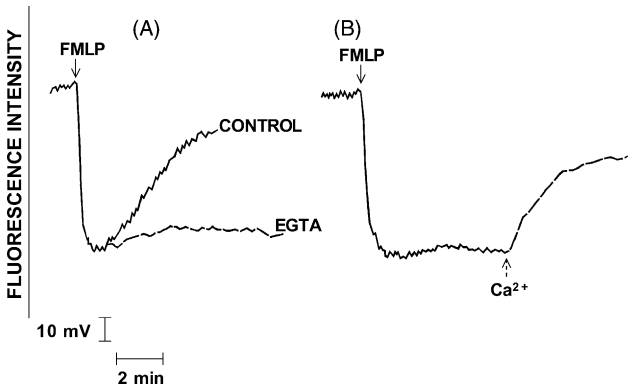

Fig. 4. Effects of EGTA on FMLP-activated alterations in neutrophil membrane potential. The traces shown are those from a single representative experiment (six in the series). The trace on the left side of the figure (A) shows the alterations in neutrophil membrane potential following the addition of FMLP (↓) to cells suspended in Ca^{2+}-replete HBSS in the absence (—) and presence of 10 mM EGTA (- - -), while the corresponding responses of neutrophils suspended in nominally Ca^{2+}-free HBSS containing 100 μM EGTA, to which $CaCl_2$ (1 mM) was added (↑) 5 min after FMLP, are shown on the right (B).

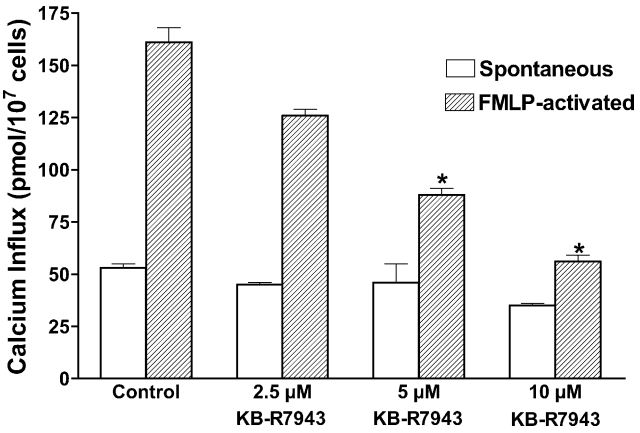

Fig. 6. Effects of KB-R7943 (2.5–10 μM) on the magnitude of both the spontaneous and FMLP-activated influx of $^{45}Ca^{2+}$ into neutrophils. The results of 6–21 experiments are expressed as the mean uptake of $^{45}Ca^{2+}$ in pmol/10^7 cells for unstimulated and FMLP-activated systems at 5 min after the addition of the chemoattractant; vertical lines represent S.E.M.s. $^{*}P < 0.001$ for comparison with the FMLP-activated control system. $^{45}Ca^{2+}$ uptake by resting and FMLP-activated control cells at 5 min was 53 ± 2 pmol/10^7 cells and 161 ± 7 pmol/10^7 cells, respectively.

for the unstimulated (FMLP-free) control system (data from six experiments).

3.3. Effects of DPI, KB-R7943 and SKF 96365 on transmembrane Ca^{2+} fluxes

The effects of DPI (10 μM) and KB-R7943 (10 μM), added 1 min following FMLP, on Ca^{2+} influx using the Mn^{2+} quenching of fura-2 fluorescence assay are shown in Fig. 5. DPI caused a marked acceleration in the rate of Ca^{2+} influx that levelled off earlier than that observed in the control system, while KB-R7943 (10 μM) attenuated the rate of Ca^{2+} influx.

Pretreatment of neutrophils with DPI (5 μM) significantly accelerated the rate of influx of $^{45}Ca^{2+}$ into

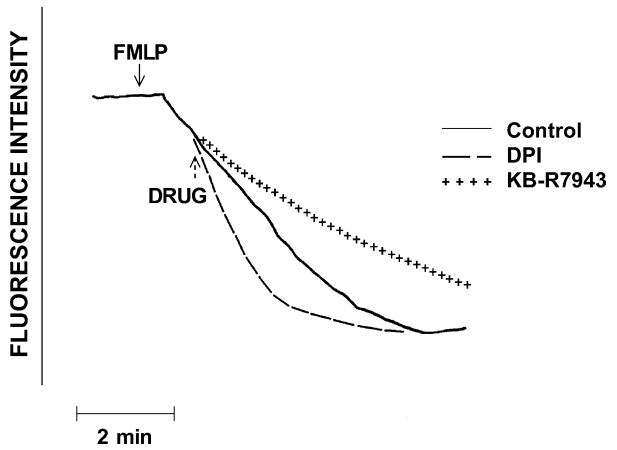

Fig. 5. Effects of DPI (10 μM) and KB-R7943 (10 μM) added as indicated (↑) 1 min after FMLP (↓), on the Mn^{2+} quenching of fura-2 fluorescence responses in FMLP-activated neutrophils. The results shown are typical traces of six replicates.

neutrophils, without affecting the magnitude of uptake. At 1.5, 3 and 5 min after addition of FMLP to control neutrophils, the values for net uptake of $^{45}Ca^{2+}$ were 38 ± 14 pmol, 178 ± 8 pmol and 242 pmol $^{45}Ca^{2+}/10^7$ cells, respectively, while the corresponding values for DPI-treated cells were 114 ± 13 pmol ($P < 0.001$), 216 ± 5 pmol and 245 ± 13 pmol $^{45}Ca^{2+}/10^7$ cells. Similar results were observed when DPI was added to the cells 1 min after FMLP (not shown).

The effects of KB-R7943 (2.5–10 μM) on the magnitude of both spontaneous and FMLP-activated uptake of Ca^{2+} by neutrophils are shown in Fig. 6, and demonstrate significant, dose-related suppression of the influx of $^{45}Ca^{2+}$ into FMLP-activated cells. Treatment of neutrophils from CGD subjects with KB-R7943 (5 μM) did not however, affect the magnitude of influx of $^{45}Ca^{2+}$ into the cells following activation with FMLP (1 μM), the values being 150 ± 15 pmol and 148 ± 16 pmol $Ca^{2+}/10^7$ cells in the absence and presence of KB-R7943, respectively ($n = 9$; measured 5 min after the addition of FMLP). These results were confirmed using the Mn^{2+} quenching of fura-2 fluorescence assay (not shown).

At the fixed concentration used (10 μM), SKF 96365 significantly ($P < 0.05$) inhibited the uptake of $^{45}Ca^{2+}$ by FMLP-activated neutrophils, with values of 160 ± 11 pmol and 51 ± 3 pmol $^{45}Ca^{2+}/10^7$ cells/5 min for the control and drug-treated systems respectively (data from five experiments).

Importantly, DPI and KB-R7943 (both at 5 μM) did not affect the abruptly occurring increases in cytosolic Ca^{2+} concentrations in FMLP-activated neutrophils. These were 317 ± 14 nM, 312 ± 20 nM and 292 ± 5 nM in control systems, and those treated with DPI and KB-R7943, respectively, rising from a basal value of 74 ± 4 nM (data from 3–4 experiments).

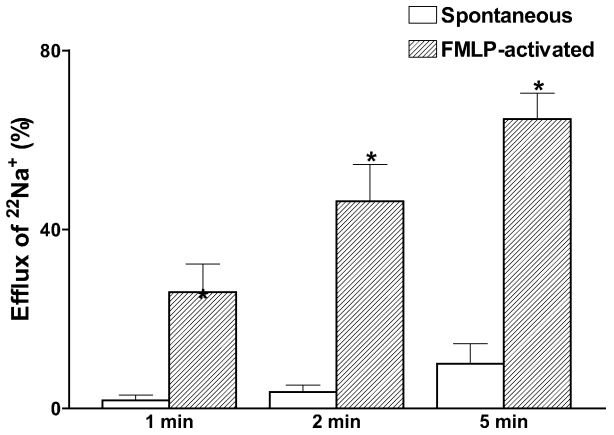

Fig. 7. Measurement of the time course of efflux of $^{22}Na^+$ from resting and FMLP-activated neutrophils. The results of six experiments are expressed as the mean percentage ± S.E.M. of $^{22}Na^+$ discharged from the cells over a 5-min time course following the addition of FMLP. $^*P < 0.05$ for comparison with the corresponding value for unstimulated cells.

3.4. Transmembrane fluxes of Na^+

Treatment of neutrophils with ouabain (50 μM) and KB-R7943 (5 μM) individually and in combination (in the absence of FMLP), significantly increased the uptake of $^{22}Na^+$ by the cells, with an interactive effect observed in the presence of the combination (data not shown), confirming the efficiency of the $^{22}Na^+$ loading procedure, as well as the efficacy of the inhibitors.

Activation of neutrophils with FMLP did not result in detectable influx of extracellular $^{22}Na^+$ at any of the times investigated (10, 20, 30 and 60 s). The values at 60 s for control and FMLP-activated neutrophils were 428 ± 31 cpm and 412 ± 24 cpm, respectively ($n = 2$). However, exposure of neutrophils to FMLP resulted in significant efflux of the cation from the cells, and these results are shown in Fig. 7. Efflux of $^{22}Na^+$ from FMLP-activated neutrophils was detectable at 1 min and maximal after 5 min, resulting in an average loss of 65% of cell-associated cation. The effects of KB-R7943 (2.5–10 μM) on $^{22}Na^+$ efflux from FMLP-activated neutrophils are shown in Fig. 8. Inclusion of KB-R7943, inhibited the efflux of $^{22}Na^+$ from FMLP-activated neutrophils in a dose-dependent manner.

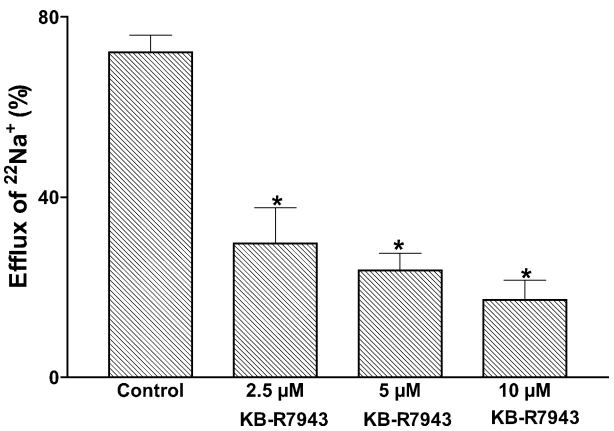

Fig. 8. Effects of KB-R7943 (2.5–10 μM) on $^{22}Na^+$ efflux from FMLP-activated neutrophils. The results of 6–18 experiments are expressed as the mean percentage ± S.E.M. of $^{22}Na^+$ discharged from the cells over a 5-min time course following the addition of FMLP. $^*P < 0.001$ for comparison with control, untreated cells.

3.5. Transmembrane fluxes of K^+

Relative to untreated control cells, addition of FMLP to neutrophils did not cause detectable efflux of $^{86}Rb^+$. At 60 s after addition of the chemoattractant to ^{86}Rb-loaded neutrophils, the amounts of $^{86}Rb^+$ associated with unstimulated and FMLP-activated cells were 26,145 ± 394 cpm and 27,286 ± 903 cpm, respectively. Likewise, activation of neutrophils with FMLP was not accompanied by detectable ouabain-inhibitable influx of $^{86}Rb^+$, the amounts of cell-associated $^{86}Rb^+$ at 1, 2, 3 and 5 min after addition of FMLP being 2593 ± 330 cpm, 2541 ± 322 cpm, 2254 ± 314 cpm and 3304 ± 405 cpm, respectively, while the corresponding values for unstimulated cells were 2485 ± 140 cpm, 2877 ± 209 cpm, 2342 ± 248 cpm and 3109 ± 281 cpm, respectively.

4. Discussion

The store-operated influx of Ca^{2+} into FMLP-activated neutrophils is carefully regulated, proceeding over a time course of several minutes, and is superimposable on the time course of membrane repolarisation [5–8]. In the current study, we have demonstrated that this relationship is not coincidental, and that influx of Ca^{2+} into FMLP-activated neutrophils is dependent on membrane repolarisation, the rate of which is determined, at least in part, by the respective restraining and driving actions of NADPH oxidase and the Na^+/Ca^{2+} exchanger.

The restraining action of NADPH oxidase on membrane repolarisation was supported by data from experiments in which neutrophils were treated with the selective NADPH oxidase inhibitor, DPI [16], either prior to activation with FMLP, or subsequent to exposure to the chemoattractant, at the time of maximum membrane depolarisation. In both cases, treatment of the cells with DPI accelerated the rates of both membrane repolarisation and store-operated influx of Ca^{2+}. These observations confirm and extend previous studies which have demonstrated accelerated influx of Ca^{2+} into neutrophils from CGD subjects in which the FMLP-activated membrane depolarisation response is markedly attenuated due to deficiency of NADPH oxidase [6,8,18]. Following activation of normal neutrophils with FMLP, maximal activity of the up-regulated NADPH oxidase occurs at 30–60 s and results in the vectorial, outward flux of electrons and abrupt membrane depolarisation. Although the activity of the oxidase subsides thereafter, residual, albeit steadily-dissipating activity

(of the oxidase) appears to be the primary, negative influence which restrains membrane repolarisation and store-operated influx of Ca^{2+}.

The existence of a Na^+/Ca^{2+} exchange mechanism on the plasma membrane of neutrophils [23] and lymphocytes [24] has been described previously. Simchowitz et al. [25] reported a 20-fold increase in the activity of the exchanger (operating in Ca^{2+} entry mode) following activation of human neutrophils with calcium-mobilising formyl-peptides. Binding of Ca^{2+} to a high-affinity regulatory site on the cytosolic side of the exchanger upregulates its activity, promoting sodium efflux and calcium influx following Ca^{2+} release from storage vesicles [26].

Although proton extrusion has been proposed to be the major charge compensation mechanism in activated neutrophils [27], the inhibitory effects of KB-R7943, on membrane repolarisation and Ca^{2+} influx observed in the current study suggest that the Na^+/Ca^{2+}-exchanger, operating in reverse mode, may also contribute to the electrogenic recovery of the membrane potential towards pre-activation levels. Several additional lines of evidence lend support to this contention: (i) the Na^+/Ca^{2+} exchanger, operating in reverse mode is electrogenic, mediating the net movement of a positive charge when three Na^+ are exchanged for one Ca^{2+} [26]; (ii) Na^+ efflux (sensitive to KB-R7943) was observed in FMLP-activated neutrophils and was not coupled to detectable alterations in ouabain-inhibitable uptake of K^+ ($^{86}Rb^+$), reducing the likelihood of involvement of Na^+, K^+-ATPase (iii) KB-R7943 caused spontaneous membrane depolarisation (in the absence of FMLP) of neutrophils which was accompanied by influx of Na^+, compatible with the involvement of the Na^+/Ca^{2+} exchanger, operating in reverse mode, in maintaining resting membrane potential; and (iv) membrane repolarisation was almost completely abolished in the presence of EGTA suggesting an absolute requirement for extracellular Ca^{2+} in this process, a requirement shared by the Na^+/Ca^{2+} exchanger operating in reverse mode [26]. The effect of EGTA on membrane repolarisation was reversed by subsequent addition of excess Ca^{2+}, underscoring the functional dependency of membrane repolarisation on the availability of extracellular Ca^{2+}. Although an alternative extracellular Ca^{2+}-dependent process may be activated by FMLP to expel intracellular Na^+, our data strongly implicate the Na^+/Ca^{2+} exchanger in contributing to the restoration of membrane potential in neutrophils. A similar observation that EGTA inhibits membrane repolarisation has been reported for activated platelets [28].

Sodium efflux from FMLP-activated neutrophils is unlikely to be mediated by upregulation of the Na^+/H^+ antiporter, as this mechanism participates in restoring intracellular pH by extruding protons in exchange for Na^+ [25,29]. Moreover, in agreement with previous reports [23,30] we were unable to detect net influx of Na^+ into FMLP-activated neutrophils during the early efflux of Ca^{2+} from the cytosol.

It has previously been suggested that the Na^+/Ca^{2+} exchanger in neutrophils may function as a primary transporter of extracellular Ca^{2+} for refilling of stores [25]. However, the observations of the current study suggest that the primary role of the exchanger is to mediate membrane repolarisation required to drive the influx of Ca^{2+} through store-operated Ca^{2+} channels, into FMLP-activated neutrophils, as opposed to being a major transporter of extracellular Ca^{2+} for store refilling. This contention is supported by two lines of evidence. Firstly the insensitivity of CGD neutrophils, which undergo only modest membrane depolarisation on exposure to FMLP, to the inhibitory actions of KB-R7943 on Ca^{2+} influx, underscores the primary involvement of the exchanger in mediating membrane repolarisation, and also excludes possible channel blocking activity of this agent [31]. Secondly, the attenuation of influx of Ca^{2+} mediated by SKF 96365, a selective inhibitor of store-operated Ca^{2+} channels [17,32], in the absence of effects on membrane repolarisation, supports the contention that the primary role of the Na^+/Ca^+ exchanger is to drive membrane repolarisation, with minimal, if any, involvement in promoting Ca^{2+} uptake for store refilling.

The results of the current study therefore reinforce the proposed role for NADPH oxidase as a negative regulator of influx of Ca^{2+} into chemoattractant-activated neutrophils [6,7,9,10]. The membrane depolarising action of the oxidase restricts influx of extracellular Ca^{2+}, optimising the efficiency of clearance of store-derived Ca^{2+} from the cytosol of activated neutrophils. Residual activity of the oxidase also restrains membrane repolarisation, allowing gradual influx of Ca^{2+} which can be efficiently diverted into stores. The Na^+/Ca^{2+} exchanger, on the other hand, drives membrane repolarisation, facilitating uptake of Ca^{2+} through store-operated Ca^{2+} channels. It is, however, unlikely to be the sole contributor to membrane repolarisation [27,33]. Although we have gone to considerable effort to ensure specificity, we do concede that the validity of these conclusions is dependent on the respective specificities of DPI and KB-R7943 for NADPH oxidase and the Na^+/Ca^{2+} exchanger.

Physiological mechanisms which regulate the store-operated influx of Ca^{2+} into activated neutrophils are potential targets for anti-inflammatory chemotherapy since the influx of Ca^{2+} is necessary not only for reactivation of the cells, but also to sustain certain pro-inflammatory functions such as activation of β_2-integrins [3,4]. In this context, plausible pharmacological strategies are those which augment and inhibit the activities of NADPH oxidase and the Na^+/Ca^{2+} exchanger respectively. However, enthusiasm for these approaches has to be tempered by an awareness of the potential for the risk of oxidant-mediated toxicity in the case of potentiation of NADPH oxidase, and lack of tissue specificity in the case of inhibitors of the Na^+/Ca^{2+} exchanger.

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