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Effect of SK&F 96365 on extracellular Ca^{2+}-dependent O_2^- production in neutrophil-like HL-60 cells

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Abstract

Store-operated Ca^{2+} entry is referred to a capacitative current activated by Ca^{2+}-stores depletion in various non-excitable cells. Neutrophil-like HL-60 cells responded to *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP) by an early O_2^- production preceded by a $[Ca^{2+}]_i$ rise. Cell stimulation in the absence of extracellular Ca^{2+} resulted in a major reduction of $[Ca^{2+}]_i$ rise and O_2^- production. A purported inhibitor of store-operated Ca^{2+} entry, SK&F 96365 (1-(β-(3-(4-methoxy-phenyl)propoxyl)-4-methoxy-phenetyl)-1*H*-imidazole hydrochloride), inhibited extracellular Ca^{2+}-dependent $[Ca^{2+}]_i$ rise by 30% but did not alter O_2^- production. In conclusion, SK&F 96365 did not modify extracellular Ca^{2+}-dependent O_2^- production, despite a significant but limited reduction in fMLP-activated membrane Ca^{2+} fluxes which can be ascribed to store-operated Ca^{2+} entry. Furthermore, Ca^{2+} influx is necessary for a full induction and maintenance of the biological response. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: fMLP (*N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine); Ca^{2+}, intracellular; Superoxide; Neutrophil; Respiratory burst

1. Introduction

Stimulation of neutrophils with the chemotactic bacterial peptide *N*-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP) results in an increase in $[Ca^{2+}]_i$ provided both by intracellular Ca^{2+} stores release and Ca^{2+} entry (Pozzan et al., 1983). While the mechanism of intracellular Ca^{2+} release is well-established, the mechanism and regulation of Ca^{2+} influx, generally referred to as store-operated Ca^{2+} entry, have remained elusive. There is strong evidence that the latter is a consequence of the former as hypothesized in the capacitance model introduced by Putney (Putney, 1986, 1990). Some neutrophil responses, notably implicated in microbial killing, are thought to be dependent on the Ca^{2+} entry component of the $[Ca^{2+}]_i$ rise (Boucek and Snyderman, 1976; Lew et al., 1984).

The method most frequently used to investigate receptor-triggered Ca^{2+} influx, in isolation from the concomitant intracellular Ca^{2+} release, has been fluorescent dye analysis according to a Ca^{2+}-free/Ca^{2+} reintroduction protocol. A more recent refinement of such fluorescent dye techniques consists in measuring fura-2 fluorescence quenching triggered by the influx of Mn^{2+} through Ca^{2+}-permeable channels (Demaurex et al., 1992), where Mn^{2+} has been shown to be a good Ca^{2+} surrogate for the tracing of unidirectional divalent cation movements in human neutrophils (Montero et al., 1991, 1992).

An organic compound, SK&F 96365 (1-(β-(3-(4-methoxy-phenyl)propoxyl)-4-methoxy-phenetyl)-1*H*-imidazole hydrochloride), was introduced as a suitable inhibitor of store-operated Ca^{2+} entry in platelets and neutrophils (Merritt et al., 1990). It appears as a useful tool to gain insights into the mechanism of Ca^{2+} entry in non-excitable cells and to probe the role of store-operated Ca^{2+} entry in mediating functional responses shown to be dependent on Ca^{2+} influx. In the present study, we have used the newly developed double labelling fluorescent assay (Bueb et al., 1995) for the simultaneous measurement of $[Ca^{2+}]_i$ and O_2^- production in neutrophil-like differentiated HL-60 cells in order to investigate the effect of SK&F 96365 on

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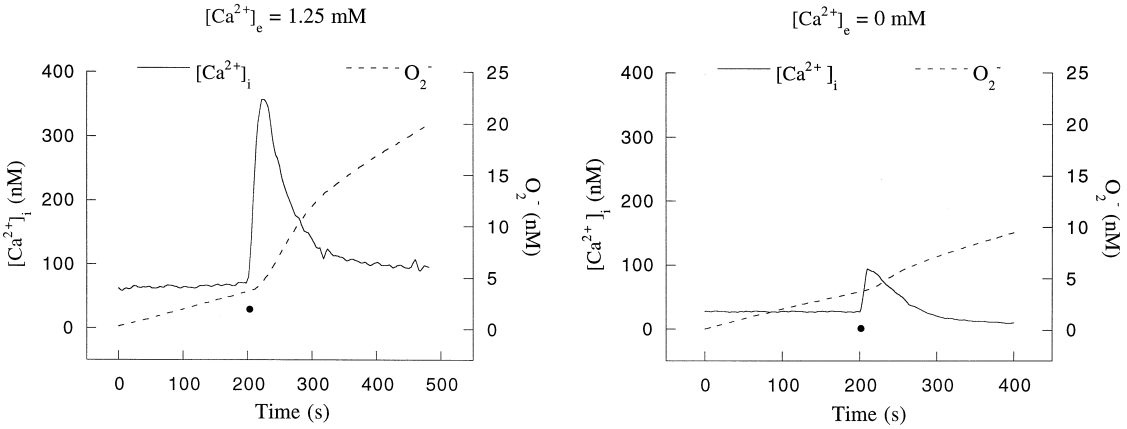

Fig. 1. fMLP-induced $[Ca^{2+}]_i$ and O_2^- responses in differentiated HL-60 cells. Assays were done in the presence (left panel) or in the absence (right panel) of extracellular Ca^{2+}. fMLP (1 μM) was added at the time indicated by the bullet. Experiments representative out of five are shown.

respiratory burst with respect to its action on store-operated Ca^{2+} entry.

2. Materials and methods

2.1. Materials

N-formyl-L-Methionyl-L-Leucyl-L-Phenylalanine (fMLP), ethylene glycol-bis(beta-amino-ethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and ionomycin were purchased from Sigma (St. Louis, MO), fura-2 acetoxymethylester (fura-2/AM) and dihydrorhodamine-123 from Molecular Probes (Eugene, OR), Iscove's modified Eagle medium, fetal calf serum and newborn calf serum from Gibco Life Technologies (Merelbeke, Belgium), and SK&F 96365 from Calbiochem (La Jolla, CA). SK&F 96365 was prepared extemporaneously in aqueous solution (30 mM). All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany). The physiological salt solution used throughout this study had the following composition (mM): NaCl 115, KCl 5, KH_2PO_4 1, $MgSO_4$ 1, Glucose 10, HEPES–Na 25, $CaCl_2$ 1.25, bovine serum albumin 0.1%, pH 7.4. Where indicated, $CaCl_2$ was omitted in the physiological salt solution.

2.2. Cells

HL-60 cells (Collins et al., 1977) were grown in Iscove's modified Eagle medium supplemented with fetal calf serum and newborn calf serum, 15% and 5%, respectively, and differentiation towards neutrophil-like cells was induced by dimethylsulfoxide (1.3%) for 4 days (Harris and Ralph, 1985).

2.3. Methods

2.3.1. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured concomitantly with O_2^- production by the use of a double labelling fluorescent technique (Bueb et al., 1995). Briefly, cells (2×10^6, 2 ml) were incubated with 2.5 μM fura-2/AM in physiological salt solution for 30 min at 37°C and then washed three times by centrifugation in physiological salt solution at 4°C. The final suspension of cells was done in physiological salt solution with or without Ca^{2+}. $[Ca^{2+}]_i$ was monitored with a Hitachi F-2000 spectrofluorimeter (10 nm slits, excitation 340 nm and 380 nm, emission 510 nm) and calculated according to Grynkiewicz et al. (1985).

2.3.2. Measurement of O_2^- production

O_2^- production was monitored with dihydrorhodamine-123 (1 μM) on fura-2 loaded cells, as previously described (Bueb et al., 1995). This dye becomes fluorescent in its oxidised state (rhodamine-123) and allows a direct monitoring of O_2^- production (Rothe et al., 1988; Emmendörffer et al., 1990). After addition of dihydrorhodamine-123 to the cell preparation 50–60 s before stimulation, the generation of rhodamine-123 was quantified by fluorescence measurements at 534 nm after excitation at 505 nm, con-

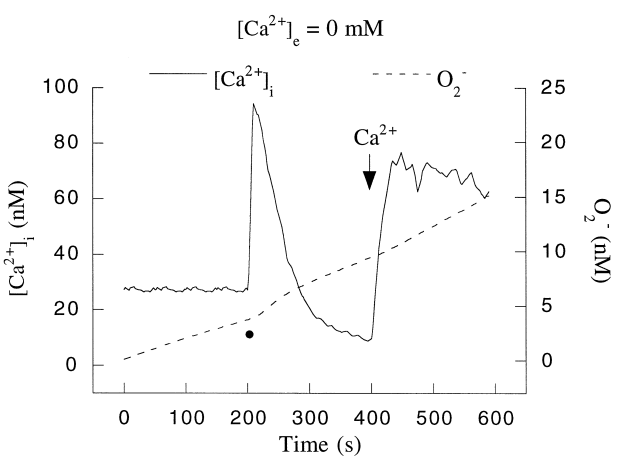

Fig. 2. Effect of Ca^{2+} addition following activation of differentiated HL-60 cells in the absence of extracellular Ca^{2+}. fMLP (1 μM, bullet) and $CaCl_2$ (1.25 mM, arrow) were added at the indicated times. Experiments representative out of five are shown.

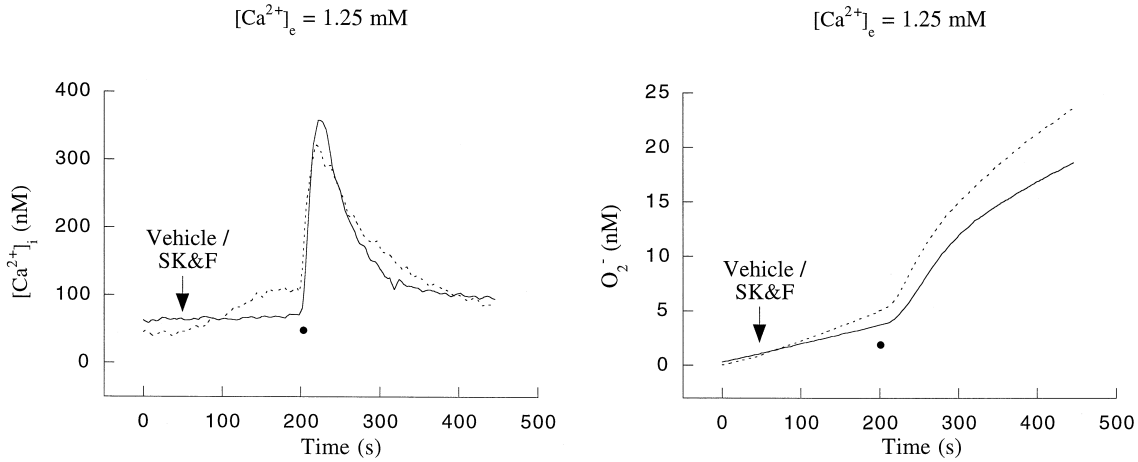

Fig. 3. Effect of SK&F 96365 on fMLP-induced responses in differentiated HL-60 cells in presence of extracellular Ca^{2+} (left panel: $[Ca^{2+}]_i$ variation; right panel: O_2^- production). Vehicle (plain line) or SK&F 96365 (30 μM, dotted line) was added to the cell suspension (arrow), followed by fMLP (1 μM, bullet). Experiments representative out of five are shown.

comitantly with the $[Ca^{2+}]_i$ measurements. Basal and stimulated production of O_2^- ions were determined by calculating the initial and maximal slopes of the tangent (dimension: M/s) to the tracings of the apparition of rhodamine-123 fluorescence before and after stimulation of the cells with the chemotactic peptide (fMLP-stimulated production). Net production of O_2^- was finally calculated by subtracting basal from fMLP-stimulated production of O_2^-.

2.3.3. Mn^{2+} influx studies

Fluorescence measurements were monitored on fura-2 loaded cells at 510 nm for the two excitation wavelengths of 340 and 380 nm, and at the isofluorescent point (360 nm). Mn^{2+} (0.2 mM) was added to the cell suspension prior to SK&F 96365 (30 μM) or vehicle in Ca^{2+}-free physiological salt solution in order to avoid competition with Ca^{2+} for fura-2 binding sites. Then, cells were stimulated with fMLP (1 μM) and finally ionomycin (10 μM) was added. Maximal decay in fura-2 fluorescence was calculated as dF/dt to analyse SK&F 96365 effects. Differences between control and treated cells were assessed by Student's *t*-test for paired observations ($p < 0.05$).

3. Results

3.1. Effect of extracellular Ca^{2+} deprivation on fMLP-induced $[Ca^{2+}]_i$ rise and O_2^- production

Dimethylsulfoxide-differentiated HL-60 cells responded to fMLP (1 μM) by an immediate and transient rise in $[Ca^{2+}]_i$, which was followed by an early O_2^- production (Fig. 1, left panel). $[Ca^{2+}]_i$ peaked at 30 s, and the

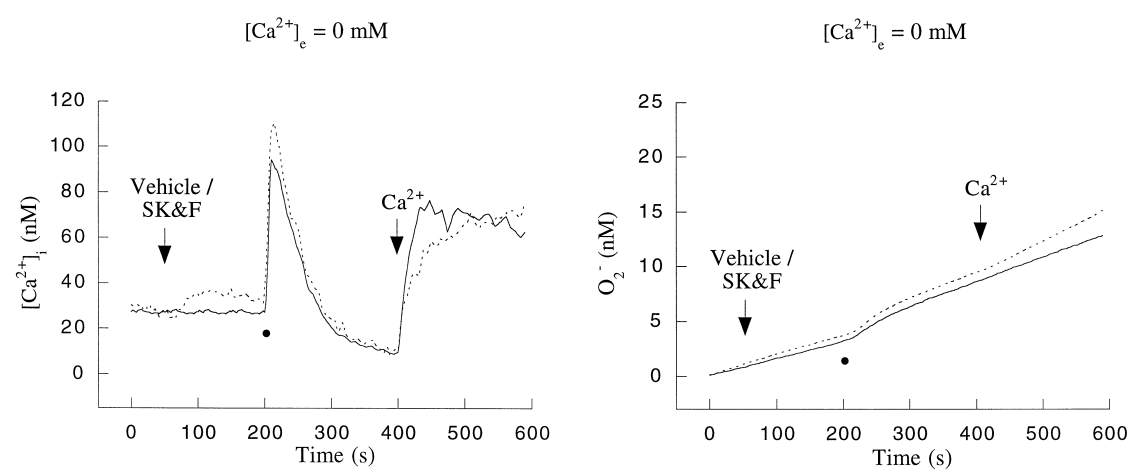

Fig. 4. Effect of SK&F 96365 on fMLP-induced responses in differentiated HL-60 cells in the absence of extracellular Ca^{2+} (left panel: $[Ca^{2+}]_i$ variation; right panel: O_2^- production). Vehicle (plain line) or SK&F 96365 (30 μM, dotted line) was added to the cell suspension (arrow), followed by fMLP (1 μM, bullet) and $CaCl_2$ (1.25 mM, arrow). Experiments representative out of five are shown.

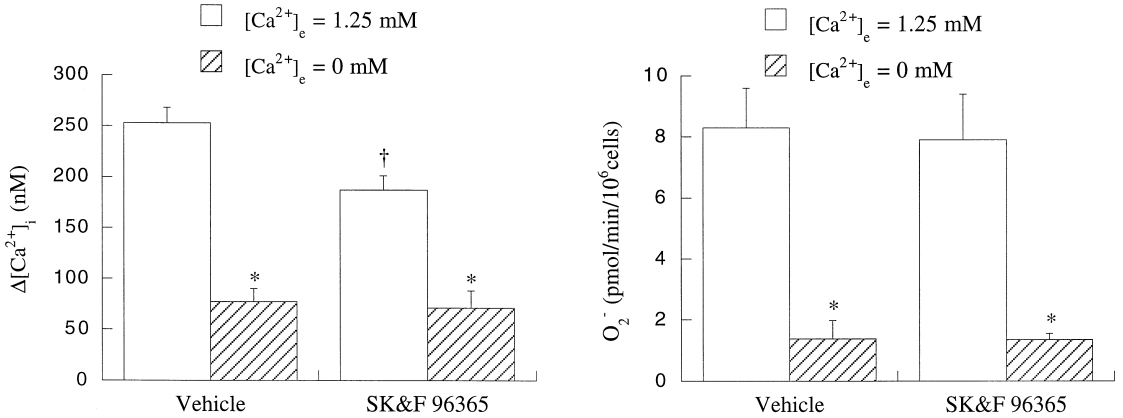

Fig. 5. Effect of SK&F 96365 on fMLP-induced responses in differentiated HL-60 cells in the presence or absence of extracellular Ca^{2+} (left panel: $[Ca^{2+}]_i$ variation; right panel: O_2^- production). The responses to fMLP (1 μM) are shown for both vehicle and SK&F 96365 (30 μM). Means ± S.E.M. of five experiments are shown. *Significantly different from response obtained in presence of extracellular Ca^{2+}. †Significantly different from the response obtained with vehicle.

maximal rate of O_2^- production was reached at 40 to 50 s. Removal of extracellular Ca^{2+} significantly reduced fMLP-induced $[Ca^{2+}]_i$ rise and O_2^- production (Fig. 1, right panel). Similar results were obtained when a Ca^{2+} chelator (EGTA, 2.5 mM) was added in physiological salt solution prior to fMLP-activation (not shown). Ca^{2+} re-addition augmented $[Ca^{2+}]_i$ (Fig. 2), at a level which was also reached when cells where not stimulated with fMLP (not shown). Ca^{2+} re-addition failed to restore a significant O_2^- production (Fig. 2).

3.2. Effect of SK&F 96365 on fMLP-induced responses in differentiated HL-60 cells

SK&F 96365 by itself induced a slight, unexpected increase in $[Ca^{2+}]_i$ (10–50 nM) (Fig. 3, left panel). SK&F 96365 inhibited approximately 30% of the $[Ca^{2+}]_i$ rise due to fMLP (Fig. 3, left panel), while slightly delaying return to baseline of $[Ca^{2+}]_i$. However, the area under the $[Ca^{2+}]_i$ curve also remained smaller in the presence of SK&F 96365. SK&F 96365 had no significant effect on fMLP-induced immediate increase in O_2^- production (Fig. 3, right panel). In the absence of extracellular-free Ca^{2+}, SK&F 96365 still induced a slight increase in $[Ca^{2+}]_i$ (5–15 nM) (Fig. 4, left panel). fMLP-activated $[Ca^{2+}]_i$ rise in the absence of extracellular Ca^{2+} was not affected by SK&F 96365, as the area under $[Ca^{2+}]_i$ curve was not modified in comparison to control curve. Neither was fMLP-induced O_2^- production (Fig. 4, right panel). In the presence of SK&F 96365, Ca^{2+} re-addition did not modified $[Ca^{2+}]_i$ rise and O_2^- production (Fig. 4). Fig. 5 summarises the results of all the experiments ($n = 5$).

3.3. Effect of SK&F 96365 on Mn^{2+} influx induced by fMLP in differentiated HL-60 cells

SK&F 96365 was able to slightly reduce basal Mn^{2+} influx (Fig. 6, insert). fMLP addition led to an increase in Mn^{2+} influx, as observed by the pronounced quenching of fura-2 fluorescence in cells not treated with SK&F 96365. As shown in Fig. 6, this fura-2 quenching was of limited duration and amplitude. Addition of ionomycin further quenched fura-2 fluorescence, indicating that these limitations after fMLP activation were due to divalent cation entry inactivation along time, rather than saturation of fura-2 binding sites for Mn^{2+}. The fMLP-activated Mn^{2+} entry was much slower in onset and quantitatively smaller in presence of SK&F 96365 (Fig. 6, insert).

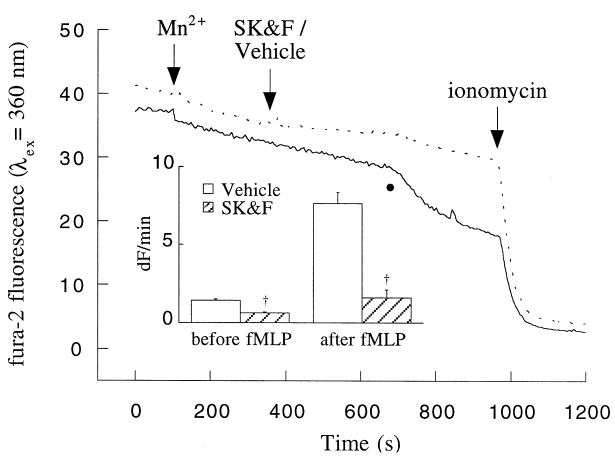

Fig. 6. Effect of SK&F 96365 on fMLP-induced Mn^{2+} entry in differentiated HL-60 cells. Mn^{2+} was added at the concentration of 0.2 mM, followed by SK&F 96365 (30 μM, dotted line) or vehicle (plain line), fMLP (1 μM, bullet) and ionomycin (10 μM). Experiments representative out of five are shown. *Insert*: effect of SK&F 96365 (30 μM) on Mn^{2+}-induced fura-2 fluorescence quenching kinetics before and after fMLP-stimulation. Means ± S.E.M. of five experiments are shown. †Significantly different from the response obtained with vehicle.

4. Discussion

The results presented here show that fMLP-induced early O_2^- production in dimethylsulfoxide-differentiated HL-60 cells is highly dependent upon external Ca^{2+} for optimal activation but is not inhibited by the purported store-operated Ca^{2+} entry inhibitor SK&F 96365, despite the fact that functioning of the store-operated Ca^{2+} entry pathway has been demonstrated in neutrophil-like HL-60 cells (Demaurex et al., 1992). Also, the relevance of extracellular Ca^{2+} for fMLP-induced $[Ca^{2+}]_i$ rise and O_2^- production was demonstrated by the major reduction of both responses in Ca^{2+}-free physiological salt solution. It appears, therefore, that extracellular Ca^{2+} is a requirement for a full biological response, i.e., O_2^- production in differentiated HL-60 neutrophil-like cells. Furthermore, the fact that Ca^{2+} re-addition is not sufficient for the restoration of the biological response indicates that Ca^{2+} has to be present at the very beginning of the activation process.

Although SK&F 96365 was not able to modify fMLP-induced O_2^- production, it had a significant inhibitory effect on fMLP-induced $[Ca^{2+}]_i$ rise. Similar results using leukotriene B_4 were reported in other cell types like guinea-pig eosinophils (Subramanian, 1992) where no relation was found between $[Ca^{2+}]_i$ elevation and the biological response. In neutrophil-like differentiated HL-60 cells, the inhibitory action of SK&F 96365 was abolished in absence of extracellular Ca^{2+}, demonstrating that SK&F 96365 was exerting its effect on Ca^{2+} influx rather than on Ca^{2+} store release or Ca^{2+} efflux in accordance with the results of Merritt et al. (1990). The influx of extracellular Ca^{2+} ions after Ca^{2+} addition in the medium was not inhibited by SK&F 96365, phenomenon which could be explained by a rapid inactivation of store-operated Ca^{2+} entry in our cell model. This concept is explicated by the dampening of the Mn^{2+} influx following fMLP-stimulation, verifying the inactivation of store-operated Ca^{2+} entry along time. This implies that extracellular Ca^{2+} has to be present at the initiation of fMLP activation to ensure a full biochemical response, i.e., $[Ca^{2+}]_i$ variation, in accordance with the biological response.

An unexpected effect of SK&F 96365 was its ability to slightly increase $[Ca^{2+}]_i$ by itself, although this increase was slow in onset and very moderate in amplitude. On our cell model, it is likely that SK&F 96365 exerted its direct effect on internal Ca^{2+} stores release or Ca^{2+} efflux rather than on Ca^{2+} influx since this phenomenon was observed in the presence and in the absence of extracellular Ca^{2+}, and since Mn^{2+}-mediated fura-2 fluorescence quenching was diminished. This SK&F 96365-induced moderate $[Ca^{2+}]_i$ increase has also been reported in platelets and in neutrophils, where it was able to cause a transient increase in $[Ca^{2+}]_i$ (100–200 nM) possibly due to an emptying of intracellular Ca^{2+} stores (Merritt et al., 1990), independently of extracellular Ca^{2+}. These extracellular Ca^{2+}-independent effects of SK&F 96365 question the selectivity and the potency of this molecule in inhibiting store-operated Ca^{2+} entry in our neutrophil-like HL-60 cell model.

Since SK&F 96365 effects were modest, we used an alternative approach, i.e., the analysis of Mn^{2+}-induced fura-2 fluorescence quenching. Indeed, the increase in bivalent cation permeability induced by fMLP was shown to concern both Ca^{2+} and Mn^{2+} ions (Demaurex et al., 1992). We could then demonstrate a clear inhibitory action of SK&F 96365 on fMLP-induced Mn^{2+} entry. Considering this result, a discrepancy between Ca^{2+}-induced and Mn^{2+}-induced fura-2 fluorescence appears. SK&F 96365 may not be effective in inhibiting large influxes of Ca^{2+} ions through store-operated Ca^{2+} entry. On the other hand, Mn^{2+}-induced fura-2 quenching studies are more efficient at studying discrete phenomenons, since Mn^{2+} displays a much higher affinity for fura-2 than Ca^{2+} (Grynkiewicz et al., 1985), giving a direct observation of divalent ions fluxes through the membrane. Therefore, these results confirm that SK&F 96365 is a weak inhibitor of store-operated Ca^{2+} entry in differentiated HL-60 cells using our experimental set-up. This result is cautioned by a high IC_{50} and a limited selectivity, as discussed previously in granulocytes and platelets by Merritt et al. (1990).

In conclusion, our results indicate that SK&F 96365, despite its ability to partially inhibit store-operated Ca^{2+} entry, is not capable of modifying the fMLP-activated resulting biological response in neutrophil-like HL-60 cells. However, in dimethylsulfoxide-differentiated neutrophil-like HL-60 cells, O_2^- production is essentially dependent upon the presence of extracellular Ca^{2+} and an influx of Ca^{2+} ions is necessary for the full induction and the maintenance of this biological response.

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References

Boucek, M.M., Snyderman, R., 1976. Calcium influx requirement for human neutrophil chemotaxis: inhibition by lanthanum chloride. Science 193, 905–907.

Bueb, J.-L., Gallois, A., Schneider, J.-C., Parini, J.-P., Tschirhart, E., 1995. A double labelling fluorescent assay for concomitant measurements of $[Ca^{2+}]_i$ and $O_2^{\cdot}$ production in human macrophages. Biochim. Biophys. Acta 1244, 79–84.

Collins, S.J., Gallo, R.C., Gallagher, R.E., 1977. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. Nature 270, 347–349.

Demaurex, N., Schlegel, W., Varnai, P., Mayr, G., Lew, D.P., Krause,

K.H., 1992. Regulation of Ca^{2+} influx in myeloid cells. J. Clin. Invest. 90, 830–839.

Emmendörffer, A., Hecht, M., Lohmann-Matthes, M.L., Roesler, J., 1990. A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. J. Immunol. Methods 131, 269–275.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3446.

Harris, P., Ralph, P., 1985. Human leukemic models of myelomonocytic development: a review of the HL-60 and U-937 cell lines. J. Leukocyte Biol. 37, 407–422.

Lew, D.P., Wollheim, C.B., Waldvogel, F.A., Pozzan, T., 1984. Modulation of cytosolic free Ca^{2+} transients by changes in intracellular Ca^{2+} buffering capacity: correlation with exocytosis and $O_2^{\cdot}$ production in human neutrophils. J. Cell. Biol. 99, 1212–1220.

Merritt, J.E., Armstrong, W.P., Benham, C.D., Hallam, T.J., Jacob, R., Jaxa-Chamiec, A., Leigh, B.K., McCarthy, S.A., Moores, K.E., Rink, T.J., 1990. SK&F 96365, a novel inhibitor of receptor-mediated Ca^{2+} entry. Biochem. J. 271, 515–522.

Montero, M., Alvarez, J., Garcia-Sancho, J., 1991. Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular Ca^{2+} stores. Biochem. J. 277, 73–79.

Montero, M., Alvarez, J., Garcia-Sancho, J., 1992. Control of plasma-membrane Ca^{2+} entry by the intracellular Ca^{2+} stores—kinetic evidence for a short-lived mediator. Biochem. J. 288, 519–525.

Pozzan, T., Lew, D.P., Wollheim, C.B., Tsien, R.Y., 1983. Is cytosolic ionized Ca^{2+} regulating neutrophil activation?. Science 221, 1413–1415.

Putney, J.W., 1986. A model for receptor-regulated Ca^{2+} entry. Cell Calcium 7, 1–12.

Putney, J.W., 1990. Capacitative Ca^{2+} entry revisited. Cell Calcium 11, 611–624.

Rothe, G., Oser, A., Valet, G., 1988. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. Naturwissenschaften 75, 354–355.

Subramanian, N., 1992. Leukotriene B_4-induced steady state Ca^{2+} rise and superoxide anion generation in guinea-pig eosinophils are not related events. Biochem. Biophys. Res. Commun. 167, 670–676.