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Are Ca^{2+} channels in neutrophils activated by a rise in cytosolic free Ca^{2+}?

Patricia E. Nasmith and Sergio Grinstein

Division of Cell Biology, Research Institute, Hospital for Sick Children, 555 University Ave, Toronto M5G 1X8 and *Department of Biochemistry, University of Toronto, Toronto, Canada*

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It was recently suggested that the opening of neutrophil plasma membrane Ca^{2+} channels by chemotactic agents is mediated by a rise in free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). This hypothesis was tested in human cells monitoring $[Ca^{2+}]_i$ with the indicator indo-1. In cells loaded with the Ca^{2+}-chelating agent bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*',*N*'-tetraacetate, transmembrane Ca^{2+} uptake could be stimulated by formyl-methionyl-leucyl-phenylalanine (fMLP) even when $[Ca^{2+}]_i$ was at or below the resting level. In contrast, simply elevating $[Ca^{2+}]_i$ in unstimulated cells failed to increase transmembrane uptake. It was concluded either that Ca^{2+} uptake across the plasma membrane is activated directly by the formation of the chemotactic factor-receptor complex or, more likely, that a transduction mechanism distinct from changes in $[Ca^{2+}]_i$ is involved.

Ca^{2+} channel; Indo-1; Chemotactic factor; Signal transduction; Polymorphonuclear leukocyte; (Human)

1. INTRODUCTION

Activation of neutrophils by chemotactic agents is accompanied by a rapid rise in cytoplasmic free Ca^{2+} level ($[Ca^{2+}]_i$[1,2], which is thought to be essential for many of the subsequent reactions, including degranulation and superoxide generation [1-3]. The change in $[Ca^{2+}]_i$ is biphasic, being composed of a large, transient rise and a more sustained, but smaller increase. The latter is believed to represent entry of extracellular Ca^{2+} across the plasma membrane, inasmuch as it is stringently dependent on the availability of external Ca^{2+}. In contrast, the transient early phase persists in cells activated in Ca^{2+}-free solutions and has therefore been interpreted as mobilization of intracellular Ca^{2+} stores, such as the endoplasmic reticulum [4,5].

Correspondence address: S. Grinstein, Division of Cell Biology, Research Institute, Hospital for Sick Children, 555 University Ave, Toronto M5G 1X8, Canada

Whereas the release of Ca^{2+} from intracellular stores is known to be activated by inositol 1,4,5-trisphosphate (IP_3) [5], the mechanism mediating the activation of Ca^{2+} uptake across the plasma membrane is not understood. In fact, the nature of the pathway responsible for transmembrane Ca^{2+} flux has not been defined. Recently, Ca^{2+}-transporting channels were identified in the plasma membrane of human neutrophils using the patch-clamp technique [6]. These channels were not directly affected by either IP_3 or chemotactic agents, but could be activated by increasing the Ca^{2+} concentration of the medium bathing the cytoplasmic side of the patch. It was therefore suggested that, when intact cells are stimulated, transmembrane Ca^{2+} uptake develops in response to the initial rise in $[Ca^{2+}]_i$, which results from mobilization of internal stores [6].

The purpose of the present experiments was to test experimentally whether the opening of plasma membrane Ca^{2+} channels by chemotactic agents is mediated by a rise in $[Ca^{2+}]_i$. This hypothesis

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predicts that the plasmalemmal channels will fail to open if the initial increase in $[Ca^{2+}]_i$ induced by the stimulus is prevented. Moreover, it also implies that channel opening should be observed whenever $[Ca^{2+}]_i$ is elevated, regardless of the method used for Ca^{2+} mobilization. These predictions were tested by measuring $[Ca^{2+}]_i$ with the fluorescent indicator indo-1 in human neutrophils activated with the chemotactic tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP).

2. MATERIALS AND METHODS

2.1. *Reagents*

Powdered RPMI-1640 medium (with 25 mM Hepes, HCO_3^--free) and fMLP were from Sigma (St. Louis, MO). Ionomycin and nigericin were from Calbiochem (San Diego, CA). The acetoxy methyl esters of indo-1 and bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA) were obtained from Molecular Probes (Eugene, OR).

2.2. *Solutions*

During fluorescence measurements, cells were suspended in a medium containing (in mM) 140 NaCl, 5 KCl, 10 glucose, 10 Hepes, and 2 $CaCl_2$. Solutions were made Ca^{2+}-free by omission of $CaCl_2$ and, where indicated, 1 mM EGTA or 1 mM $MnCl_2$ was added. The osmolarity of all solutions was adjusted to 290 ± 5 mosM.

2.3. *Methods*

Neutrophils were isolated from fresh heparinized human blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation [7]. Contaminating red cells were removed by ammonium chloride lysis. Cells were maintained in RPMI (10^7 cells/ml) at room temperature until use.

For $[Ca^{2+}]_i$ determinations, neutrophils were loaded with indo-1 by incubation in RPMI with 1 μM of the precusor acetoxy methyl ester for 30 min at 37°C. Where indicated, 10 μM BAPTA acetoxy methyl ester was also added. After washing, 3×10^6 cells were suspended in 1 ml of the indicated medium at 37°C with continuous magnetic stirring and fluorescence was determined using a Perkin-Elmer 650-40 fluorimeter with excitation at 331 nm and emission at 410 nm, using 3 and 15 nm slits, respectively. Calibration was made using ionomycin and Mn^{2+} as described [8], using a K_d of 250 nM and an F_{max}/F_{min} ratio of 12. At the concentrations used, neither fMLP nor nigericin was fluorescent at the wavelengths measured. Data are presented as means ± SE of the indicated number of experiments.

3. RESULTS AND DISCUSSION

Human neutrophils respond with a biphasic $[Ca^{2+}]_i$ change when challenged with fMLP (fig.1A). The resting $[Ca^{2+}]_i$, which averaged 170 ± 15 nM ($n=7$) when measured with indo-1, increased within 15 s to a peak value of 1900 ± 200 nM ($n=7$), and then rapidly subsided giving way to a more slowly decaying secondary phase. The latter was absent when the cells were activated in Ca^{2+}-free medium (fig.1B), in which case only the initial transient phase was detected. Similar results were obtained whether the medium was nominally Ca^{2+}-free or if, in addition, 1 mM EGTA was added. These data are consistent with the existence of two components of the $[Ca^{2+}]_i$ change: a rapid mobilization of intracellular Ca^{2+} stores and a more prolonged influx of extracellular Ca^{2+}.

We investigated whether the large intial $[Ca^{2+}]_i$ increase was required to trigger Ca^{2+} uptake across the plasma membrane. For this purpose, the transient was obliterated by pre-loading the cells with BAPTA, an efficient Ca^{2+}-chelating agent that does not interfere with the indo-1 determinations. As shown in fig.1C (bottom trace), stimulation of BAPTA-loaded cells suspended in Ca^{2+}-free medium with fMLP produced only a marginal increase in $[Ca^{2+}]_i$ (20 ± 5 nM, $n=14$). Note that $[Ca^{2+}]_i$ never exceeded the level normally observed in untreated cells suspended in medium with Ca^{2+} (e.g.fig.1A). However, subsequent addition of extracellular Ca^{2+} resulted in a marked $[Ca^{2+}]_i$ increase (top trace, fig.1C), attaining levels comparable to that of the sustained phase recorded in intact cells (cf. fig.1A and C). Approximately the same $[Ca^{2+}]_i$ level was reached when BAPTA-loaded cells were stimulated by fMLP in Ca^{2+}-containing medium (fig.1D). In this case the stimulus elicited a very small rapid increase, reflecting the residual effect of mobilization of stores on the heavily buffered $[Ca^{2+}]_i$, followed by a

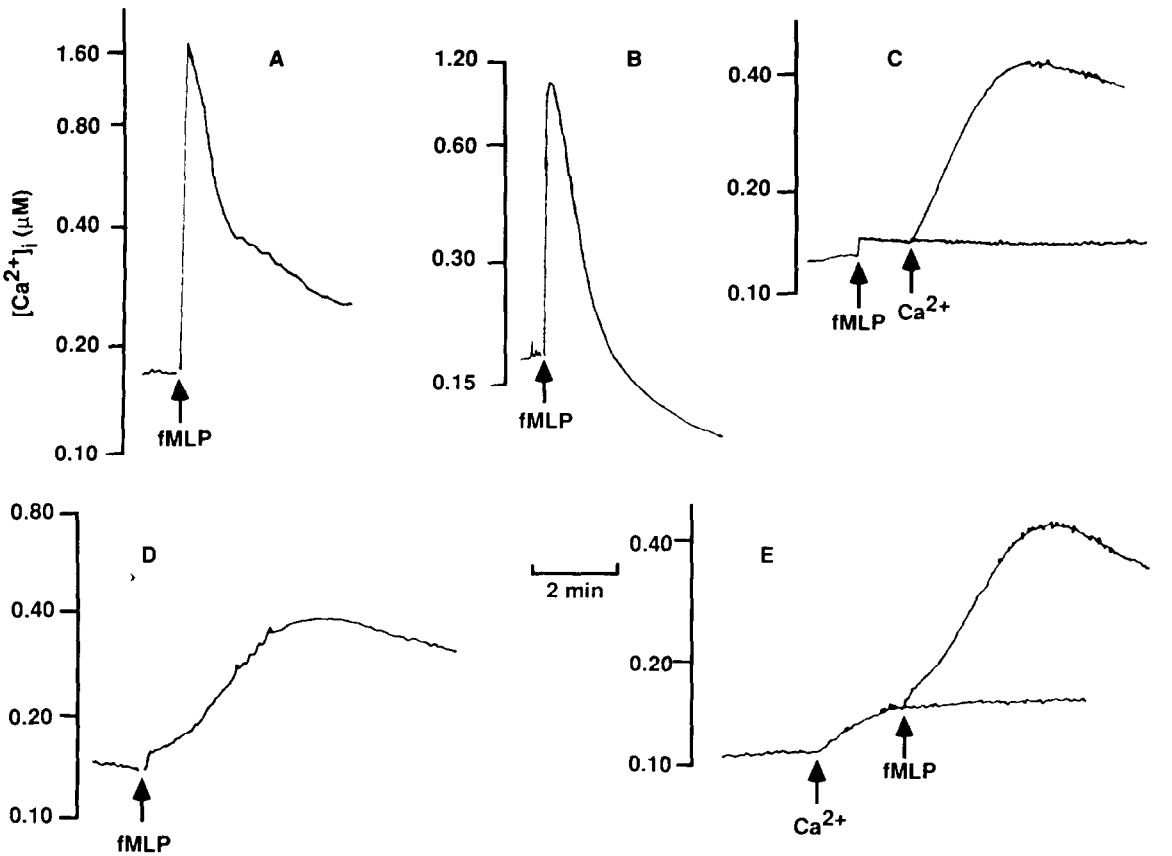

Fig.1. Free cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) changes induced by fMLP in human neutrophils. Cells were loaded with indo-1 and used for $[Ca^{2+}]_i$ determinations as described in section 2. In C-E the cells were also loaded with BAPTA by pre-incubation with 10 μM acetoxy methyl ester precursor for 30 min at 37°C. (A) Indo-1 loaded cells suspended in Ca^{2+}-containing medium; (B) indo-1-loaded cells suspended in Ca^{2+}-free medium with 1 mM EGTA; (C) cells loaded with both BAPTA and indo-1, suspended in nominally Ca^{2+}-free medium; (D) BAPTA- and indo-1-loaded cells suspended in Ca^{2+}-containing medium; (E) BAPTA- and indo-1-loaded cells suspended in nominally Ca^{2+}-free medium. Where indicated, fMLP (10^{-7} M) or $CaCl_2$ (2 mM) was added to the medium. In C and E, the second addition was made to the top trace only. The traces are representative of at least 4 experiments. The time scale is applicable to all the traces. Temperature, 37°C.

slower, larger rise. Note that, unlike the top trace in fig.1C, the increase in fig.1D is sigmoidal, attaining maximal rate approx. 1–2 min after addition of fMLP. This lag is inherent to the transduction process and does not reflect the kinetics of binding of fMLP to its receptor, since most of the biological responses induced by the tripeptide, including Ca^{2+} mobilization (e.g. fig.1A), are evident much earlier. The similarity between the traces in fig.1C (top) and D suggests that the slow, sustained phase of $[Ca^{2+}]_i$ change is due to uptake of extracellular Ca^{2+}. Fig.1E shows that the marked $[Ca^{2+}]_i$ change observed upon reintroduction of Ca^{2+} in fig.1C was due to the pretreatment with fMLP, and was not the result of cell damage incurred by loading with BAPTA or during incubation in Ca^{2+}-free medium. When Ca^{2+} was added to BAPTA-loaded cells suspended in Ca^{2+}-free medium, only a small increase (40 ± 8 nM, $n = 6$) was recorded, bringing $[Ca^{2+}]_i$ to a level similar to that observed in untreated cells maintained continuously in Ca^{2+}-containing media (e.g. fig.1A). Subsequent addition of fMLP induced the expected response (top trace, fig.1E). Taken

together, these results suggest that the large initial transient $[Ca^{2+}]_i$ change induced by fMLP is not required to induce the transmembrane net Ca^{2+} uptake.

A rise in $[Ca^{2+}]_i$ like those reported in fig.1 could result from an increased rate of Ca^{2+} influx, decreased rate of efflux or a combination of both. The experiments in fig.2 suggest that the principal effect of fMLP is to increase the rate of unidirectional Ca^{2+} uptake. This was demonstrated using Mn^{2+}, a substituent that can permeate Ca^{2+}-selective channels in other cell types [9]. Unlike Ca^{2+}, which enhances indo-1 fluorescence at the wavelengths studied, Mn^{2+} binds to the probe with high affinity and quenches its fluorescence. Thus, the rate of indo-1 fluorescence quenching can be used as a measure of Mn^{2+} uptake. As shown in fig.2A, when indo-1-loaded cells are suspended in nominally Ca^{2+}-free media containing 1 mM Mn^{2+}, the fluorescence intensity declines gradually, indicating a continuous influx of Mn^{2+}. Chelation of extracellular Mn^{2+} with excess EGTA largely stops the progression of quenching, but no reversal (i.e. fluorescence increase) was detected, indicating that efflux of Mn^{2+} from the cells is negligible. Thus, monitoring indo-1 quenching by Mn^{2+} is a convenient method to measure unidirectional divalent cation uptake. Accordingly, the divalent cation ionophore, ionomycin, induced a drastic increase in the rate of indo-1 quenching. This was preceded by a brief fluorescence increase, likely due to Ca^{2+} release from internal stores.

Fig.2B shows the effect of fMLP on the rate of indo-1 quenching by Mn^{2+} in nominally Ca^{2+}-free medium. The chemotactic peptide induced a small fluorescence increase like that in fig.1C, probably induced by mobilization of intracellular Ca^{2+}. Unlike a previous report using quin2 [10], we minimized this component by pre-loading the cells with BAPTA. After a lag of about 1 min, the rate of quenching rose significantly, consistent with an increased rate of Mn^{2+} (divalent cation) uptake. A similar lag was observed for the increase in $[Ca^{2+}]_i$ (fig.1D), confirming that Mn^{2+} enters the cells via the receptor-activated Ca^{2+} pathway. As before EGTA greatly reduced the rate of quenching but did not induce recovery of fluorescence, indicating minimal Mn^{2+} efflux. These data indicate that the effect of fMLP on net Ca^{2+} uptake is mainly due to an increase in the influx of Ca^{2+} (Mn^{2+}), which is consistent with reports of increased $^{45}Ca^{2+}$ influx in stimulated cells [11], rather than to a decrease in the rate of efflux.

The preceding data indicate that Ca^{2+} (Mn^{2+}) uptake across the plasma membrane is stimulated by fMLP even under conditions where the $[Ca^{2+}]_i$ increase resulting from intracellular Ca^{2+} mobilization is largely obliterated. It could be argued that the very small residual increase observed in BAPTA-loaded cells (fig.1C,D) suffices to elicit the response. However, two lines of evidence rule out this possibility. First, because the baseline $[Ca^{2+}]_i$ tends to drop when cells are suspended in Ca^{2+}-free media, the small change induced by fMLP in BAPTA-loaded cells generally does not exceed the level normally observed in untreated cells suspended in medium with Ca^{2+} (cf. fig.1A and C). Second, increasing $[Ca^{2+}]_i$ to com-

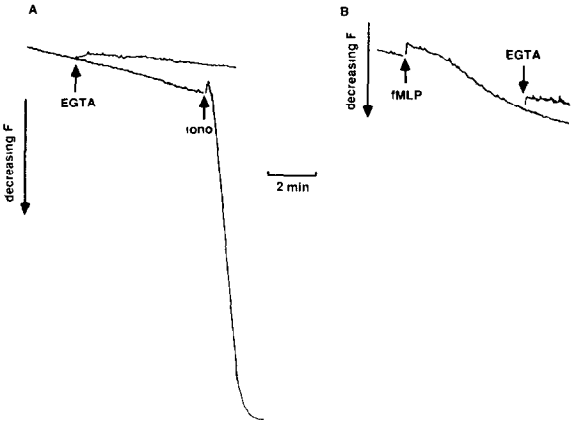

Fig.2. Mn^{2+} uptake by neutrophils, measured by the rate of indo-1 fluorescence quenching. Neutrophils were double-loaded with indo-1 and BAPTA, washed and suspended in nominally Ca^{2+}-free medium containing 1 mM Mn^{2+}. Indo-1 fluorescence was recorded as described in section 2. (A) Where indicated EGTA (1.5 mM, top trace) or ionomycin (1 μM, bottom trace) was added. (B) 10^{-7} fMLP and EGTA (1.5 mM, top trace only) were added where indicated. A and B are composites of two separate traces. The composites are representative of at least three experiments. Temperature, 37°C.

parable or even higher levels by means other than fMLP failed to increase the permeability of the membrane to divalent cations. This is illustrated in fig.3, where nigericin was used to elevate $[Ca^{2+}]_i$. Treatment with 2 μM nigericin increased $[Ca^{2+}]_i$ by 40 ± 5 nM ($n=6$). The mechanism whereby nigericin increases $[Ca^{2+}]_i$ is not understood, but must involve mobilization of internal Ca^{2+} stores, inasmuch as the effect is observed in nominally Ca^{2+}-free media (fig.3A). As shown in fig.3B, the $[Ca^{2+}]_i$ increase induced by nigericin is also detectable in cells suspended in Ca^{2+}-free medium with Mn^{2+}. Significantly, the rate of indo-1 quenching by Mn^{2+} was not affected by treatment with nigericin, indicating that elevating $[Ca^{2+}]_i$ by 40 nM does not suffice to increase the divalent cation permeability of the plasma membrane. Therefore, the hypothesis that the small (≤ 20 nM) residual $[Ca^{2+}]_i$ increase in fig.1C is sufficient to elicit transmembrane Ca^{2+} (Mn^{2+}) uptake is not tenable.

In summary, the evidence presented here is not consistent with the hypothesis of Von Tscharner et al. [6] that an increase in $[Ca^{2+}]_i$ is the signal that mediates opening of Ca^{2+} channels in the plasma membrane of neutrophils activated with chemotactic agents. Our evidence indicates that transmembrane Ca^{2+} uptake can be stimulated by chemoattractants even when $[Ca^{2+}]_i$ is at or below the resting level. Moreover, in unstimulated cells, increasing $[Ca^{2+}]_i$ by mobilization of internal stores was not sufficient to activate the plasma membrane pathway. It must be concluded that the channels are activated directly by the receptor-ligand complex or, alternatively, that a transduction mechanism distinct from changes in $[Ca^{2+}]_i$ is involved. Accumulation of a second messenger is suggested by the time required to attain maximal rates of Ca^{2+} uptake following interaction of the ligand with its receptor (figs.1D,E,2B). In lymphocytes, IP_3 has been proposed as the putative messenger mediating the opening of Ca^{2+} channels by mitogens [12]. However, IP_3 was unable to activate the Ca^{2+}-transporting channels in neutrophils [6]. In these cells the signal may be conveyed instead by inositol 1,3,4,5-tetrakisphosphate, which was recently suggested to activate Ca^{2+} uptake in sea urchin eggs [13]. Alternatively, a different and novel transduction mechanism may be involved.

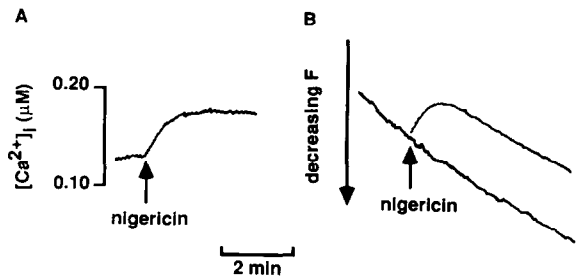

Fig.3. Effects of nigericin on $[Ca^{2+}]_i$ and on the rate of Mn^{2+} uptake. (A) Indo-1-loaded cells were suspended in nominally Ca^{2+}-free medium and $[Ca^{2+}]_i$ recorded as described in section 2. Where indicated, 2 μM nigericin was added to the cuvette. (B) Indo-1-loaded cells were suspended in nominally Ca^{2+}-free medium containing 1 mM Mn^{2+}. Fluorescence quenching was recorded as in fig.2. Where indicated by the arrow, 2 μM nigericin was added to the top trace. The untreated cells illustrated by the bottom trace are included for comparison. The traces are representative of at least 3 experiments. Temperature, 37°C.

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