

Regulation of Ca²⁺ influx in myeloid cells. Role of plasma membrane potential, inositol phosphates, cytosolic free [Ca²⁺]_i, and filling state of intracellular Ca²⁺ stores.

N Demaurex, ... , D P Lew, K H Krause

J Clin Invest. 1992;**90**(3):830-839. <https://doi.org/10.1172/JCI115958>.

Research Article

To study the mediation of Ca²⁺ influx by second messengers in myeloid cells, we have combined the whole-cell patch clamp technique with microfluorimetric measurements of [Ca²⁺]_i. Me₂SO-differentiated HL-60 cells were loaded with the fluorescent Ca²⁺ indicator Indo-1, allowed to adhere to glass slides, and patch-clamped. Receptor agonists and Ca(2+)-ATPase inhibitors were applied by superfusion and inositol phosphates by microperfusion through the patch pipette. In voltage-clamped cells, [Ca²⁺]_i elevations with a sustained phase could be induced by (a) the chemoattractant receptor agonist FMLP, (b) the Ca(2+)-releasing second messenger myo-inositol(1,4,5)trisphosphate [Ins(1,4,5)P₃], as well as its nonmetabolizable analogues, and (c) the Ca(2+)-ATPase inhibitor cyclopiazonic acid, which depletes intracellular Ca²⁺ stores. In the absence of extracellular Ca²⁺, responses to all stimuli were short-lasting, monophasic transients; however, subsequent addition of Ca²⁺ to the extracellular medium led to an immediate [Ca²⁺]_i increase. In all cases, the sustained phase of the [Ca²⁺]_i elevations could be inhibited by millimolar concentrations of extracellular Ni²⁺, and its amplitude could be decreased by depolarization of the plasma membrane. Thus, the sustained phase of the Ca²⁺ elevations was due to Ca²⁺ influx through a pathway sensitive to the electrical driving force and to Ni²⁺. No Ca²⁺ influx could be observed after (a) plasma membrane depolarization in resting cells, (b) an imposed [Ca²⁺]_i transient independent of receptor activation, or (c) microperfusion of myo-inositol(1,3,4,5)tetrahisphosphate [...]

Find the latest version:

<https://jci.me/115958/pdf>



Regulation of Ca²⁺ Influx in Myeloid Cells

Role of Plasma Membrane Potential, Inositol Phosphates, Cytosolic Free [Ca²⁺]_i, and Filling State of Intracellular Ca²⁺ Stores

Nicolas Demaurex,* Werner Schlegel,† Peter Varnai,§ Georg Mayr,|| Daniel P. Lew,* and Karl-Heinz Krause*

*Infectious Diseases Division, University Hospital Geneva, CH-1211 Geneva 4, Switzerland; †Fondation pour

Recherches Medicales, CH-1211 Geneva 4, Switzerland; §Semmelweis University Medical School, H-1444 Budapest 8, Hungary;

and ||Ruhr Universität, D-4680 Bochum 1, Federal Republic of Germany

Abstract

To study the mediation of Ca²⁺ influx by second messengers in myeloid cells, we have combined the whole-cell patch clamp technique with microfluorimetric measurements of [Ca²⁺]_i. Me₂SO-differentiated HL-60 cells were loaded with the fluorescent Ca²⁺ indicator Indo-1, allowed to adhere to glass slides, and patch-clamped. Receptor agonists and Ca²⁺-ATPase inhibitors were applied by superfusion and inositol phosphates by microperfusion through the patch pipette. In voltage-clamped cells, [Ca²⁺]_i elevations with a sustained phase could be induced by (a) the chemoattractant receptor agonist FMLP, (b) the Ca²⁺-releasing second messenger myo-inositol(1,4,5)trisphosphate [Ins(1,4,5)P₃], as well as its nonmetabolizable analogues, and (c) the Ca²⁺-ATPase inhibitor cyclopiazonic acid, which depletes intracellular Ca²⁺ stores. In the absence of extracellular Ca²⁺, responses to all stimuli were short-lasting, monophasic transients; however, subsequent addition of Ca²⁺ to the extracellular medium led to an immediate [Ca²⁺]_i increase. In all cases, the sustained phase of the [Ca²⁺]_i elevations could be inhibited by millimolar concentrations of extracellular Ni²⁺, and its amplitude could be decreased by depolarization of the plasma membrane. Thus, the sustained phase of the Ca²⁺ elevations was due to Ca²⁺ influx through a pathway sensitive to the electrical driving force and to Ni²⁺. No Ca²⁺ influx could be observed after (a) plasma membrane depolarization in resting cells, (b) an imposed [Ca²⁺]_i transient independent of receptor activation, or (c) microperfusion of myo-inositol(1,3,4,5)tetrahydrophosphate (Ins(1,3,4,5)P₄). Also, Ins(1,3,4,5)P₄ did not have additive effects when co-perfused with a submaximal concentration of Ins(1,4,5)P₃. Our results suggest that, in myeloid cells, activation of chemoattractant receptors induces an electrogenic, Ni²⁺-sensitive Ca²⁺ influx via generation of Ins(1,4,5)P₃. Ins(1,4,5)P₃ might activate Ca²⁺ influx directly, or by depletion of intracellular Ca²⁺ stores, but not via [Ca²⁺]_i increase or Ins(1,3,4,5)P₄ generation. (*J. Clin. Invest.* 1992;90:830–839.) Key words: Ca²⁺ regulation • ion channels • inositol phosphates • patch clamp • phagocytes

Address reprint requests to Dr. Demaurex, Infectious Diseases Division, University Hospital Geneva, CH1211 Geneva 4, Switzerland.

Received for publication 27 January 1992 and in revised form 7 April 1992.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/92/09/0830/10 \$2.00

Volume 90, September 1992, 830–839

Introduction

Activation of myeloid cells by chemoattractants leads to biphasic [Ca²⁺]_i increases, an initial transient due to Ca²⁺ release from internal stores, and a sustained phase due to Ca²⁺ influx across the plasma membrane (1–3). It has been clearly demonstrated (4–6) that agonist-induced Ca²⁺ release is mediated by myo-inositol (1,4,5) trisphosphate [Ins(1,4,5)P₃].¹ In contrast, the mechanism and mediation by second messengers of chemoattractant-induced Ca²⁺ influx in myeloid cells remains a controversial issue (for review, see reference 7). Previous studies did not find evidence for the existence of voltage-dependent Ca²⁺ channels (2, 8). Although scarcely any studies have directly investigated the role of second messengers for Ca²⁺ influx in myeloid cells, its mediation by a second messenger is generally assumed. Based on the very close correlation among Ca²⁺ influx, inositol phosphate generation, Ins(1,4,5)P₃-induced Ca²⁺ release, and increase in [Ca²⁺]_i, the following hypotheses have received most attention: (a) the product of Ins(1,4,5)P₃ phosphorylation, Myo-inositol(1,3,4,5)tetrahydrophosphate (Ins(1,3,4,5)P₄), mediates Ca²⁺ influx (3, 9), (b) a rise in [Ca²⁺]_i mediates Ca²⁺ influx (8), (c) Ins(1,4,5)P₃ itself activates a Ca²⁺ channel in the plasma membrane, in addition to its activation of the Ca²⁺ release channel of internal Ca²⁺ stores, and (d) the filling state of the intracellular Ca²⁺ stores regulates Ca²⁺ influx (capacitative regulation of Ca²⁺ influx; see references 10 and 11).

In this study, we have characterized the regulation of Ca²⁺ influx in dimethylsulfoxide (DMSO)-differentiated HL-60 cells at the single-cell level by using the patch clamp technique combined with microfluorimetric measurements of [Ca²⁺]_i. Our results show that, in myeloid cells, Ins(1,4,5)P₃ is a powerful regulator of Ca²⁺ influx. Ins(1,4,5)P₃-induced Ca²⁺ influx is mediated, at least in part, by depletion of intracellular Ca²⁺ stores.

Methods

Materials. N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), cyclopiazonic acid (CPA), and DMS were purchased from Sigma Chemical Co. (St. Louis, MO), fetal calf serum from Gibco (Paisley, Scotland), Indo-1 acetoxymethylester (AM) and Indo-1-free acid from Molecular Probes, Inc. (Eugene, OR). Myo-inositol(1,4,5)trisphosphatathioate (Ins(1,4,5)P₃S₃) from New England Nuclear (Dreieich, FRG), Ins(1,4,5)P₃, and other inositol phosphates (> 99% pure) were isolated and purified by one of us (Dr. Mayr), according to reference

1. **Abbreviations used in this paper:** CPA, cyclopiazonic acid; Ins(1,4,5)P₃, myo-inositol(1,4,5)trisphosphate; Ins(1,3,4,5)P₄, myo-inositol(1,3,4,5)tetrahydrophosphate; Ins(2,4,5)P₃, myo-inositol(2,4,5)trisphosphate; Ins(1,4,5)P₃S₃, myo-inositol(1,4,5)trisphosphatathioate; Ins(3,4,5,6)P₄, myo-inositol(3,4,5,6)tetrahydrophosphate.

12. All other chemicals were of analytical grade and obtained from Sigma Chemical Co., Merck (Darmstadt, FRG) and Fluka (Buchs, Switzerland). The medium referred to as "Ca²⁺ medium" contained (in mM): NaCl 138; KCl 6; CaCl₂ 1.1; MgCl₂ 1; EGTA 0.1; glucose 20; Hepes 20. The Ca²⁺-free medium has the same ionic composition; however, it contained 1 mM EGTA, and CaCl₂ was omitted.

Culture of HL-60 cells. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (5 U/ml) and streptomycin (50 µg/ml). The cells were replated twice per week and differentiated by adding DMSO (final concentration 1.3% vol/vol) to the cell suspension 7 d before experiments.

Measurement of cytosolic free Ca²⁺. Cells (2 × 10⁷/ml) were incubated for 30 min at room temperature with 5 µM Indo-1/AM, centrifuged, resuspended in a Ca²⁺-containing medium, and kept on ice. Just before use, an aliquot of 10⁶ cells was allowed to adhere on a glass coverslip and experiments were performed within 1 h. Basal [Ca²⁺]_i levels were identical in cells kept on ice when compared to freshly prepared cells. We used an inverted microscope equipped for Indo-1 measurements (Nikon Diaphot, all optics are from Nikon Corp., Tokyo, Japan). The excitation light, provided by a Hg lamp, was first attenuated 256 times (two neutral density filters), then passed through a 355 ± 5 nm interference filter, and was reflected to the stage (dichroic mirror 380 nm). The emitted fluorescence was split in two on a second dichroic mirror (dichroic mirror 455 nm), the light > 455 nm passing through a 480 ± 5 nm interference filter, the light < 455 nm through a 405 ± 5 nm interference filter. The light intensity was measured simultaneously at 405 and 480 nm on two P1 photometers (Hamamatsu, Tokyo, Japan). The photometric data were recorded at a rate of 50 Hz, using a 12-bit analog/digital (A/D) interface (Acqui, Sicomu, Geneva), filtered with a moving average procedure, and stored on an IBM computer. [Ca²⁺]_i was calculated using the equation: [Ca²⁺]_i = $Kd^*\beta^*(R - R_{min}) / (R_{max} - R)$; $R = F_{405} / F_{480} \cdot R_{min} \cdot R_{max}$, and $Kd^*\beta$ were determined in dye-loaded cells according to reference 13. $R_{min} = 0.025 \pm 0.005$ ($n = 6$), measured as the R value following break-in with a pipette solution containing 10 mM EGTA, $R_{max} = 0.35 \pm 0.01$ ($n = 8$), determined as the R value of intact cells incubated with 2 µM ionomycin in the presence of 10 mM CaCl₂, and $Kd^*\beta = 1280 \pm 40$ ($n = 6$), determined after break-in with a pipette solution containing a fixed Ca²⁺ concentration of 300 nM (9.2 mM EGTA and 5.4 mM CaCl₂). FMLP and CPA were applied to the cell through a perfusion pipette (10 µm inner diameter) located 30 µm from the cell by applying a 1 psi pressure pulse for the indicated duration. We have chosen supra-maximal concentrations of FMLP (10⁻⁶), in order to reproducibly induce maximal [Ca²⁺]_i responses. We tested lower FMLP concentrations (10⁻⁸) and found that (a) the percentage of responding cells was lower and (b) a part of the responding cells showed [Ca²⁺]_i oscillations rather than a biphasic [Ca²⁺]_i transient.

Whole-cell patch clamp technique and microperfusion of inositol phosphates. We used the whole-cell patch clamp technique to voltage clamp cells and to microperfuse inositol phosphates. Whole cell patch clamp was performed as described (14, 15). Patch clamp electrodes were pulled from borosilicate glass using a BB-CH-PC puller (Mechanex, CH-1260 Nyon, Switzerland). Pipettes were filled with (in mM) 140 KCl, 5 NaCl, 1 MgCl₂, 0.2 EGTA, 20 Hepes, and 0.01 Indo-1 free acid (pH 7.2). The inositol isomer to be tested was added in the pipette solution before the experiment. Under these conditions, [Ca²⁺]_i in the pipette was 120 nM, measured with a Ca²⁺ electrode. Pipette resistance varied between 5 and 15 MΩ, seal resistance between 5 and 50 GΩ. After achieving the seal, a burst of suction caused the appearance of capacitive currents, indicating that the whole-cell configuration had been obtained. Diffusion of the pipette content to the cytosol of the patched cell was verified by inclusion of excess Indo-1 free acid in the pipette solution. An immediate increase in fluorescence without any change in fluorescence ratio was observed after obtaining the whole-cell configuration. The rapid diffusion of the pipette contents into the cytosol after achieving the whole-cell configuration was used to introduce inositol phosphates into the patched cells. This method of intracellular application of membrane impermeant com-

pounds will be referred to throughout this paper as "microperfusion." Patch clamp recordings were performed using an EPC-7 amplifier (List Medical, Darmstadt, FRG) in the voltage clamp mode. Stimulation and recording of electrophysiological data were performed with the same A/D interface used for [Ca²⁺]_i measurements.

Results

Plasma membrane potential and agonist-induced Ca²⁺ influx. To understand the relationship between plasma membrane potential and agonist-induced Ca²⁺ influx in myeloid cells, we studied the effect of the chemotactic peptide FMLP on [Ca²⁺]_i in Indo-1-loaded HL-60 cells voltage-clamped at -60 mV in the whole-cell configuration. Similar to results obtained in single non-patch-clamped HL-60 cells, FMLP superfusion (1 µM) of voltage-clamped HL-60 cells induced an increase in [Ca²⁺]_i whose delayed phase was dependent on the presence of extracellular Ca²⁺ (Fig. 1 a) and could be blocked by 5 mM extracellular Ni²⁺ (Fig. 1 b). Thus, in HL-60 cells, FMLP induced Ca²⁺ influx at an electrically imposed constant plasma membrane potential. We then studied the effects of a plasma membrane depolarization on [Ca²⁺]_i in resting and stimulated HL-60 cells by applying 15-s depolarizing steps before, during, and after the stimulation of the cell with FMLP. A depolarizing step to +60 mV did not alter [Ca²⁺]_i in unstimulated cells; however, when applied during the delayed phase of the FMLP-induced [Ca²⁺]_i increase, it caused a rapid decrease in [Ca²⁺]_i (a typical trace is shown in Fig. 1 c). Upon return to the holding voltage (-60 mV), a small but clearly noticeable reincrease of [Ca²⁺]_i was observed, and a subsequent depolarizing step caused a small and reversible decrease in [Ca²⁺]_i from suprabasal levels to basal [Ca²⁺]_i values (Fig. 1 c). In a different set of experiments, cells stimulated with FMLP were held at hyperpolarized or depolarized potentials throughout the experiment. Under these conditions, hyperpolarization increased, while depolarization decreased, the duration of the delayed phase of the FMLP-induced [Ca²⁺]_i increase (not shown). Thus, in unstimulated HL-60 cells no voltage activation of Ca²⁺ influx could be detected. However, the amplitude of the FMLP-induced Ca²⁺ influx was dependent on the imposed plasma membrane potential and followed the electrochemical driving force for Ca²⁺. This suggests that agonist-induced Ca²⁺ influx occurs through either a plasma membrane Ca²⁺ channel or an electrogenic transporter with net transfer of positive charges from the outside to the inside of the cell.

Inositol phosphates and Ca²⁺ influx. The response of myeloid cells to chemoattractants is characterized by a rapid generation of inositol phosphates (3, 6). Inhibition of inositol phosphate generation by pertussis toxin is paralleled by an inhibition of chemoattractant-induced Ca²⁺ influx (6). We next studied the role of inositol phosphates in the mediation of Ca²⁺ influx. Various inositol phosphates were applied intracellularly through microperfusion by a patch pipette. Microperfusion of Ins(1,4,5)P₃ caused immediate, dose-dependent [Ca²⁺]_i elevations (Fig. 2). In the presence of external Ca²⁺, [Ca²⁺]_i first increased within 5–20 s from resting levels of ~ 120 nM to a transient peak, then slowly declined to a new steady-state [Ca²⁺]_i, above basal [Ca²⁺]_i values. This sustained phase of [Ca²⁺]_i increase was stable for the period of observation (up to 20 min). A threshold concentration of 1 µM Ins(1,4,5)P₃ in the pipette solution had to be exceeded in order to elicit [Ca²⁺]_i increases. In the range of 2–10 µM Ins(1,4,5)P₃ in the pipette

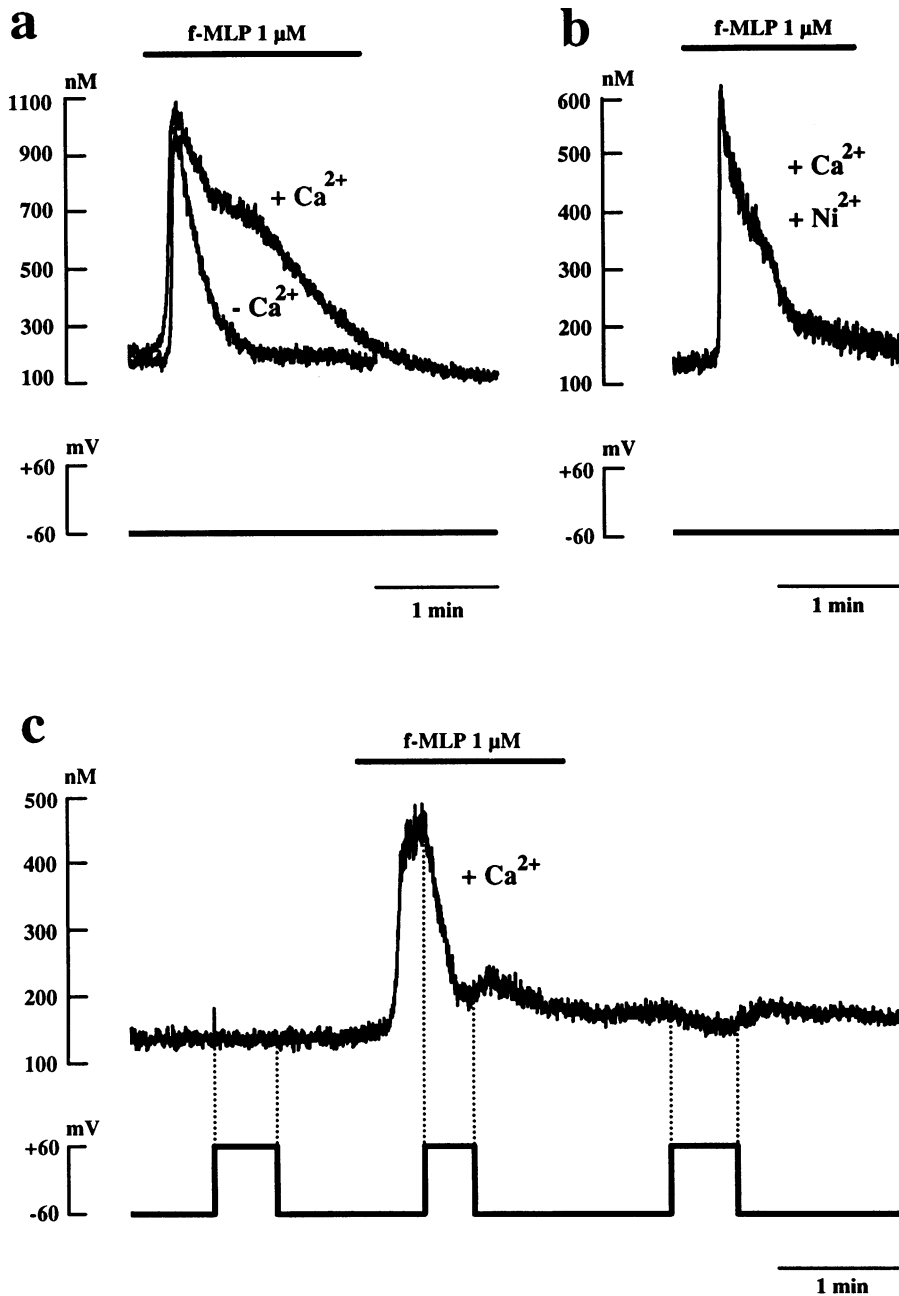


Figure 1. $[Ca^{2+}]_i$ changes induced by FMLP in voltage-clamped HL-60 cells: effects of extracellular Ca^{2+} , Ni^{2+} , and plasma membrane depolarization. Indo-1-loaded cells were voltage-clamped in the whole cell configuration and superfused with FMLP (1 μ M). The voltage protocols of the respective experiments are shown under the $[Ca^{2+}]_i$ traces. (a) FMLP-induced $[Ca^{2+}]_i$ changes in the presence (upper trace) and absence (lower trace) of extracellular Ca^{2+} , at a constant holding potential of -60 mV. Traces have been superimposed for comparison. (b) FMLP-induced $[Ca^{2+}]_i$ increase in the presence of extracellular Ca^{2+} and Ni^{2+} (5 mM), at a constant holding potential of -60 mV. (c) Effects on $[Ca^{2+}]_i$ of a depolarization to +60 mV before, during, and after FMLP superfusion in the presence of extracellular Ca^{2+} .

solution, a dose-dependent effect of $Ins(1,4,5)P_3$ on $[Ca^{2+}]_i$ peak height, slope of the initial $[Ca^{2+}]_i$ rise, and on the $[Ca^{2+}]_i$ level of the delayed phase was observed (Table I). Higher doses did not further increase the response (not shown).

In the absence of external Ca^{2+} , the $Ins(1,4,5)P_3$ -induced $[Ca^{2+}]_i$ increase was transient, returning to levels close to basal $[Ca^{2+}]_i$ levels within 2 min (Fig. 3 a). Similar to the chemoattractant-induced Ca^{2+} increase, the sustained phase of the $Ins(1,4,5)P_3$ -induced $[Ca^{2+}]_i$ increase could be blocked by 5 mM Ni^{2+} (Fig. 3 b) and was reduced by plasma membrane depolarization (Fig. 3 c), indicating that it is also due to Ca^{2+} influx across an electrogenic transmembrane pathway. In contrast, the slope of the initial $[Ca^{2+}]_i$ rise and the peak $[Ca^{2+}]_i$ levels were similar in a Ca^{2+} -containing and a Ca^{2+} -free medium (Table I), suggesting that the initial $[Ca^{2+}]_i$ rise is due to

Ca^{2+} release from internal stores and that Ca^{2+} influx is delayed with respect to Ca^{2+} release.

To determine whether induction of Ca^{2+} influx by $Ins(1,4,5)P_3$ necessitates its metabolism, we microperfused cells with $Ins(1,4,5)P_3S_3$ and $Ins(2,4,5)P_3$, two poorly metabolizable, synthetic $Ins(1,4,5)P_3$ analogues. $Ins(1,4,5)P_3S_3$ and $Ins(2,4,5)P_3$ caused immediate $[Ca^{2+}]_i$ elevations, which were sustained in the presence of external Ca^{2+} (Fig. 4). Maximal effects were obtained with 20 μ M $Ins(1,4,5)P_3S_3$ or $Ins(2,4,5)P_3$ in the pipette. As observed with $Ins(1,4,5)P_3$, the sustained phase of $Ins(2,4,5)P_3$ - and $Ins(1,4,5)P_3S_3$ -induced $[Ca^{2+}]_i$ increase could be blocked by 5 mM Ni^{2+} and was reduced by plasma membrane depolarization (not shown). At maximal concentration, both analogs produced $[Ca^{2+}]_i$ increases whose initial slope, peak $[Ca^{2+}]_i$ levels, and $[Ca^{2+}]_i$

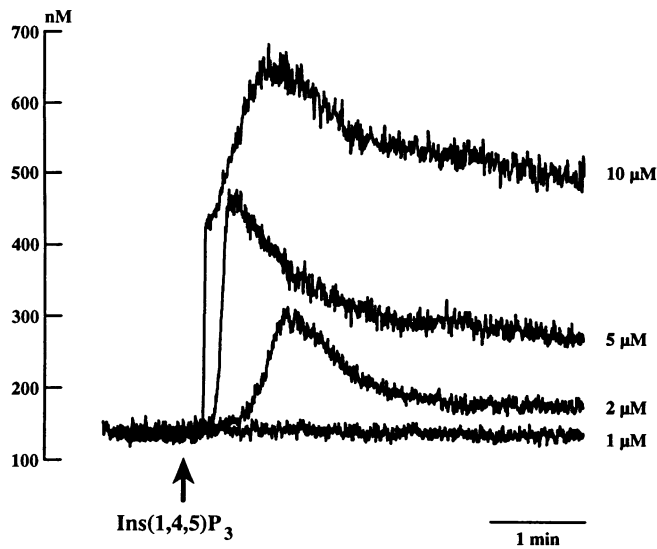


Figure 2. Effects of Ins(1,4,5)P₃ microperfusion on [Ca²⁺]_i in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were kept in the cell-attached patch clamp configuration (holding voltage -60 mV), in the presence of extracellular Ca²⁺. When indicated by the arrow, the whole-cell configuration was achieved. The cell was thereby microperfused with the pipette solution which contained the indicated concentration of Ins(1,4,5)P₃. The [Ca²⁺]_i responses to increasing concentrations of Ins(1,4,5)P₃ are shown. Traces have been superimposed for comparison.

levels during the delayed phase were similar to values observed during microperfusion with a maximal dose of Ins(1,4,5)P₃ (Table I).

It has been proposed that Ins(1,3,4,5)P₄, the product of Ins(1,4,5)P₃ phosphorylation, acts in synergy with Ins(1,4,5)P₃ to induce Ca²⁺ influx (9, 16, 17). To study this question, we microperfused cells either with various concentrations of Ins(1,3,4,5)P₄ alone or with Ins(1,3,4,5)P₄ added to a submaximal dose of Ins(1,4,5)P₃. Ins(1,3,4,5)P₄ microperfusion by itself did not have an effect on Ca²⁺ homeostasis at a range of concentrations of 1 to 20 μM (Fig. 5 a and Table I).² The co-microperfusion of 20 μM Ins(1,3,4,5)P₄ with 2 μM Ins(1,4,5)P₃ produced [Ca²⁺]_i increases indistinguishable from those induced by microperfusion of 2 μM Ins(1,4,5)P₃ alone (Fig. 5 b and c, Table I).

[Ca²⁺]_i elevations and Ca²⁺ influx. As both the physiological agonist FMLP and the intracellular messenger Ins(1,4,5)P₃ induced an initial [Ca²⁺]_i rise due to Ca²⁺ release from intracellular stores, they might induce Ca²⁺ influx via activation of plasma membrane Ca²⁺ channels which are sensitive to [Ca²⁺]_i increases (8). To test this hypothesis, we pro-

2. In 3 out of 39 cells, Ins(1,3,4,5)P₄ (8–20 μM) caused delayed and minor [Ca²⁺]_i elevations. These elevations were independent of the extracellular Ca²⁺ concentration and were observed in a similar percentage of cells microperfused with control intracellular solution (Table I).

Table I. [Ca²⁺]_i Changes Induced by FMLP and Inositol Phosphates in Voltage-Clamped HL-60 Cells

Stimulus	Concentration	Ca ²⁺ ext	Basal [Ca ²⁺] _i	Peak [Ca ²⁺] _i	120-s [Ca ²⁺] _i	Lag time	Initial slope	Responding cells	Tested cells
	μM			nM		s	nM/s	%	n
Ins(1,4,5)P ₃	1	+	100±20	110±20	110±20	>30	<1	0	4
Ins(1,4,5)P ₃	2	+	120±15	390±60	260±50	11±1	30±10	78	18
Ins(1,4,5)P ₃	5	+	100±20	460±40	240±10	7±2	80±20	100	4
Ins(1,4,5)P ₃	10	+	130±10	680±60	570±70	4±1	220±20	100	24
Ins(1,4,5)P ₃	10	-	130±30	610±140	190±50	5±1	210±40	100	5
Ins(2,4,5)P ₃	20	+	120±20	640±140	630±130	9±2	210±30	100	5
Ins(2,4,5)P ₃	20	-	140±20	520±70	220±40	15±4	200±40	100	7
Ins(1,4,5)P ₃ S ₃	20	+	130±20	610±170	390±60	8±1	230±10	80	5
Ins(1,4,5)P ₃ S ₃	20	-	110±10	530±70	180±20	14±2	210±20	100	7
Ins(1,3,4,5)P ₄	8–20	+	110±10	220±40	170±10	>30	<1	11	19
Ins(1,3,4,5)P ₄	8–20	-	90±10	130±40	130±40	>30	<1	10	10
Ins(1,4,5)P ₃ + Ins(1,3,4,5)P ₄	2 + 20	+	110±10	370±50	270±30	7±1	40±10	76	17
Ins(3,4,5,6)P ₄	20	+	90±10	170±40	170±40	>30	<1	13	8
Control	—	+	130±10	220±60	190±40	>30	<1	10	10
Control	—	-	110±10	120±10	120±10	>30	<1	11	9
F-MLP	1	+	140±20	1010±170	380±60	17±3	200±20	86	14
F-MLP	1	-	130±10	960±130	140±10	16±5	210±10	80	10

Indo-1-loaded HL-60 cells in a Ca²⁺-containing or Ca²⁺-free extracellular solution were voltage-clamped at -60 mV and microperfused with various inositol phosphates or superfused with FMLP at the indicated concentrations. The following parameters were determined: (a) the basal [Ca²⁺]_i, measured before application of the stimulus; (b) the peak [Ca²⁺]_i, measured during the 30-s interval, after the application of the stimulus; (c) the delayed phase of the [Ca²⁺]_i increase, measured at 120 s (inositol phosphates) or 60 s (FMLP) after the application of the stimulus; (d) the lag time between the application of the stimulus and the [Ca²⁺]_i increase; (e) the slope of the initial 10 s of the [Ca²⁺]_i increase; (f) the number of responding cells (defined as cells showing an increase in [Ca²⁺]_i > 100 nM in the 30-s interval after the application of the stimulus; and (g) the number of cells tested. The values shown are mean±SEM.

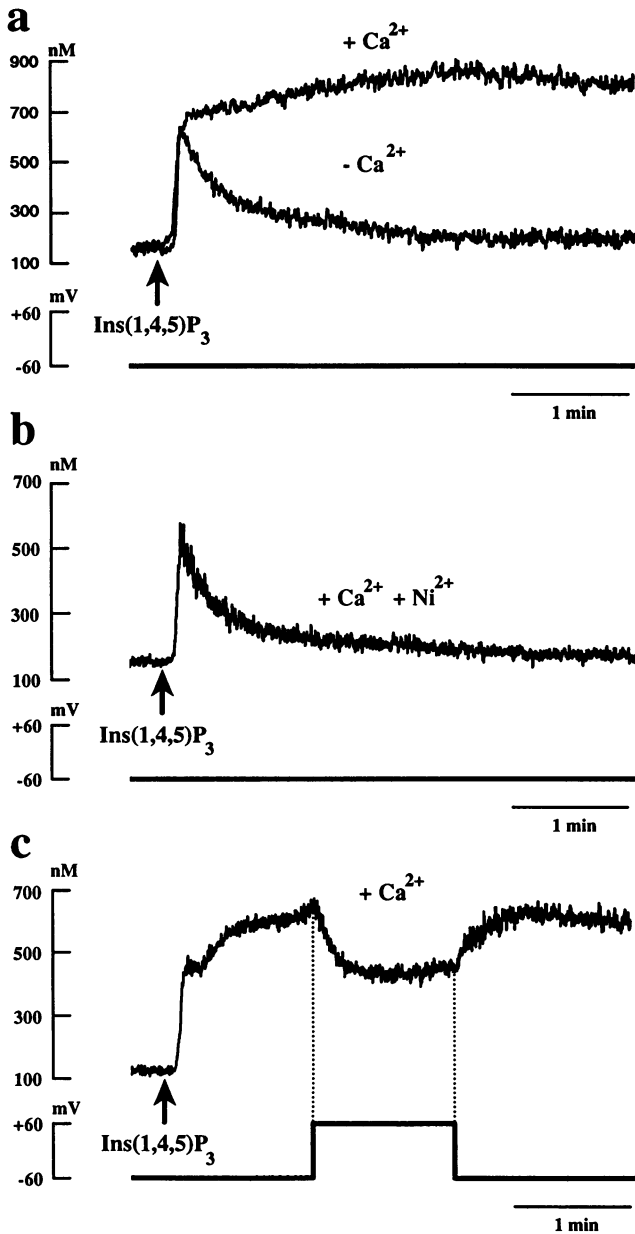


Figure 3. Effects of extracellular Ca^{2+} , Ni^{2+} , and plasma membrane depolarization on the $[\text{Ca}^{2+}]_i$ changes induced by $\text{Ins}(1,4,5)\text{P}_3$. Indo-1-loaded HL-60 cells were voltage-clamped in the whole cell configuration. The pipette contained $10 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$; arrows indicate when the whole cell configuration was achieved. The voltage protocols of the respective experiments are shown under the $[\text{Ca}^{2+}]_i$ traces. (a) $\text{Ins}(1,4,5)\text{P}_3$ -induced $[\text{Ca}^{2+}]_i$ changes in the presence (upper trace) and absence (lower trace) of extracellular Ca^{2+} , at a constant holding potential of -60 mV . Traces have been superimposed for comparison. (b) $\text{Ins}(1,4,5)\text{P}_3$ -induced $[\text{Ca}^{2+}]_i$ changes in the presence of extracellular Ca^{2+} and Ni^{2+} (5 mM), at a constant holding potential of -60 mV . (c) Effect on $[\text{Ca}^{2+}]_i$ of a depolarization to $+60 \text{ mV}$ during the sustained phase of $\text{Ins}(1,4,5)\text{P}_3$ -induced $[\text{Ca}^{2+}]_i$ increase in the presence of extracellular Ca^{2+} .

duced a $[\text{Ca}^{2+}]_i$ increase independent of receptor activation and $\text{Ins}(1,4,5)\text{P}_3$ microperfusion by deliberately wounding the cell with a microelectrode in the presence of external Ca^{2+} (as described in reference 18). This procedure transiently

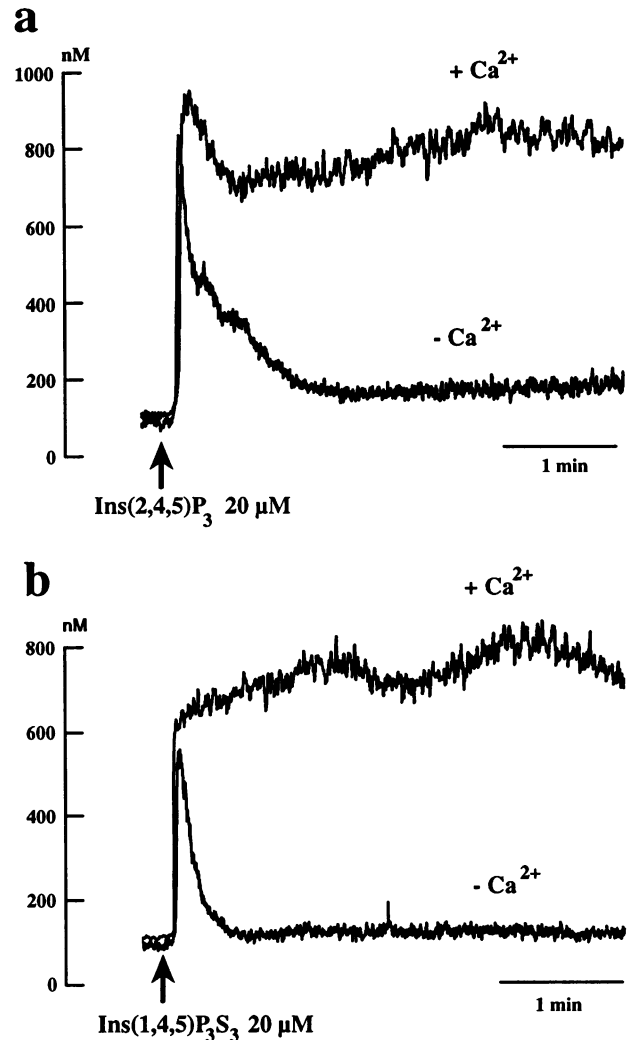


Figure 4. Effects of $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3\text{S}_3$ microperfusion on $[\text{Ca}^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were voltage-clamped at -60 mV . The pipette contained the respective $\text{Ins}(1,4,5)\text{P}_3$ analogue; arrows indicate when the whole cell configuration was achieved. (a) $[\text{Ca}^{2+}]_i$ response to $20 \mu\text{M}$ $\text{Ins}(2,4,5)\text{P}_3$ in the presence (upper trace) and absence (lower trace) of extracellular Ca^{2+} . (b) $[\text{Ca}^{2+}]_i$ response to $20 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3\text{S}_3$ in the presence (upper trace) and absence (lower trace) of extracellular Ca^{2+} . Traces have been superimposed for comparison.

disrupted the plasma membrane and caused a rapid elevation of $[\text{Ca}^{2+}]_i$, which was not due to Ca^{2+} release, but due to a transient Ca^{2+} influx through the membrane leak created by the patch pipette. This $[\text{Ca}^{2+}]_i$ elevation, albeit of large amplitude, was short lasting (2 min) and monophasic. Subsequent superfusion of the same cell with FMLP induced a biphasic $[\text{Ca}^{2+}]_i$ transient (Fig. 6), indicating that the cell was capable of responding with Ca^{2+} influx to FMLP stimulation after the wounding procedure. Comparison of this protocol with the $\text{Ins}(1,4,5)\text{P}_3$ -induced $[\text{Ca}^{2+}]_i$ changes (see Figs. 3 and 4) is very striking. Wounding of the cell caused a $[\text{Ca}^{2+}]_i$ peak comparable to the one observed with microperfusion of $10 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$. However, microperfusion of $\text{Ins}(1,4,5)\text{P}_3$ caused a permanent opening of the Ca^{2+} influx pathway, while the wounding caused only a short lasting monophasic peak.

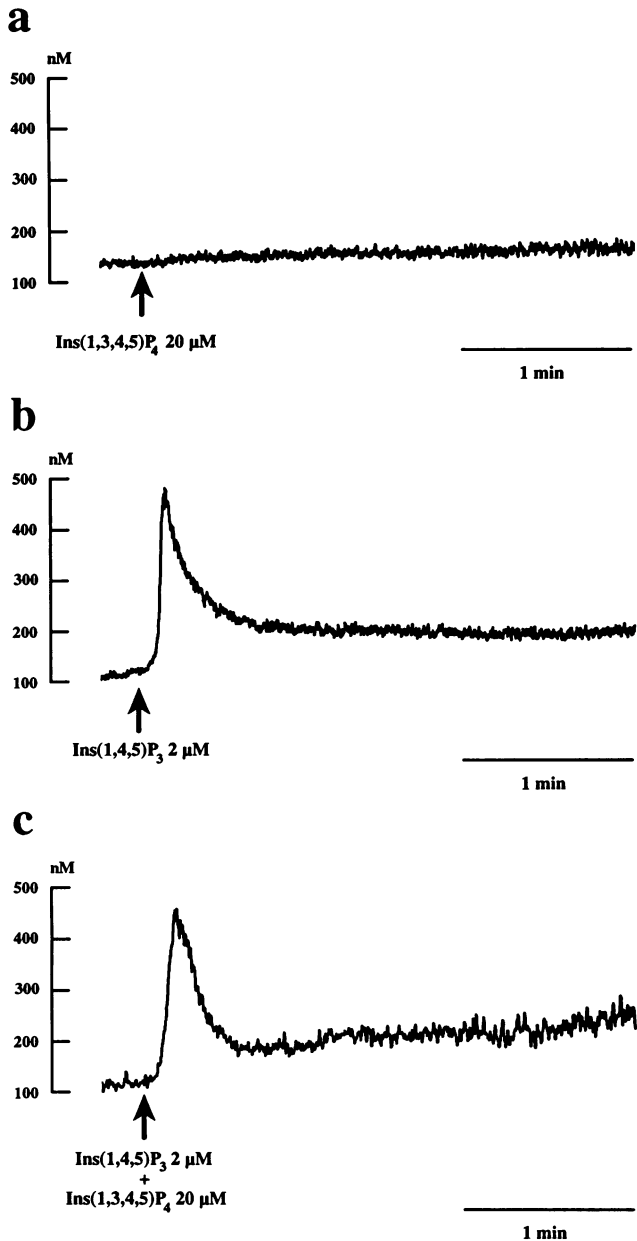


Figure 5. Effects of $\text{Ins}(1,3,4,5)\text{P}_4$ microperfusion on $[\text{Ca}^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were voltage-clamped at -60 mV in the presence of external Ca^{2+} . The pipette contained the indicated inositol phosphate(s); arrows indicate when the whole-cell configuration was achieved. (a) $[\text{Ca}^{2+}]_i$ response to $20 \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$. (b) $[\text{Ca}^{2+}]_i$ response to $2 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$. (c) $[\text{Ca}^{2+}]_i$ response to the combination of $2 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ and $20 \mu\text{M}$ $\text{Ins}(1,3,4,5)\text{P}_4$.

Thus, while $\text{Ins}(1,4,5)\text{P}_3$ is a potent intracellular activator of Ca^{2+} influx, a sudden increase in $[\text{Ca}^{2+}]_i$ is not sufficient to induce a sustained Ca^{2+} influx. However, both FMLP and $\text{Ins}(1,4,5)\text{P}_3$ produce an elevated $[\text{Ca}^{2+}]_i$ due to Ca^{2+} release that occurs prior to the induction of Ca^{2+} influx. This elevated $[\text{Ca}^{2+}]_i$ might be required in addition to $\text{Ins}(1,4,5)\text{P}_3$ for the activation of the Ca^{2+} influx pathway. To study this question, we microperfused cells with $\text{Ins}(1,4,5)\text{P}_3$ in the absence of extracellular Ca^{2+} , then added 2 mM Ca^{2+} to the extracellular

medium 7 min after return of $[\text{Ca}^{2+}]_i$ to basal levels. In control cells, the addition of Ca^{2+} did not alter $[\text{Ca}^{2+}]_i$, whereas in $\text{Ins}(1,4,5)\text{P}_3$ microperfused cells, the addition of Ca^{2+} did cause an immediate and sustained increase in $[\text{Ca}^{2+}]_i$ from 135 nM to steady-state $[\text{Ca}^{2+}]_i$ levels of 800 nM (Fig. 7), i.e., $[\text{Ca}^{2+}]_i$ values comparable to the one observed when cells were microperfused with $\text{Ins}(1,4,5)\text{P}_3$ in a Ca^{2+} -containing medium (Table I). Thus, the activity of $\text{Ins}(1,4,5)\text{P}_3$ on the Ca^{2+} influx pathway does not appear to require a concomitant high $[\text{Ca}^{2+}]_i$.

Induction of Ca^{2+} influx by CPA. The Ca^{2+} -ATPase inhibitor, CPA, has recently been described to empty agonist sensitive intracellular Ca^{2+} stores independently of inositol phosphate production and to induce Ca^{2+} influx when studied in populations of HL-60 cells or lymphocytes (19, 20). To study the effects of depletion of intracellular Ca^{2+} stores on Ca^{2+} influx, we measured the $[\text{Ca}^{2+}]_i$ changes induced by CPA superfusion to single adherent HL-60 cells. In the absence of extracellular Ca^{2+} , CPA superfusion produced a transient $[\text{Ca}^{2+}]_i$ increase, and a subsequent microperfusion of the cell with a maximal dose of $\text{Ins}(1,4,5)\text{P}_3$ could not release additional Ca^{2+} (Fig. 8 a). In the presence of extracellular Ca^{2+} , CPA induced a sustained increase in $[\text{Ca}^{2+}]_i$ whose prolonged phase could be blocked by 5 mM Ni^{2+} (Fig. 8 b) and was reduced by plasma membrane depolarization (not shown). Thus, in HL-60 cells, CPA empties $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores³ and induces a Ca^{2+} influx with characteristics similar to the Ca^{2+} influx induced by FMLP and $\text{Ins}(1,4,5)\text{P}_3$.

Discussion

Although it is now generally accepted that myeloid cells possess a Ca^{2+} influx pathway, both the nature of the influx pathway and its regulation by second messengers remain obscure (7). In this study we have investigated the regulation of Ca^{2+} influx by second messengers in HL-60 cells at the single cell level using the combination of the patch clamp technique and double emission wavelength microfluorimetry.

As a technical approach we used the whole-cell patch clamp technique to voltage clamp and to microperfuse the cells. However, we used microfluorimetry and not current measurements to detect Ca^{2+} influx for the following reasons: (a) Current measurements preferentially detect ion fluxes through channels, while it is not yet known if Ca^{2+} influx in myeloid cells involves conductive pathways (i.e., ion channels), transport pathways, or both. (b) By analogy to results obtained in mast cells (21), net Ca^{2+} fluxes across the plasma membrane during stimulation of HL-60 cells might be small and difficult to detect by current measurements, even if they involve channels. (c) Detection of Ca^{2+} currents usually necessitates the use of nonphysiological ionic conditions as well as blockers of other ion channels. In contrast, microfluorimetry allows study of the regulation of Ca^{2+} influx under physiological ionic conditions, and detection of Ca^{2+} influx independently of its electrical properties with a high sensitivity. However, the present experimental approach was not designed to study the putative con-

3. This is, to the best of our knowledge, the first demonstration that depletion of intracellular Ca^{2+} stores by cyclopiazonic acid prevents $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release.

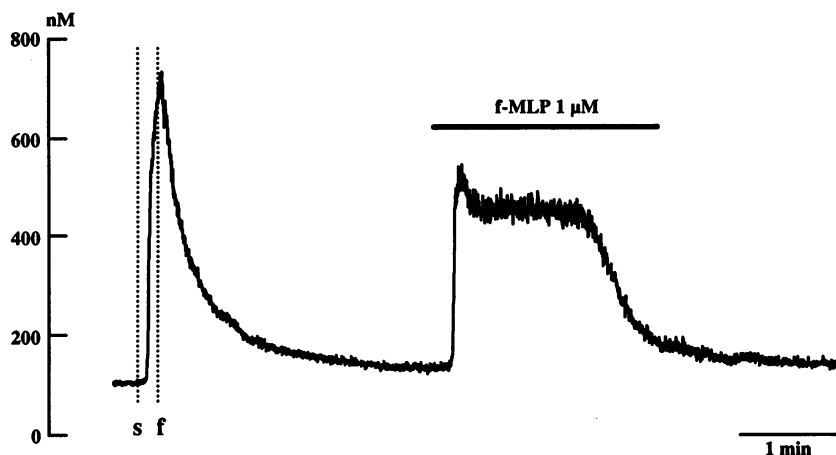


Figure 6. Effects of an imposed rapid $[Ca^{2+}]_i$ transient on $[Ca^{2+}]_i$ in an adherent HL-60 cell. An Indo-1-loaded HL-60 cell was allowed to adhere on a glass coverslip in the presence of extracellular Ca^{2+} . The cell was transiently impaled with a micropipette (s = start, f = finish). As a control, the same cell was subsequently superfused with $1 \mu M$ FMLP (bar) after $[Ca^{2+}]_i$ had returned to basal levels. The $[Ca^{2+}]_i$ response to this protocol is shown.

ductive properties of the influx pathway(s), and future studies measuring Ca^{2+} currents are required to answer this question.

With this limitation in mind, several conclusions concerning the nature and the electrical properties of the influx pathway can be drawn. First, plasma membrane depolarization did not induce Ca^{2+} influx in HL-60 cells. Thus, in agreement with results obtained by a different approach in human neutrophils (2, 8), our results rule out the presence of classical voltage-acti-

vated Ca^{2+} channels. They also exclude Ca^{2+} influx through putative atypical voltage-activated Ca^{2+} channels that may be difficult to detect by current measurements. This question is important, as the chemotactic peptide FMLP depolarizes the plasma membrane and induces Ca^{2+} influx with very similar time courses (22, 23). As depolarization did not induce $[Ca^{2+}]_i$ elevations, and FMLP was able to induce Ca^{2+} influx in voltage-clamped HL-60 cells, our results exclude a role of the

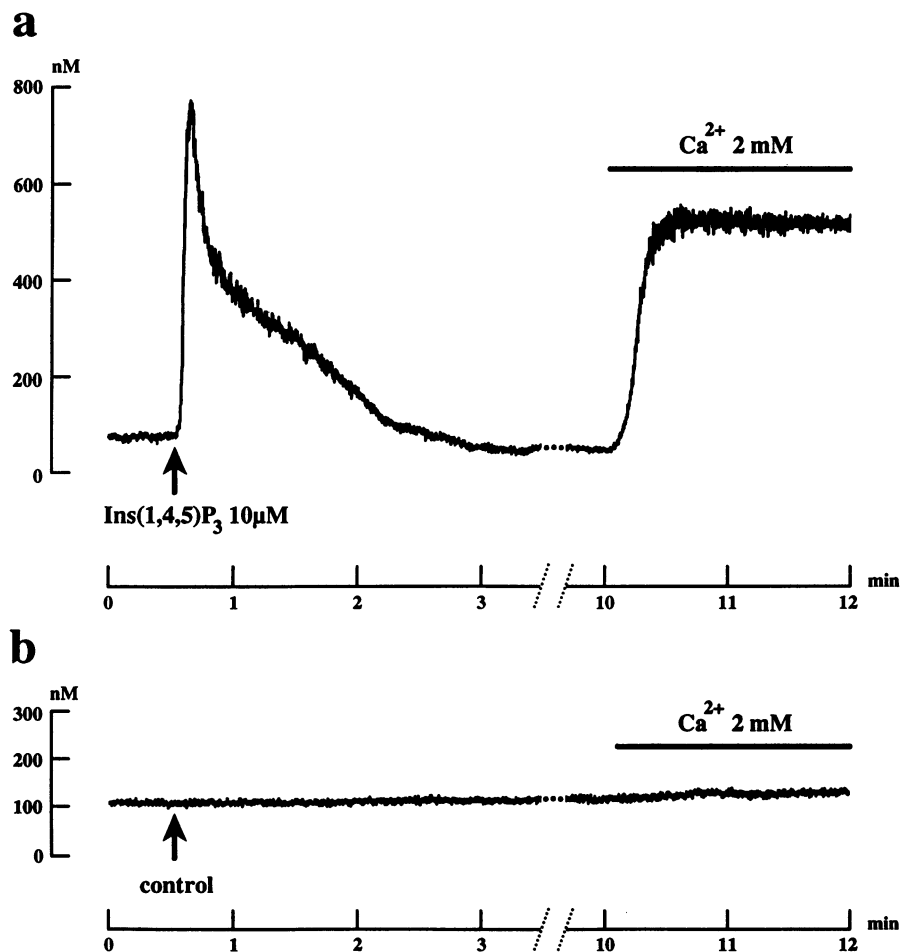


Figure 7. Effects on $[Ca^{2+}]_i$ of extracellular Ca^{2+} addition to cells microperfused with Ins(1,4,5) P_3 in the absence of extracellular Ca^{2+} . Indo-1-loaded HL-60 cells were voltage-clamped at -60 mV in the absence of extracellular Ca^{2+} . The pipette contained either $10 \mu M$ Ins(1,4,5) P_3 or standard intracellular solution as a control. Arrows indicate when the whole cell configuration was achieved; after 10 min 2 mM Ca^{2+} was added to the extracellular solution ($bars$). (a) Effects on $[Ca^{2+}]_i$ of extracellular Ca^{2+} addition to a cell microperfused with Ins(1,4,5) P_3 . (b) Effects on $[Ca^{2+}]_i$ of extracellular Ca^{2+} addition to a control cell. For better representation, the display has been cut for the time comprised between the dotted lines.

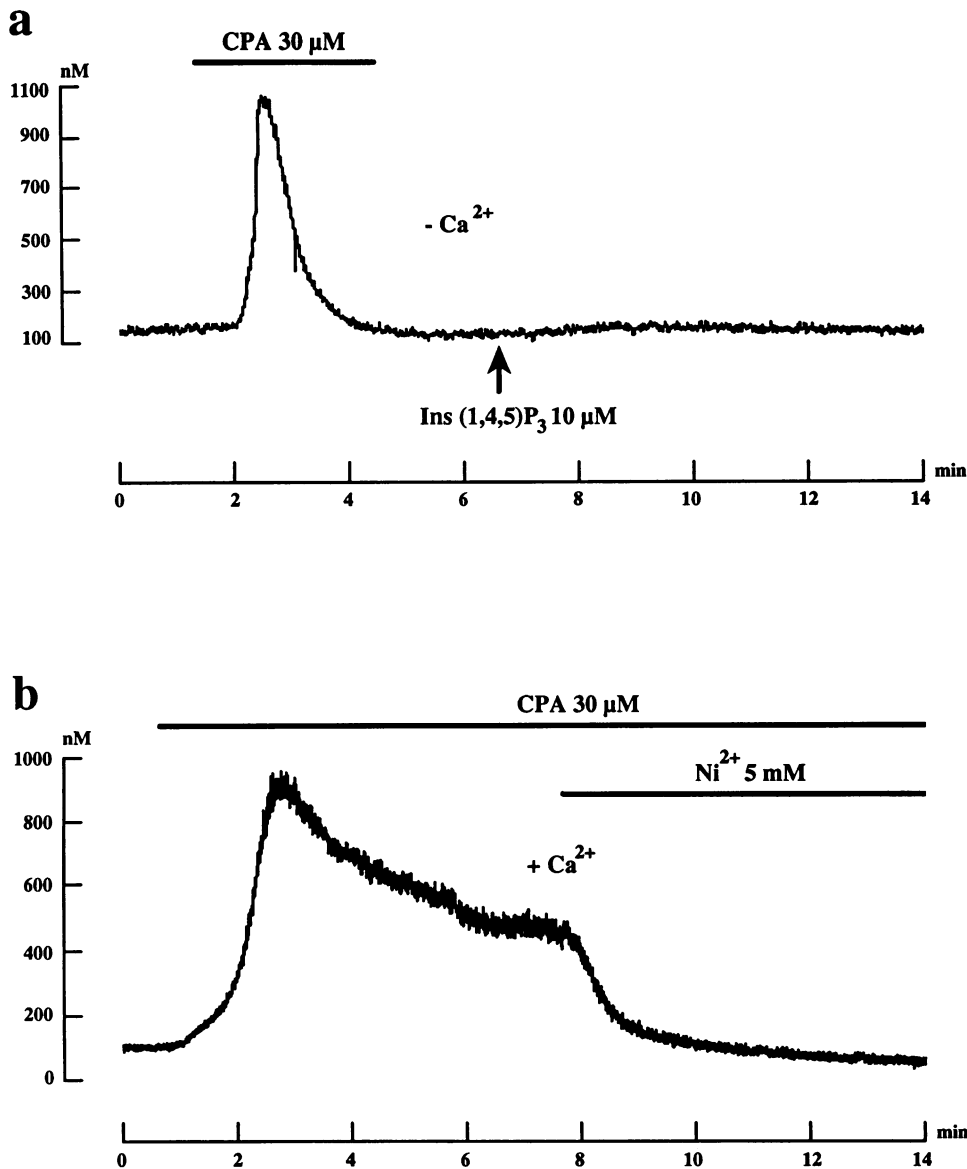


Figure 8. Effects of the Ca^{2+} -ATPase inhibitor CPA on $[\text{Ca}^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were voltage-clamped at -60 mV and superfused with $10 \mu\text{M}$ CPA (an inhibitor of Ca^{2+} -ATPases of muscle of sarcoplasmic reticulum and intracellular Ca^{2+} -ATPase of various nonmuscle cells). The pipette contained either $10 \mu\text{M}$ $\text{Ins}(1,4,5)\text{P}_3$ or standard intracellular solution; arrows indicate when the whole cell configuration was achieved. (a) $[\text{Ca}^{2+}]_i$ responses to CPA superfusion and subsequent $\text{Ins}(1,4,5)\text{P}_3$ microperfusion in the absence of extracellular Ca^{2+} . (b) $[\text{Ca}^{2+}]_i$ responses to CPA superfusion in the presence of extracellular Ca^{2+} . When indicated, the extracellular medium was exchanged for a solution containing 5 mM Ni^{2+} .

FMLP-induced depolarization for the stimulation of Ca^{2+} influx. Second, the stimulated Ca^{2+} influx was inhibited by depolarization. These results are in agreement with a previous study using populations of HL-60 cells pharmacologically depolarized with the ionophore gramicidin (23). This shows that Ca^{2+} influx follows the electrochemical driving force for Ca^{2+} , suggesting that Ca^{2+} enters the cell through a conductive pathway, i.e., a channel. Alternatively however, Ca^{2+} influx might occur through an electrogenic transporter or an electrogenic antiporter with a net transfer of positive charges from the outside to the inside of the cell. Our results are not compatible with the proposed role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger as the pathway of Ca^{2+} influx (24, 25), because the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is an electrogenic transporter with a net transfer of positive charges from the inside to the outside of the cell (3 Na^+ for 1 Ca^{2+}).

Thus, our approach allows some new insights into the electrical properties of the Ca^{2+} influx pathway of myeloid cells. However, the main focus of this study was the regulation of Ca^{2+} influx by second messengers. Our study clearly demon-

strates that $\text{Ins}(1,4,5)\text{P}_3$ is a powerful activator of Ca^{2+} influx in myeloid cells. This finding differs from the conclusion of a previous patch clamp study in human neutrophils, where no effects of $\text{Ins}(1,4,5)\text{P}_3$ on the proposed Ca^{2+} influx pathway were found (8). Although this difference might be explained by the different cell types studied, we think that the negative finding of the latter study is most likely explained by the technical obstacles of current measurements in the case of receptor-mediated Ca^{2+} influx, as discussed above. In our system, the $\text{Ins}(1,4,5)\text{P}_3$ concentrations required to induce Ca^{2+} release and Ca^{2+} influx were very similar. For both processes the threshold concentration was $\sim 1 \mu\text{M}$ and the half-maximal concentration $\sim 5 \mu\text{M}$. Thus, there is no indication for a regulation of Ca^{2+} influx and Ca^{2+} release by $\text{Ins}(1,4,5)\text{P}_3$ receptors with different affinities.

Mass concentration of $\text{Ins}(1,4,5)\text{P}_3$ were $0.3 \mu\text{M}$ in unstimulated HL-60 cells, and $2.2 \mu\text{M}$ in HL-60 cells stimulated with 10^{-6} M FMLP for 30 s (26). Thus, the $\text{Ins}(1,4,5)\text{P}_3$ concentrations used in this study ($1\text{--}10 \mu\text{M}$) are within a biologically

relevant range. Similar to results from studies performed in other cell types (21, 27, 28), the Ins(1,4,5)P₃ concentrations necessary in the patch pipette for maximal effects on [Ca²⁺]_i were around 10 μM, i.e., ~ 5–10 times higher than half-maximal concentrations for Ca²⁺ release in permeabilized cells or in homogenates (4). Two explanations seem to be possible for these differing results. Cells might be less sensitive to Ins(1,4,5)P₃ under the whole-cell patch clamp condition. This is conceivable, as the Ins(1,4,5)P₃ binding to its receptor depends on many cofactors, such as pH, and concentrations of Ca²⁺, Mg²⁺, and ATP (29). Alternatively, however, the effective Ins(1,4,5)P₃ concentration within the patched cell is lower than within the patch pipette. In the case of Indo-1 free acid, routinely added to the pipette in our study (see Methods), we observed a rapid equilibration (1 min) of cell and pipette concentrations. However, as Ins(1,4,5)P₃ is metabolized, a permanent gradient between the pipette and the intracellular space cannot be excluded. Interestingly, in whole cell patch-clamped HL-60 cells, concentrations necessary for maximal effects of nonhydrolyzable Ins(1,4,5)P₃ analogs are only double of the respective concentrations of Ins(1,4,5)P₃, while in permeabilized cells or in homogenates these compounds are usually 5–10 times less potent than Ins(1,4,5)P₃. This observation indirectly suggests that Ins(1,4,5)P₃ metabolism may play a role for the relatively low Ins(1,4,5)P₃ sensitivity of whole cell patch-clamped cells.

How might Ins(1,4,5)P₃ induce Ca²⁺ influx? So far, four major hypotheses have received most attention: First, inositol tetrakis phosphate-activated Ca²⁺ influx: According to this hypothesis, Ca²⁺ influx is due to the combined action of Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄ (9, 16, 17). Indeed, in HL-60 cells, there is an excellent temporal correlation between Ins(1,3,4,5)P₄ generation and Ca²⁺ influx (3). Here we show that microperfusion of Ins(1,3,4,5)P₄ is neither necessary nor sufficient for the induction of Ca²⁺ influx in HL-60 cells. These results are in agreement with recent studies in other cellular systems (28, 30). In particular, no additive effect of Ins(1,3,4,5)P₄ on submaximal concentrations of Ins(1,4,5)P₃ could be found. Thus, our results are not compatible with the inositol tetrakis phosphate hypothesis. Second, [Ca²⁺]_i-activated Ca²⁺ influx: In this hypothesis, Ca²⁺ influx is activated by cytosolic Ca²⁺ ions. Neutrophils possess non-selective Ca²⁺-activated cation channels that can conduct Ca²⁺. The activation of these channels by the initial Ca²⁺ release from internal stores has been proposed to mediate Ca²⁺ influx (8). In this study, we could not detect, in HL-60 cells, a sustained Ca²⁺ influx in response to [Ca²⁺]_i increases that were induced independently from emptying of intracellular Ca²⁺ stores. In addition, Ins(1,4,5)P₃-induced Ca²⁺ release and Ca²⁺ influx could be temporarily dissociated (Fig. 7). Thus, our results argue against a central role of a [Ca²⁺]_i elevation in the mediation of Ca²⁺ influx. Third, directly Ins(1,4,5)P₃-mediated Ca²⁺ influx and fourth, capacitative Ca²⁺ influx: Having excluded a role for Ins(1,3,4,5)P₄ and [Ca²⁺]_i, the most likely explanations for the induction of Ca²⁺ influx by Ins(1,4,5)P₃ are either a direct effect of Ins(1,4,5)P₃ on the Ca²⁺ influx pathway in the plasma membrane, or mediation of Ca²⁺ influx by the filling state of the intracellular Ca²⁺ store. As the Ca²⁺-ATPase inhibitor, cyclopiazonic acid, induces Ca²⁺ influx without raising levels of Ins(1,4,5)P₃ (20), our results strongly suggest that the emptying of intracellular Ca²⁺ pools by Ins(1,4,5)P₃ is a factor that

regulates the Ca²⁺ permeability of the plasma membrane (10, 11). However, we cannot exclude an additional direct effect of Ins(1,4,5)P₃ on the Ca²⁺ influx pathway. Recent studies in hepatocytes support such a concept of a dual action of Ins(1,4,5)P₃ (27). Thus, further studies are necessary to determine whether the capacitative mechanism of Ca²⁺ influx is the only, or one of several mechanisms that regulate receptor-mediated Ca²⁺ influx in myeloid cells.

Acknowledgments

We thank Antoinette Monod for help with the culture of HL-60 cells, Drs. C. Chiavaroli, R. Krause-Miyazaki, and R. A. Clark for discussions.

These studies were supported by a grant from the Swiss National Foundation, No. 32-30161.90; Dr. Demaurex was supported by the Swiss League against Rheumatism, the Reuter Foundation, Geneva, and the Clöetta Foundation, Geneva.

References

- Pozzan, T., D. P. Lew, C. B. Wollheim, and R. Y. Tsiens. 1983. Is cytosolic inorganic calcium regulating neutrophil activation? *Science (Wash. DC)* 221:1413–1415.
- Andersson, T., C. Dahlgren, T. Pozzan, O. Stendahl, and D. P. Lew. 1986. Characterization of fMet-Leu-Phe receptor-mediated Ca²⁺ influx across the plasma membrane of human neutrophils. *Mol. Pharmacol.* 30:437–443.
- Pittet, D., D. P. Lew, G. W. Mayr, A. Monod, and W. Schlegel. 1989. Chemoattractant receptor promotion of Ca²⁺ influx across the plasma membrane of HL-60 cells. *J. Biol. Chem.* 264:7251–7261.
- Prentki, M., C. B. Wollheim, and D. P. Lew. 1984. Ca²⁺ homeostasis in permeabilized human neutrophils: characterization of Ca²⁺-sequestering pools and the action of inositol 1,4,5-trisphosphate. *J. Biol. Chem.* 259:13 777–13 782.
- Burgess, G. M., J. S. McKinney, R. F. Irvine, M. J. Berridge, P. C. Hoyle, and J. W. Putney, Jr. 1984. Inositol 1,4,5-trisphosphate may be a signal for f-Met-Leu-Phe-induced intracellular Ca²⁺ mobilisation in human leucocytes (HL-60 cells). *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 176:193–196.
- Krause, K. H., W. Schlegel, C. B. Wollheim, T. Andersson, F. A. Waldvogel, and D. P. Lew. 1985. Chemotactic peptide activation of human neutrophils and HL60 cells: pertussis toxin reveals correlation between inositol 1,4,5-trisphosphate generation, Ca²⁺ transients, and cellular activation. *J. Clin. Invest.* 76:1348–1354.
- Gallin, E. K. 1991. Ion channels in leukocytes. *Physiol. Rev.* 71:775–811.
- von Tscherner, V., B. Prod'hom, M. Baggolini, and H. Reuter. 1986. Ion channels in human neutrophils activated by a rise in free cytosolic calcium concentration. *Nature (Lond.)* 324:369–372.
- Morris, A. P., D. V. Gallacher, R. F. Irvine, and O. H. Petersen. 1987. Synergism of inositol trisphosphate and tetrakisphosphate in activating Ca²⁺-dependent K⁺ channels. *Nature (Lond.)* 330:653–655.
- Alvarez, J., M. Montero, and J. Garcia-Sancho. 1991. Cytochrome P-450 may link intracellular Ca²⁺ stores with plasma membrane Ca²⁺ influx. *Biochem. J.* 274:193–197.
- Putney, J. W., Jr. 1990. Capacitative calcium entry revisited. *Cell Calcium* 11:611–624.
- Henne, V., G. W. Mayr, B. Grabowski, B. Koppitz, and H. D. Soling. 1988. Semisynthetic derivatives of inositol 1,4,5-trisphosphate substituted at the 1-phosphate group: effects on calcium release from permeabilized guinea-pig parotid acinar cells and comparison with binding to aldolase A. *Eur. J. Biochem.* 174:95–101.
- Almer, W., and E. Neher. 1985. The Ca²⁺ signal from fura-2-loaded mast cells depends strongly on the method of dye-loading. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 192:13–18.
- Hamill, O. P., A. Marty, E. Neher, B. Sakman, and F. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. Eur. J. Physiol.* 391:85.
- Krause, K. H., and M. J. Welsh. 1989. Voltage dependent and Ca²⁺-activated ion channels in human neutrophils. *J. Clin. Invest.* 85:491–498.
- Irvine, R. F., and R. M. Moor. 1986. Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca²⁺. *Biochem. J.* 240:917–920.
- Changya, L., D. V. Gallacher, R. F. Irvine, B. V. Potter, and O. H. Petersen. 1989. Inositol 1,3,4,5-tetrakisphosphate is essential for sustained activation of the Ca²⁺-dependent K⁺ current in single internally perfused mouse lacrimal acinar cells. *J. Membr. Biol.* 109:85–93.

18. Harootunian, A. T., J. P. Y. Kao, S. Paranjape, and R. Y. Tsien. 1991. Generation of calcium oscillations in fibroblasts by positive feedback between calcium and IP₃. *Science (Wash. DC)*. 251:75-78.
19. Mason, M. J., C. Garcia-Rodriguez, and S. Grinstein. 1991. Coupling between intracellular Ca²⁺ stores and the Ca²⁺ permeability of the plasma membrane: comparison of the effects of thapsigargin, 2,5-di-(tert-butyl)-1,4-hydroquinone, and cyclopiazonic acid in rat thymic lymphocytes. *J. Biol. Chem.* 266:20 856-20 862.
20. Demaurex, N., D. P. Lew, and K. H. Krause. 1991. Cyclopiazonic acid depletes intracellular Ca²⁺ stores and promotes divalent cation influx across the plasma membrane of HL-60. *J. Biol. Chem.* 267:2318-2324.
21. Penner, R., G. Matthews, and E. Neher. 1988. Regulation of calcium influx by second messengers in rat mast cells. *Nature (Lond.)*. 334:499-504.
22. Di Virgilio, F., D. P. Lew, T. Andersson, and T. Pozzan. 1986. Plasma membrane potential modulates chemotactic peptide-stimulated cytosolic free Ca²⁺ changes in human neutrophils. *J. Biol. Chem.* 262:4574-4579.
23. Pittet, D., F. Di Virgilio, T. Pozzan, A. Monod, and D. P. Lew. 1990. Correlation between plasma membrane potential and second messenger generation in the promyelocytic cell line HL-60. *J. Biol. Chem.* 265:14 256-14 263.
24. Simchowicz, L., and E. J. Cragoe, Jr. 1988. Na⁺-Ca²⁺ exchange in human neutrophils. *Am. J. Physiol.* 254:C150-C164.
25. Simchowicz, L., M. A. Foy, and E. J. Cragoe, Jr. 1991. A role for Na⁺/Ca²⁺ exchange in the generation of superoxide radicals by human neutrophils. *J. Biol. Chem.* 265:13 449-13 456.
26. Pittet, D., W. Schlegel, D. P. Lew, A. Monod, and G. Mayr. 1989. Mass changes in inositol tetrakis- and pentakisphosphate isomers induced by chemotactic peptide stimulation in HL-60 cells. *J. Biol. Chem.* 264:18 489-18 493.
27. Hansen, C. A., L. Yang, and J. R. Williamson. 1991. Mechanisms of receptor-mediated Ca²⁺ signalling in rat hepatocytes. *J. Biol. Chem.* 266:18 573-18 579.
28. Bird, G. S. J., M. F. Rossier, A. R. Hugues, S. B. Shears, D. L. Armstrong, and J. W. Putney, Jr. 1991. Activation of Ca²⁺ entry into acinar cells by a non-phosphorylatable inositol trisphosphate. *Nature (Lond.)*. 352:162-165.
29. Nunn, D. L., and C. W. Taylor. 1990. Liver inositol, 1,4,5-trisphosphate-binding sites are the Ca²⁺-mobilizing receptors. *Biochem J.* 270:227-232.
30. Snyder, P. M., K. H. Krause, and M. J. Welsh. 1988. Inositol trisphosphate isomers, but not inositol 1,3,4,5-tetrakisphosphate, induce calcium influx in *Xenopus laevis* oocytes. *J. Biol. Chem.* 263:11 048-11 051.