Regulation of Ca^{2+} Influx in Myeloid Cells

Role of Plasma Membrane Potential, Inositol Phosphates, Cytosolic Free [Ca^{2+}], and Filling State of Intracellular Ca^{2+} Stores

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Abstract

To study the mediation of Ca^{2+} influx by second messengers in myeloid cells, we have combined the whole-cell patch clamp technique with microfluorimetric measurements of $[Ca^{2+}]_i$. Me_2SO-differentiated HL-60 cells were loaded with the fluorescent Ca^{2+} 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, $[Ca^{2+}]_i$ 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)P_3], as well as its nonmetabolizable analogues, and (*c*) the Ca^{2+}-ATPase inhibitor cyclopiazonic acid, which depletes intracellular Ca^{2+} stores. In the absence of extracellular Ca^{2+}, responses to all stimuli were short-lasting, monophasic transients; however, subsequent addition of Ca^{2+} to the extracellular medium led to an immediate $[Ca^{2+}]_i$ increase. In all cases, the sustained phase of the $[Ca^{2+}]_i$ elevations could be inhibited by millimolar concentrations of extracellular Ni^{2+}, and its amplitude could be decreased by depolarization of the plasma membrane. Thus, the sustained phase of the Ca^{2+} elevations was due to Ca^{2+} influx through a pathway sensitive to the electrical driving force and to Ni^{2+}. No Ca^{2+} influx could be observed after (*a*) plasma membrane depolarization in resting cells, (*b*) an imposed $[Ca^{2+}]_i$ transient independent of receptor activation, or (*c*) microperfusion of myo-inositol(1,3,4,5)tetrahisphosphate (Ins(1,3,4,5)P_4). Also, Ins(1,3,4,5)P_4 did not have additive effects when co-perfused with a submaximal concentration of Ins(1,4,5)P_3. Our results suggest that, in myeloid cells, activation of chemoattractant receptors induces an electrogenic, Ni^{2+}-sensitive Ca^{2+} influx via generation of Ins(1,4,5)P_3. Ins(1,4,5)P_3 might activate Ca^{2+} influx directly, or by depletion of intracellular Ca^{2+} stores, but not via $[Ca^{2+}]_i$ increase or Ins(1,3,4,5)P_4 generation. (*J. Clin. Invest.* 1992.90:830–839.) Key words: Ca^{2+} regulation • ion channels • inositol phosphates • patch clamp • phagocytes

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Introduction

Activation of myeloid cells by chemoattractants leads to biphasic $[Ca^{2+}]_i$ increases, an initial transient due to Ca^{2+} release from internal stores, and a sustained phase due to Ca^{2+} influx across the plasma membrane (1–3). It has been clearly demonstrated (4–6) that agonist-induced Ca^{2+} release is mediated by myo-inositol (1,4,5) trisphosphate [Ins(1,4,5)P_3].[1] In contrast, the mechanism and mediation by second messengers of chemoattractant-induced Ca^{2+} 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 Ca^{2+} channels (2, 8). Although scarcely any studies have directly investigated the role of second messengers for Ca^{2+} influx in myeloid cells, its mediation by a second messenger is generally assumed. Based on the very close correlation among Ca^{2+} influx, inositol phosphate generation, Ins(1,4,5)P_3-induced Ca^{2+} release, and increase in $[Ca^{2+}]_i$, the following hypotheses have received most attention: (*a*) the product of Ins(1,4,5)P_3 phosphorylation, Myo-inositol(1,3,4,5)tetrahisphosphate (Ins(1,3,4,5)P_4), mediates Ca^{2+} influx (3, 9), (*b*) a rise in $[Ca^{2+}]_i$ mediates Ca^{2+} influx (8), (*c*) Ins(1,4,5)P_3 itself activates a Ca^{2+} channel in the plasma membrane, in addition to its activation of the Ca^{2+} release channel of internal Ca^{2+} stores, and (*d*) the filling state of the intracellular Ca^{2+} stores regulates Ca^{2+} influx (capacitative regulation of Ca^{2+} influx; see references 10 and 11).

In this study, we have characterized the regulation of Ca^{2+} influx in dimethylsulfoxide (DMSO)-differentiated HL-60 cells at the single-cell level by using the patch clamp technique combined with microfluorimetric measurements of $[Ca^{2+}]_i$. Our results show that, in myeloid cells, Ins(1,4,5)P_3 is a powerful regulator of Ca^{2+} influx. Ins(1,4,5)P_3-induced Ca^{2+} influx is mediated, at least in part, by depletion of intracellular Ca^{2+} 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)trisphosparathioate (Ins(1,4,5)P_3S_3) from New England Nuclear (Dreieich, FRG), Ins(1,4,5)P_3, 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)P_3, myo-inositol(1,4,5)trisphosphate; Ins(1,3,4,5)P_4,myo-inositol(1,3,4,5)tetrahisphosphate; Ins(2,4,5)P_3, myo-inositol(2,4,5)trisphosphate; Ins(1,4,5)P_3S_3, myo-inositol(1,4,5)trisphosparathioate; Ins (3,4,5,6)P_4, myo-inositol(3,4,5,6)tetrahisphosphate.

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 "Ca^{2+} medium" contained (in mM): NaCl 138; KCl 6; $CaCl_2$ 1.1; $MgCl_2$ 1; EGTA 0.1; glucose 20; Hepes 20. The Ca^{2+}-free medium has the same ionic composition; however, it contained 1 mM EGTA, and $CaCl_2$ 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 Ca^{2+}. Cells (2×10^7/ml) were incubated for 30 min at room temperature with 5 μM Indo-1/AM, centrifugated, resuspended in a Ca^{2+}-containing medium, and kept on ice. Just before use, an aliquot of 10^6 cells was allowed to adhere on a glass coverslip and experiments were performed within 1 h. Basal $[Ca^{2+}]_i$ 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, Sicmu, Geneva), filtered with a moving average procedure, and stored on an IBM computer. $[Ca^{2+}]_i$ was calculated using the equation: $[Ca^{2+}]_i = Kd^*\beta^*(R - R_{min})/(R_{max} - R)$; $R = F_{405}/F_{480}$. R_{min}, 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 $CaCl_2$, and $Kd^*\beta = 1280 \pm 40$ ($n = 6$), determined after break-in with a pipette solution containing a fixed Ca^{2+} concentration of 300 nM (9.2 mM EGTA and 5.4 mM $CaCl_2$). 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 supramaximal concentrations of FMLP (10^{-6}), in order to reproducibly induce maximal $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ oscillations rather than a biphasic $[Ca^{2+}]_i$ 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 (Mecanex, CH-1260 Nyon, Switzerland). Pipettes were filled with (in mM) 140 KCl, 5 NaCl, 1 $MgCl_2$, 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, $[Ca^{2+}]_i$ in the pipette was 120 nM, measured with a Ca^{2+} 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 compounds 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 $[Ca^{2+}]_i$ measurements.

Results

Plasma membrane potential and agonist-induced Ca^{2+} influx. To understand the relationship between plasma membrane potential and agonist-induced Ca^{2+} influx in myeloid cells, we studied the effect of the chemotactic peptide FMLP on $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ whose delayed phase was dependent on the presence of extracellular Ca^{2+} (Fig. 1 *a*) and could be blocked by 5 mM extracellular Ni^{2+} (Fig. 1 *b*). Thus, in HL-60 cells, FMLP induced Ca^{2+} influx at an electrically imposed constant plasma membrane potential. We then studied the effects of a plasma membrane depolarization on $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ in unstimulated cells; however, when applied during the delayed phase of the FMLP-induced $[Ca^{2+}]_i$ increase, it caused a rapid decrease in $[Ca^{2+}]_i$ (a typical trace is shown in Fig. 1 *c*). Upon return to the holding voltage (−60 mV), a small but clearly noticeable reincrease of $[Ca^{2+}]_i$ was observed, and a subsequent depolarizing step caused a small and reversible decrease in $[Ca^{2+}]_i$ from suprabasal levels to basal $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increase (not shown). Thus, in unstimulated HL-60 cells no voltage activation of Ca^{2+} influx could be detected. However, the amplitude of the FMLP-induced Ca^{2+} influx was dependent on the imposed plasma membrane potential and followed the electrochemical driving force for Ca^{2+}. This suggests that agonist-induced Ca^{2+} influx occurs through either a plasma membrane Ca^{2+} channel or an electrogenic transporter with net transfer of positive charges from the outside to the inside of the cell.

Inositol phosphates and Ca^{2+} 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 Ca^{2+} influx (6). We next studied the role of inositol phosphates in the mediation of Ca^{2+} influx. Various inositol phosphates were applied intracellularly through microperfusion by a patch pipette. Microperfusion of Ins(1,4,5)P_3 caused immediate, dose-dependent $[Ca^{2+}]_i$ elevations (Fig. 2). In the presence of external Ca^{2+}, $[Ca^{2+}]_i$ first increased within 5–20 s from resting levels of ~ 120 nM to a transient peak, then slowly declined to a new steady-state $[Ca^{2+}]_i$, above basal $[Ca^{2+}]_i$ values. This sustained phase of $[Ca^{2+}]_i$ increase was stable for the period of observation (up to 20 min). A threshold concentration of 1 μM Ins(1,4,5)P_3 in the pipette solution had to be exceeded in order to elicit $[Ca^{2+}]_i$ increases. In the range of 2–10 μM Ins(1,4,5)P_3 in the pipette

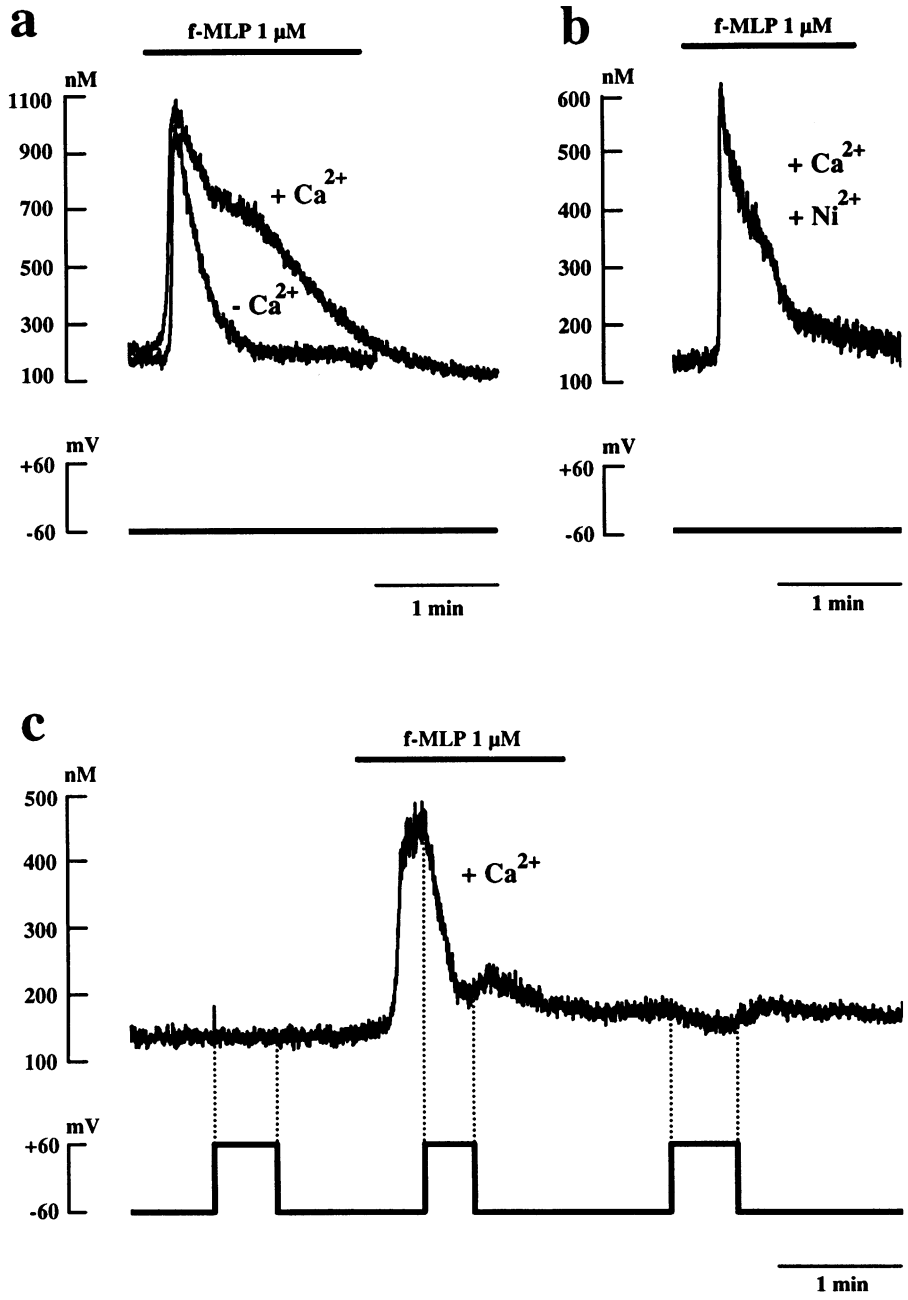

Figure 1. $[Ca^{2+}]_i$ changes induced by FMLP in voltage-clamped HL-60 cells: effects of extracellular Ca^{2+}, Ni^{2+}, and plasma membrane depolarization. Indo-1-loaded cells were voltage-clamped in the whole cell configuration and superfused with FMLP (1 μM). The voltage protocols of the respective 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 μM 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 $[Ca^{2+}]_i$

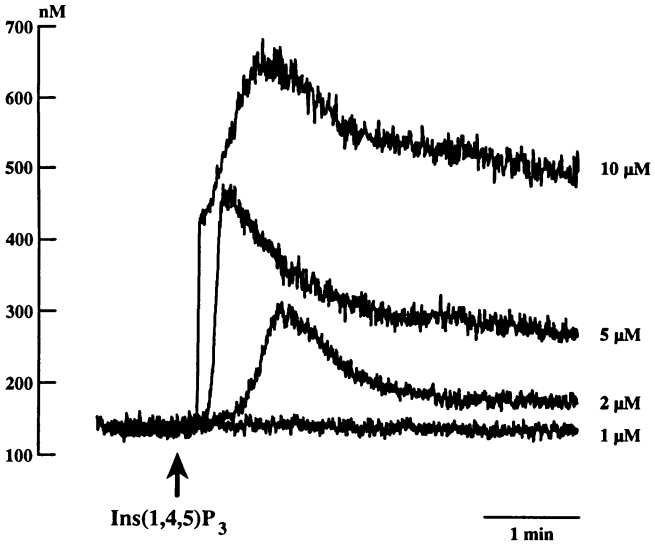

Figure 2. Effects of Ins(1,4,5)P_3 microperfusion on $[Ca^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were kept in the cell-attached patch clamp configuration (holding voltage −60 mV), in the presence of extracellular Ca^{2+}. When indicated by the arrow, the whole-cell configuration was achieved. The cell was thereby microperfused with the pipette solution which contained the indicated concentration of Ins(1,4,5)P_3. The $[Ca^{2+}]_i$ responses to increasing concentrations of Ins(1,4,5)P_3 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)P_3 (Table I).

It has been proposed that Ins(1,3,4,5)P_4, the product of Ins(1,4,5)P_3 phosphorylation, acts in synergy with Ins(1,4,5)P_3 to induce Ca^{2+} influx (9, 16, 17). To study this question, we microperfused cells either with various concentrations of Ins(1,3,4,5)P_4 alone or with Ins(1,3,4,5)P_4 added to a submaximal dose of Ins(1,4,5)P_3. Ins(1,3,4,5)P_4 microperfusion by itself did not have an effect on Ca^{2+} homeostasis at a range of concentrations of 1 to 20 μM (Fig. 5 *a* and Table 1).[2] The co-microperfusion of 20 μM Ins(1,3,4,5)P_4 with 2 μM Ins(1,4,5)P_3 produced $[Ca^{2+}]_i$ increases indistinguishable from those induced by microperfusion of 2 μM Ins(1,4,5)P_3 alone (Fig. 5 *b* and *c*, Table I).

$[Ca^{2+}]_i$ elevations and Ca^{2+} influx. As both the physiological agonist FMLP and the intracellular messenger Ins(1,4,5)P_3 induced an initial $[Ca^{2+}]_i$ rise due to Ca^{2+} release from intracellular stores, they might induce Ca^{2+} influx via activation of plasma membrane Ca^{2+} channels which are sensitive to $[Ca^{2+}]_i$ increases (8). To test this hypothesis, we pro-

2. In 3 out of 39 cells, Ins(1,3,4,5)P_4 (8–20 μM) caused delayed and minor $[Ca^{2+}]_i$ elevations. These elevations were independent of the extracellular Ca^{2+} concentration and were observed in a similar percentage of cells microperfused with control intracellular solution (Table I).

Table I. $[Ca^{2+}]_i$ 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_3S_3	20	+	130±20	610±170	390±60	8±1	230±10	80	5
Ins(1,4,5)P_3S_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 Ca^{2+}-containing or Ca^{2+}-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 $[Ca^{2+}]_i$, measured before application of the stimulus; (*b*) the peak $[Ca^{2+}]_i$ measured during the 30-s interval, after the application of the stimulus; (*c*) the delayed phase of the $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ increase; (*e*) the slope of the initial 10 s of the $[Ca^{2+}]_i$ increase; (*f*) the number of responding cells (defined as cells showing an increase in $[Ca^{2+}]_i$ > 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.

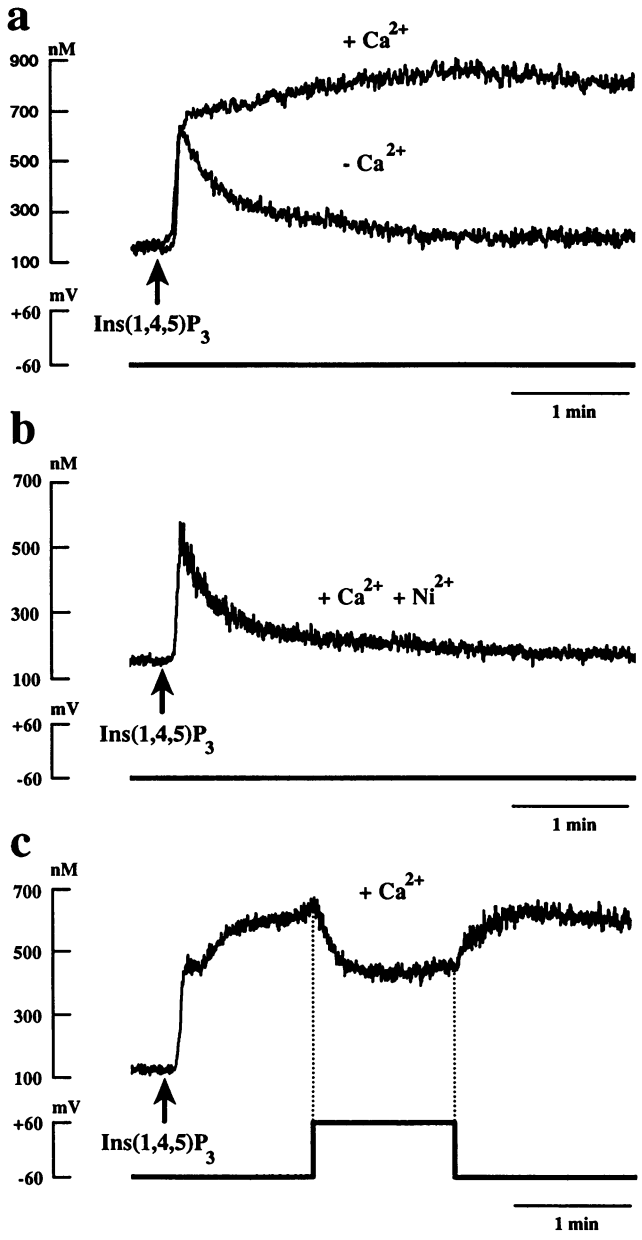

Figure 3. Effects of extracellular Ca^{2+}, Ni^{2+}, and plasma membrane depolarization on the $[Ca^{2+}]_i$ changes induced by Ins(1,4,5)P_3. Indo-1-loaded HL-60 cells were voltage-clamped in the whole cell configuration. The pipette contained 10 μM Ins(1,4,5)P_3; arrows indicate when the whole cell configuration was achieved. The voltage protocols of the respective experiments are shown under the $[Ca^{2+}]_i$ traces. (*a*) Ins(1,4,5)P_3-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*) Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ changes in the presence of extracellular Ca^{2+} and Ni^{2+} (5 mM), at a constant holding potential of −60 mV. (*c*) Effect on $[Ca^{2+}]_i$ of a depolarization to +60 mV during the sustained phase of Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ increase in the presence of extracellular Ca^{2+}.

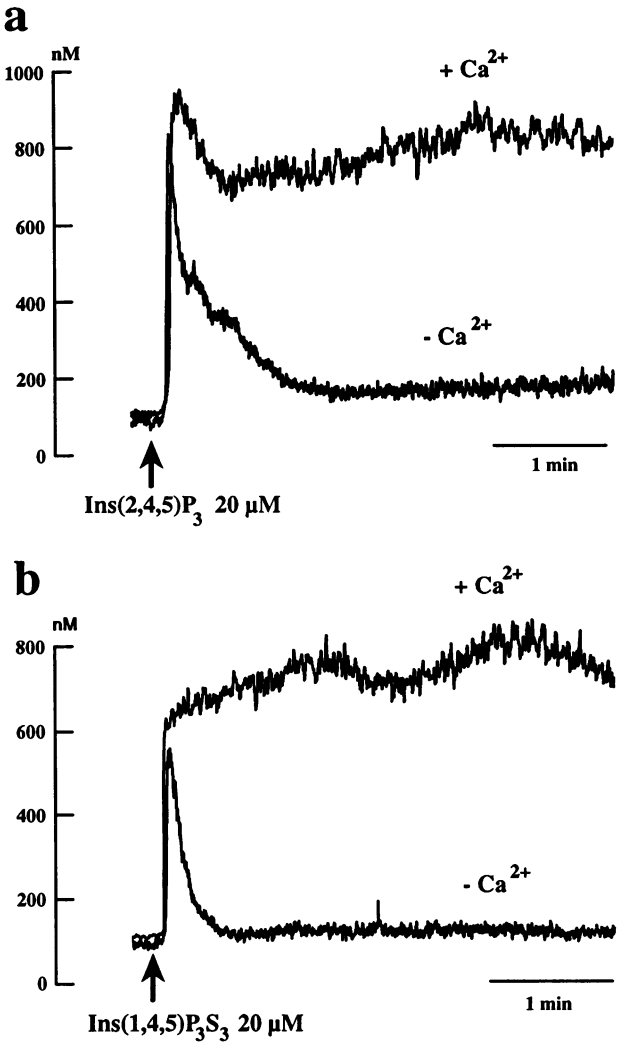

Figure 4. Effects of Ins(2,4,5)P_3 and Ins(1,4,5)P_3S_3 microperfusion on $[Ca^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were voltage-clamped at −60 mV. The pipette contained the respective Ins(1,4,5)P_3 analogue; arrows indicate when the whole cell configuration was achieved. (*a*) $[Ca^{2+}]_i$ response to 20 μM Ins(2,4,5)P_3 in the presence (*upper trace*) and absence (*lower trace*) of extracellular Ca^{2+}. (*b*) $[Ca^{2+}]_i$ response to 20 μM Ins(1,4,5)P_3S_3 in the presence (*upper trace*) and absence (*lower trace*) of extracellular Ca^{2+}. Traces have been superimposed for comparison.

duced a $[Ca^{2+}]_i$ increase independent of receptor activation and Ins(1,4,5)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 disrupted the plasma membrane and caused a rapid elevation of $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ elevation, albeit of large amplitude, was short lasting (2 min) and monophasic. Subsequent superfusion of the same cell with FMLP induced a biphasic $[Ca^{2+}]_i$ 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 Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ changes (see Figs. 3 and 4) is very striking. Wounding of the cell caused a $[Ca^{2+}]_i$ peak comparable to the one observed with microperfusion of 10 μM Ins(1,4,5)P_3. However, microperfusion of Ins(1,4,5)P_3 caused a permanent opening of the Ca^{2+} influx pathway, while the wounding caused only a short lasting monophasic peak.

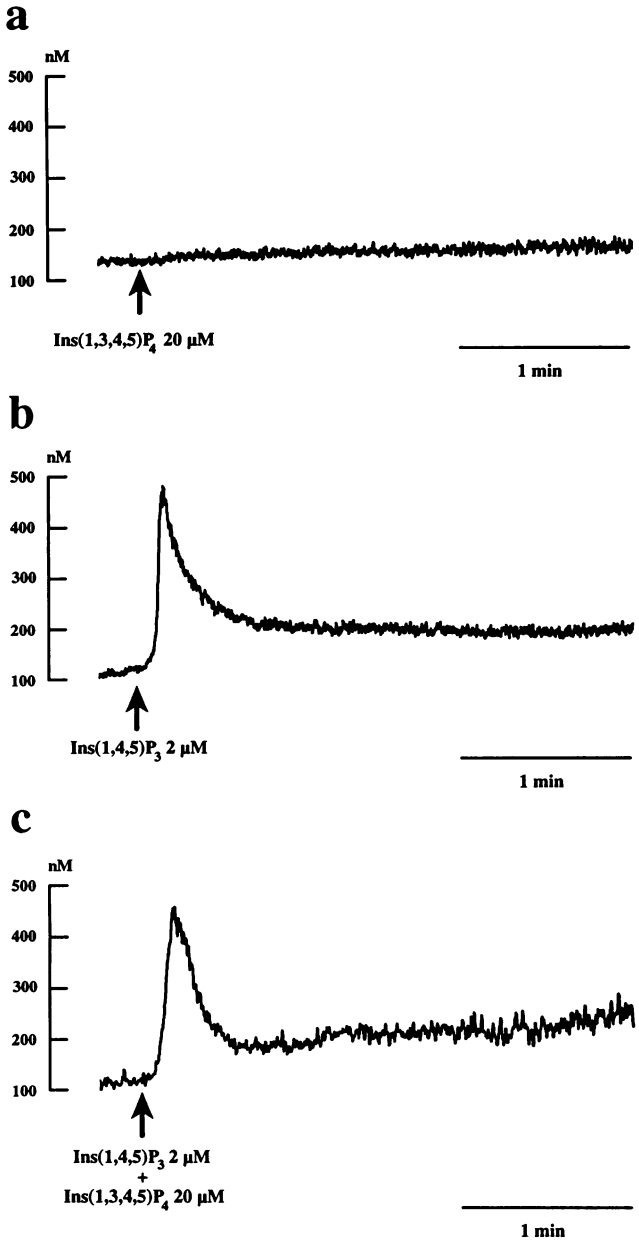

Figure 5. Effects of Ins(1,3,4,5)P_4 microperfusion on $[Ca^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were voltage-clamped at −60 mV in the presence of external Ca^{2+}. The pipette contained the indicated inositol phosphate(s); arrows indicate when the whole-cell configuration was achieved. (*a*) $[Ca^{2+}]_i$ response to 20 μM Ins(1,3,4,5)P_4. (*b*) $[Ca^{2+}]_i$ response to 2 μM Ins(1,4,5)P_3. (*c*) $[Ca^{2+}]_i$ response to the combination of 2 μM Ins(1,4,5)P_3 and 20 μM Ins(1,3,4,5)P_4.

Thus, while Ins(1,4,5)P_3 is a potent intracellular activator of Ca^{2+} influx, a sudden increase in $[Ca^{2+}]_i$ is not sufficient to induce a sustained Ca^{2+} influx. However, both FMLP and Ins(1,4,5)P_3 produce an elevated $[Ca^{2+}]_i$ due to Ca^{2+} release that occurs prior to the induction of Ca^{2+} influx. This elevated $[Ca^{2+}]_i$ might be required in addition to Ins(1,4,5)P_3 for the activation of the Ca^{2+} influx pathway. To study this question, we microperfused cells with Ins(1,4,5)P_3 in the absence of extracellular Ca^{2+}, then added 2 mM Ca^{2+} to the extracellular medium 7 min after return of $[Ca^{2+}]_i$ to basal levels. In control cells, the addition of Ca^{2+} did not alter $[Ca^{2+}]_i$, whereas in Ins(1,4,5)P_3 microperfused cells, the addition of Ca^{2+} did cause an immediate and sustained increase in $[Ca^{2+}]_i$ from 135 nM to steady-state $[Ca^{2+}]_i$ levels of 800 nM (Fig. 7), i.e., $[Ca^{2+}]_i$ values comparable to the one observed when cells were microperfused with Ins(1,4,5)P_3 in a Ca^{2+}-containing medium (Table I). Thus, the activity of Ins(1,4,5)P_3 on the Ca^{2+} influx pathway does not appear to require a concomitant high $[Ca^{2+}]_i$.

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 $[Ca^{2+}]_i$ changes induced by CPA superfusion to single adherent HL-60 cells. In the absence of extracellular Ca^{2+}, CPA superfusion produced a transient $[Ca^{2+}]_i$ increase, and a subsequent microperfusion of the cell with a maximal dose of Ins(1,4,5)P_3 could not release additional Ca^{2+} (Fig. 8 *a*). In the presence of extracellular Ca^{2+}, CPA induced a sustained increase in $[Ca^{2+}]_i$ 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 Ins(1,4,5)P_3-sensitive Ca^{2+} stores[3] and induces a Ca^{2+} influx with characteristics similar to the Ca^{2+} influx induced by FMLP and Ins(1,4,5)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 Ins(1,4,5)P_3-induced Ca^{2+} release.

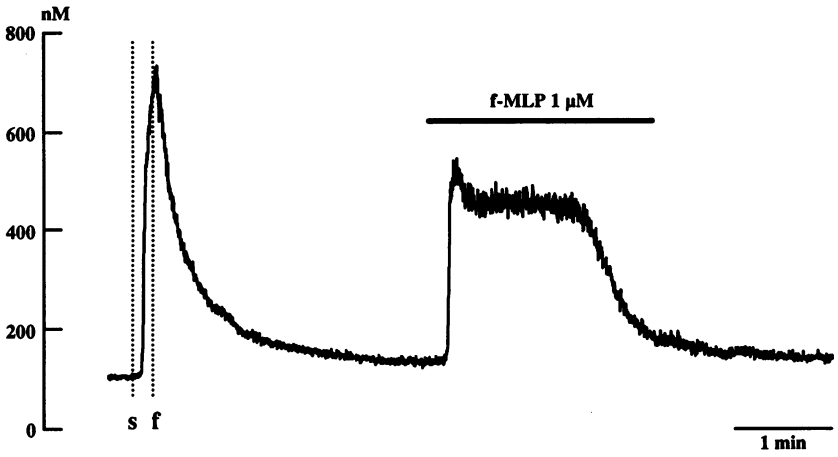

Figure 6. Effects of an imposed rapid $[Ca^{2+}]_i$ transient on $[Ca^{2+}]_i$ in an adherent HL-60 cell. An Indo-1-loaded HL-60 cell was allowed to adhere on a glass coverslip in the presence of extracellular Ca^{2+}. The cell was transiently impaled with a micropipette (s = start, f = finish). As a control, the same cell was subsequently superfused with 1 μM 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-activated 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

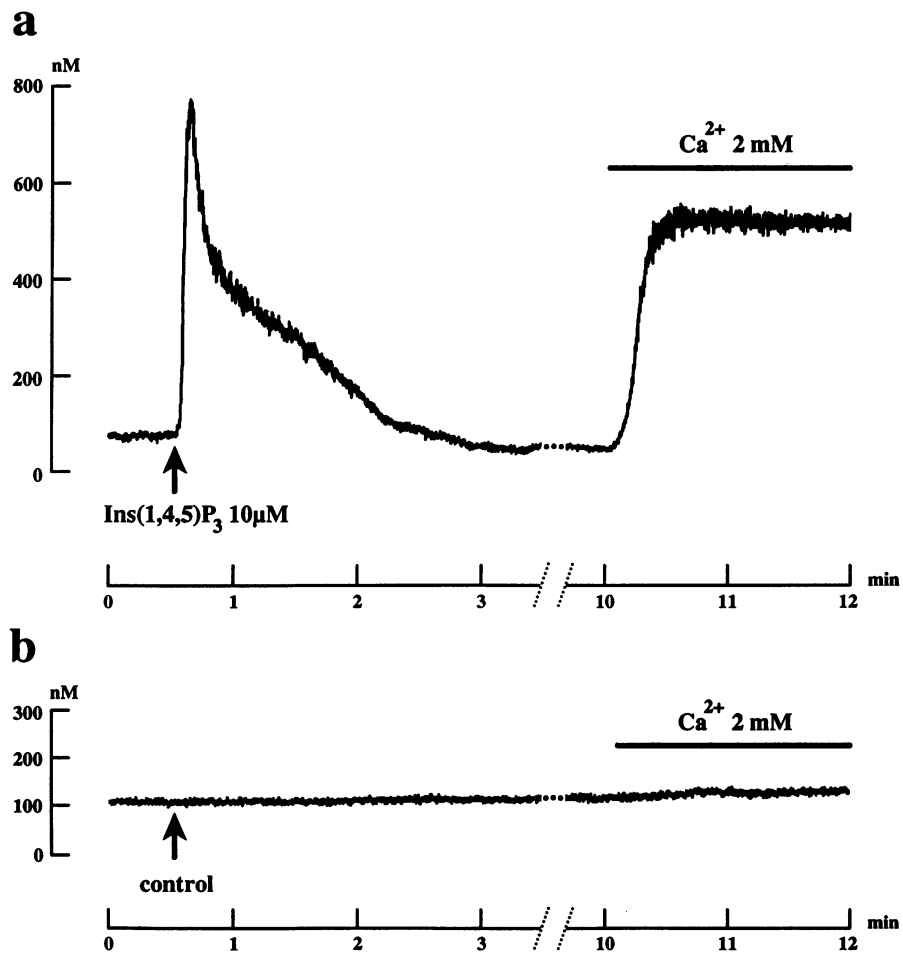

Figure 7. Effects on $[Ca^{2+}]_i$ of extracellular Ca^{2+} addition to cells microperfused with Ins(1,4,5)P_3 in the absence of extracellular Ca^{2+}. Indo-1-loaded HL-60 cells were voltage-clamped at −60 mV in the absence of extracellular Ca^{2+}. The pipette contained either 10 μM Ins(1,4,5)P_3 or 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.

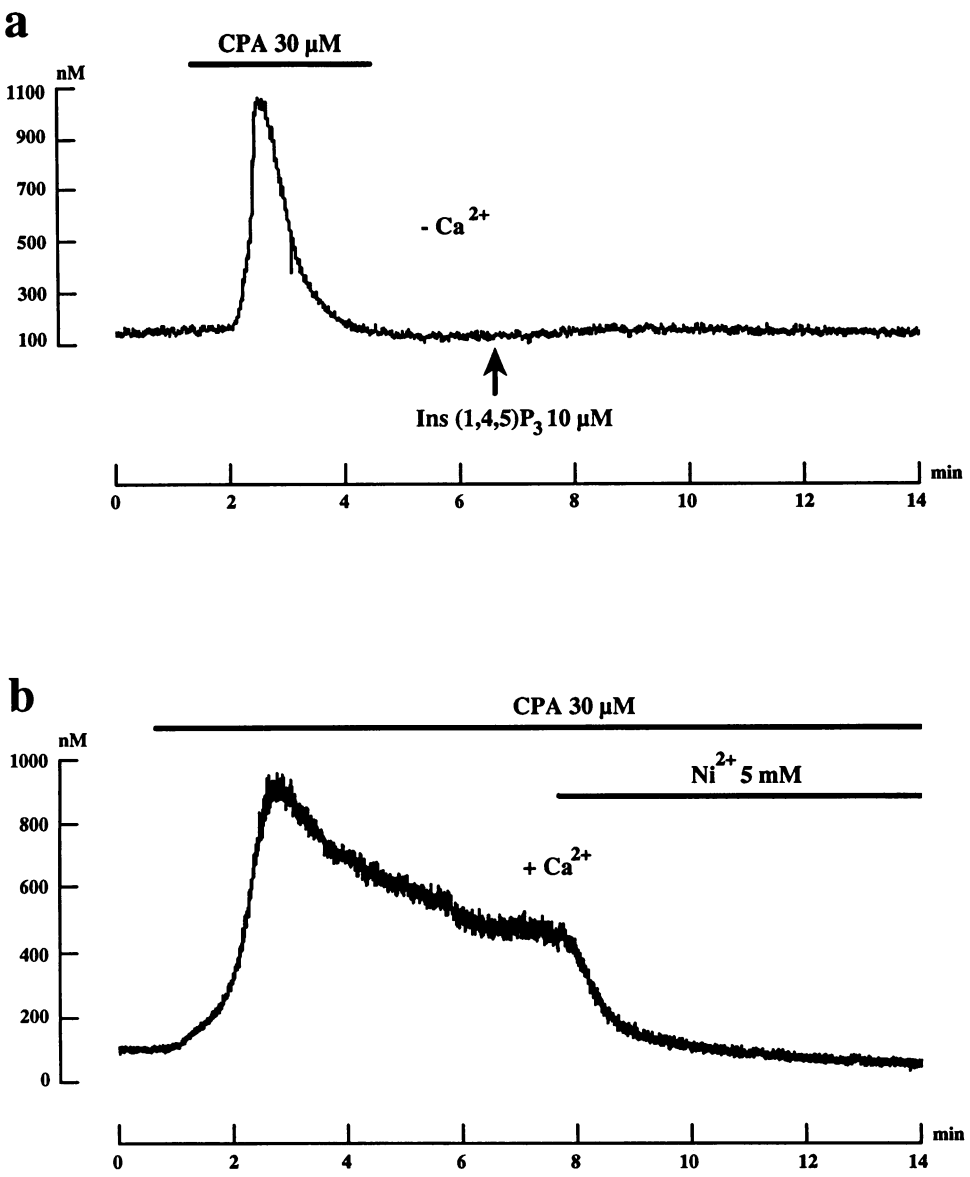

Figure 8. Effects of the Ca^{2+}-ATPase inhibitor CPA on $[Ca^{2+}]_i$ in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were voltage-clamped at −60 mV and superfused with 10 μM CPA (an inhibitor of Ca^{2+}-ATPases of muscle of sarcoplasmic reticulum and intracellular Ca^{2+}-ATPase of various nonmuscle cells). The pipette contained either 10 μM Ins(1,4,5)P_3 or standard intracellular solution; arrows indicate when the whole cell configuration was achieved. (*a*) $[Ca^{2+}]_i$ responses to CPA superfusion and subsequent Ins(1,4,5)P_3 microperfusion in the absence of extracellular Ca^{2+}. (*b*) $[Ca^{2+}]_i$ 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 Na^+/Ca^{2+} exchanger as the pathway of Ca^{2+} influx (24, 25), because the Na^+/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 demonstrates that Ins(1,4,5)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 Ins(1,4,5)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 Ins(1,4,5)P_3 concentrations required to induce Ca^{2+} release and Ca^{2+} influx were very similar. For both processes the threshold concentration was ~ 1 μM and the half-maximal concentration ~ 5 μM. Thus, there is no indication for a regulation of Ca^{2+} influx and Ca^{2+} release by Ins(1,4,5)P_3 receptors with different affinities.

Mass concentration of Ins(1,4,5)P_3 were 0.3 μM in unstimulated HL-60 cells, and 2.2 μM in HL-60 cells stimulated with 10^{-6} M FMLP for 30 s (26). Thus, the Ins(1,4,5)P_3 concentrations used in this study (1–10 μM) are within a biologically

relevant range. Similar to results from studies performed in other cell types (21, 27, 28), the Ins(1,4,5)P_3 concentrations necessary in the patch pipette for maximal effects on $[Ca^{2+}]_i$ were around 10 μM, i.e., ~ 5–10 times higher than half-maximal concentrations for Ca^{2+} 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)P_3 under the whole-cell patch clamp condition. This is conceivable, as the Ins(1,4,5)P_3 binding to its receptor depends on many cofactors, such as pH, and concentrations of Ca^{2+}, Mg^{2+}, and ATP (29). Alternatively, however, the effective Ins(1,4,5)P_3 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)P_3 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)P_3 analogs are only double of the respective concentrations of Ins(1,4,5)P_3, while in permeabilized cells or in homogenates these compounds are usually 5–10 times less potent than Ins(1,4,5)P_3. This observation indirectly suggests that Ins(1,4,5)P_3 metabolism may play a role for the relatively low Ins(1,4,5)P_3 sensitivity of whole cell patch-clamped cells.

How might Ins(1,4,5)P_3 induce Ca^{2+} influx? So far, four major hypotheses have received most attention: First, inositol tetrakis phosphate-activated Ca^{2+} influx: According to this hypothesis, Ca^{2+} influx is due to the combined action of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 (9, 16, 17). Indeed, in HL-60 cells, there is an excellent temporal correlation between Ins(1,3,4,5)P_4 generation and Ca^{2+} influx (3). Here we show that microperfusion of Ins(1,3,4,5)P_4 is neither necessary nor sufficient for the induction of Ca^{2+} 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)P_4 on submaximal concentrations of Ins(1,4,5)P_3 could be found. Thus, our results are not compatible with the inositol tetrakis phosphate hypothesis. Second, $[Ca^{2+}]_i$-activated Ca^{2+} influx: In this hypothesis, Ca^{2+} influx is activated by cytosolic Ca^{2+} ions. Neutrophils possess non-selective Ca^{2+}-activated cation channels that can conduct Ca^{2+}. The activation of these channels by the initial Ca^{2+} release from internal stores has been proposed to mediate Ca^{2+} influx (8). In this study, we could not detect, in HL-60 cells, a sustained Ca^{2+} influx in response to $[Ca^{2+}]_i$ increases that were induced independently from emptying of intracellular Ca^{2+} stores. In addition, Ins(1,4,5)P_3-induced Ca^{2+} release and Ca^{2+} influx could be temporarily dissociated (Fig. 7). Thus, our results argue against a central role of a $[Ca^{2+}]_i$ elevation in the mediation of Ca^{2+} influx. Third, directly Ins(1,4,5)P_3-mediated Ca^{2+} influx and fourth, capacitative Ca^{2+} influx: Having excluded a role for Ins(1,3,4,5)P_4 and $[Ca^{2+}]_i$, the most likely explanations for the induction of Ca^{2+} influx by Ins(1,4,5)P_3 are either a direct effect of Ins(1,4,5)P_3 on the Ca^{2+} influx pathway in the plasma membrane, or mediation of Ca^{2+} influx by the filling state of the intracellular Ca^{2+} store. As the Ca^{2+}-ATPase inhibitor, cyclopiazonic acid, induces Ca^{2+} influx without raising levels of Ins(1,4,5)P_3 (20), our results strongly suggest that the emptying of intracellular Ca^{2+} pools by Ins(1,4,5)P_3 is a factor that regulates the Ca^{2+} permeability of the plasma membrane (10, 11). However, we cannot exclude an additional direct effect of Ins(1,4,5)P_3 on the Ca^{2+} influx pathway. Recent studies in hepatocytes support such a concept of a dual action of Ins(1,4,5)P_3 (27). Thus, further studies are necessary to determine whether the capacitative mechanism of Ca^{2+} influx is the only, or one of several mechanisms that regulate receptor-mediated Ca^{2+} influx in myeloid cells.

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