

# Regulation of $\text{Ca}^{2+}$ Influx in Myeloid Cells

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

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### Abstract

To study the mediation of  $\text{Ca}^{2+}$  influx by second messengers in myeloid cells, we have combined the whole-cell patch clamp technique with microfluorimetric measurements of  $[\text{Ca}^{2+}]_i$ .  $\text{Me}_2\text{SO}$ -differentiated HL-60 cells were loaded with the fluorescent  $\text{Ca}^{2+}$  indicator Indo-1, allowed to adhere to glass slides, and patch-clamped. Receptor agonists and  $\text{Ca}^{2+}$ -ATPase inhibitors were applied by superfusion and inositol phosphates by microperfusion through the patch pipette. In voltage-clamped cells,  $[\text{Ca}^{2+}]_i$  elevations with a sustained phase could be induced by (a) the chemoattractant receptor agonist FMLP, (b) the  $\text{Ca}^{2+}$ -releasing second messenger myo-inositol(1,4,5)trisphosphate  $[\text{Ins}(1,4,5)\text{P}_3]$ , as well as its nonmetabolizable analogues, and (c) the  $\text{Ca}^{2+}$ -ATPase inhibitor cyclopiazonic acid, which depletes intracellular  $\text{Ca}^{2+}$  stores. In the absence of extracellular  $\text{Ca}^{2+}$ , responses to all stimuli were short-lasting, monophasic transients; however, subsequent addition of  $\text{Ca}^{2+}$  to the extracellular medium led to an immediate  $[\text{Ca}^{2+}]_i$  increase. In all cases, the sustained phase of the  $[\text{Ca}^{2+}]_i$  elevations could be inhibited by millimolar concentrations of extracellular  $\text{Ni}^{2+}$ , and its amplitude could be decreased by depolarization of the plasma membrane. Thus, the sustained phase of the  $\text{Ca}^{2+}$  elevations was due to  $\text{Ca}^{2+}$  influx through a pathway sensitive to the electrical driving force and to  $\text{Ni}^{2+}$ . No  $\text{Ca}^{2+}$  influx could be observed after (a) plasma membrane depolarization in resting cells, (b) an imposed  $[\text{Ca}^{2+}]_i$  transient independent of receptor activation, or (c) microperfusion of myo-inositol(1,3,4,5)tetraphosphate  $[\text{Ins}(1,3,4,5)\text{P}_4]$ . Also,  $\text{Ins}(1,3,4,5)\text{P}_4$  did not have additive effects when co-perfused with a submaximal concentration of  $\text{Ins}(1,4,5)\text{P}_3$ . Our results suggest that, in myeloid cells, activation of chemoattractant receptors induces an electrogenic,  $\text{Ni}^{2+}$ -sensitive  $\text{Ca}^{2+}$  influx via generation of  $\text{Ins}(1,4,5)\text{P}_3$ .  $\text{Ins}(1,4,5)\text{P}_3$  might activate  $\text{Ca}^{2+}$  influx directly, or by depletion of intracellular  $\text{Ca}^{2+}$  stores, but not via  $[\text{Ca}^{2+}]_i$  increase or  $\text{Ins}(1,3,4,5)\text{P}_4$  generation. (*J. Clin. Invest.* 1992;90:830–839.) Key words:  $\text{Ca}^{2+}$  regulation • ion channels • inositol phosphates • patch clamp • phagocytes

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### Introduction

Activation of myeloid cells by chemoattractants leads to biphasic  $[\text{Ca}^{2+}]_i$  increases, an initial transient due to  $\text{Ca}^{2+}$  release from internal stores, and a sustained phase due to  $\text{Ca}^{2+}$  influx across the plasma membrane (1–3). It has been clearly demonstrated (4–6) that agonist-induced  $\text{Ca}^{2+}$  release is mediated by myo-inositol (1,4,5) trisphosphate  $[\text{Ins}(1,4,5)\text{P}_3]$ .<sup>1</sup> In contrast, the mechanism and mediation by second messengers of chemoattractant-induced  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channels (2, 8). Although scarcely any studies have directly investigated the role of second messengers for  $\text{Ca}^{2+}$  influx in myeloid cells, its mediation by a second messenger is generally assumed. Based on the very close correlation among  $\text{Ca}^{2+}$  influx, inositol phosphate generation,  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release, and increase in  $[\text{Ca}^{2+}]_i$ , the following hypotheses have received most attention: (a) the product of  $\text{Ins}(1,4,5)\text{P}_3$  phosphorylation, Myo-inositol(1,3,4,5)tetraphosphate  $[\text{Ins}(1,3,4,5)\text{P}_4]$ , mediates  $\text{Ca}^{2+}$  influx (3, 9), (b) a rise in  $[\text{Ca}^{2+}]_i$  mediates  $\text{Ca}^{2+}$  influx (8), (c)  $\text{Ins}(1,4,5)\text{P}_3$  itself activates a  $\text{Ca}^{2+}$  channel in the plasma membrane, in addition to its activation of the  $\text{Ca}^{2+}$  release channel of internal  $\text{Ca}^{2+}$  stores, and (d) the filling state of the intracellular  $\text{Ca}^{2+}$  stores regulates  $\text{Ca}^{2+}$  influx (capacitative regulation of  $\text{Ca}^{2+}$  influx; see references 10 and 11).

In this study, we have characterized the regulation of  $\text{Ca}^{2+}$  influx in dimethylsulfoxide (DMSO)-differentiated HL-60 cells at the single-cell level by using the patch clamp technique combined with microfluorimetric measurements of  $[\text{Ca}^{2+}]_i$ . Our results show that, in myeloid cells,  $\text{Ins}(1,4,5)\text{P}_3$  is a powerful regulator of  $\text{Ca}^{2+}$  influx.  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  influx is mediated, at least in part, by depletion of intracellular  $\text{Ca}^{2+}$  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)trisphosphatidate  $[\text{Ins}(1,4,5)\text{P}_3\text{S}_3]$  from New England Nuclear (Dreieich, FRG),  $\text{Ins}(1,4,5)\text{P}_3$ , 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;  $\text{Ins}(1,4,5)\text{P}_3$ , myo-inositol(1,4,5)trisphosphate;  $\text{Ins}(1,3,4,5)\text{P}_4$ , myo-inositol(1,3,4,5)tetraphosphate;  $\text{Ins}(2,4,5)\text{P}_3$ , myo-inositol(2,4,5)trisphosphate;  $\text{Ins}(1,4,5)\text{P}_3\text{S}_3$ , myo-inositol(1,4,5)trisphosphatidate;  $\text{Ins}(3,4,5,6)\text{P}_4$ , myo-inositol(3,4,5,6)tetraphosphate.

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<sup>2+</sup> medium" contained (in mM): NaCl 138; KCl 6; CaCl<sub>2</sub> 1.1; MgCl<sub>2</sub> 1; EGTA 0.1; glucose 20; Hepes 20. The Ca<sup>2+</sup>-free medium has the same ionic composition; however, it contained 1 mM EGTA, and CaCl<sub>2</sub> 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<sup>2+</sup>.** Cells ( $2 \times 10^7$ /ml) were incubated for 30 min at room temperature with 5 µM Indo-1/AM, centrifuged, resuspended in a Ca<sup>2+</sup>-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<sup>2+</sup>]<sub>i</sub> 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. [Ca<sup>2+</sup>]<sub>i</sub> was calculated using the equation: [Ca<sup>2+</sup>]<sub>i</sub> =  $Kd^* \beta^* (R - R_{\min}) / (R_{\max} - R)$ ;  $R = F_{405} / F_{480} \cdot R_{\min} / R_{\max}$ , 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<sub>2</sub>, and  $Kd^* \beta^* = 1280 \pm 40$  ( $n = 6$ ), determined after break-in with a pipette solution containing a fixed Ca<sup>2+</sup> concentration of 300 nM (9.2 mM EGTA and 5.4 mM CaCl<sub>2</sub>). 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 [Ca<sup>2+</sup>]<sub>i</sub> 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<sup>2+</sup>]<sub>i</sub> oscillations rather than a biphasic [Ca<sup>2+</sup>]<sub>i</sub> 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 MgCl<sub>2</sub>, 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<sup>2+</sup>]<sub>i</sub> in the pipette was 120 nM, measured with a Ca<sup>2+</sup> 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-

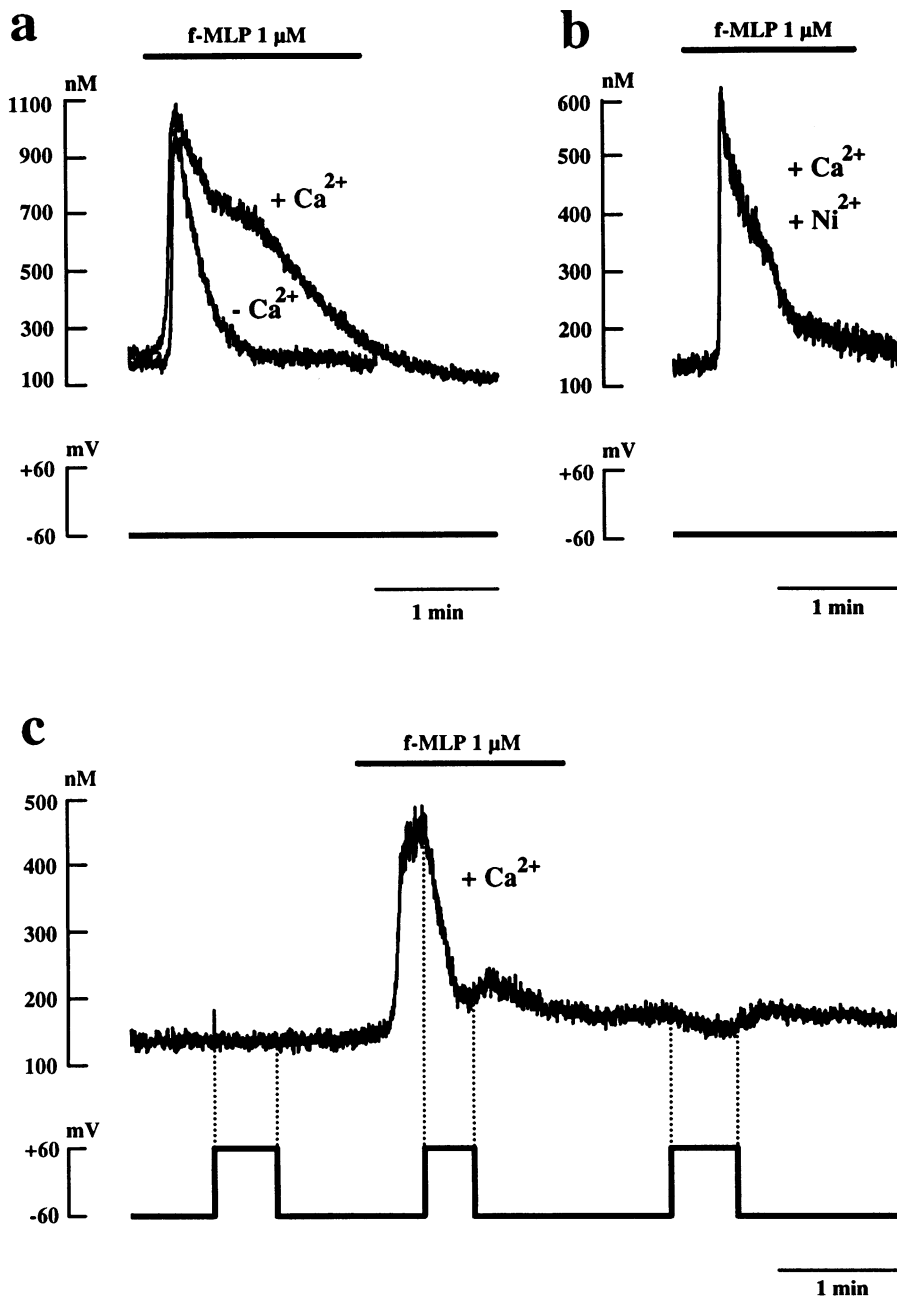
pounds will be referred to throughout this paper as "microperfusion." Patch clamp recordings were performed using an EPC-7 amplifier (List Medical, Darmstadt, FRG) in the voltage clamp mode. Stimulation and recording of electrophysiological data were performed with the same A/D interface used for [Ca<sup>2+</sup>]<sub>i</sub> measurements.

## Results

### *Plasma membrane potential and agonist-induced Ca<sup>2+</sup> influx.*

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

**Inositol phosphates and Ca<sup>2+</sup> 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<sup>2+</sup> influx (6). We next studied the role of inositol phosphates in the mediation of Ca<sup>2+</sup> influx. Various inositol phosphates were applied intracellularly through microperfusion by a patch pipette. Microperfusion of Ins(1,4,5)P<sub>3</sub> caused immediate, dose-dependent [Ca<sup>2+</sup>]<sub>i</sub> elevations (Fig. 2). In the presence of external Ca<sup>2+</sup>, [Ca<sup>2+</sup>]<sub>i</sub> 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<sup>2+</sup>]<sub>i</sub>, above basal [Ca<sup>2+</sup>]<sub>i</sub> values. This sustained phase of [Ca<sup>2+</sup>]<sub>i</sub> increase was stable for the period of observation (up to 20 min). A threshold concentration of 1 µM Ins(1,4,5)P<sub>3</sub> in the pipette solution had to be exceeded in order to elicit [Ca<sup>2+</sup>]<sub>i</sub> increases. In the range of 2–10 µM Ins(1,4,5)P<sub>3</sub> 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  $\mu$ 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  $\mu$ 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  $[Ca^{2+}]_i$

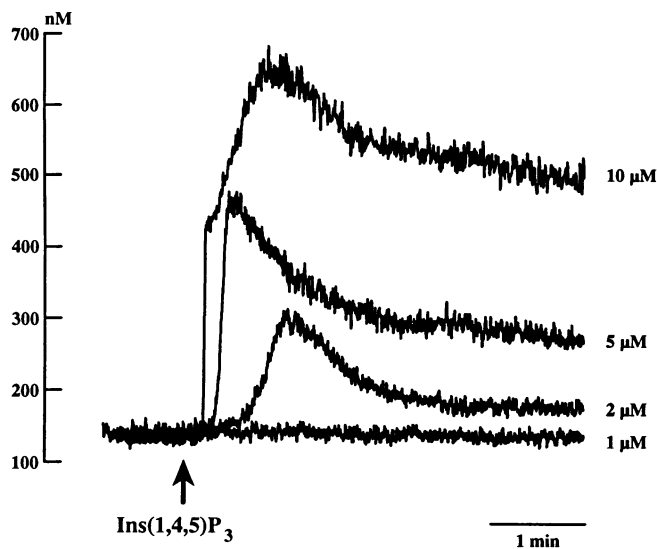


Figure 2. Effects of  $\text{Ins}(1,4,5)\text{P}_3$  microperfusion on  $[\text{Ca}^{2+}]_i$  in voltage-clamped HL-60 cells. Indo-1-loaded HL-60 cells were kept in the cell-attached patch clamp configuration (holding voltage  $-60$  mV), in the presence of extracellular  $\text{Ca}^{2+}$ . When indicated by the arrow, the whole-cell configuration was achieved. The cell was thereby microperfused with the pipette solution which contained the indicated concentration of  $\text{Ins}(1,4,5)\text{P}_3$ . The  $[\text{Ca}^{2+}]_i$  responses to increasing concentrations of  $\text{Ins}(1,4,5)\text{P}_3$  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  $\text{Ins}(1,4,5)\text{P}_3$  (Table I).

It has been proposed that  $\text{Ins}(1,3,4,5)\text{P}_4$ , the product of  $\text{Ins}(1,4,5)\text{P}_3$  phosphorylation, acts in synergy with  $\text{Ins}(1,4,5)\text{P}_3$  to induce  $\text{Ca}^{2+}$  influx (9, 16, 17). To study this question, we microperfused cells either with various concentrations of  $\text{Ins}(1,3,4,5)\text{P}_4$  alone or with  $\text{Ins}(1,3,4,5)\text{P}_4$  added to a submaximal dose of  $\text{Ins}(1,4,5)\text{P}_3$ .  $\text{Ins}(1,3,4,5)\text{P}_4$  microperfusion by itself did not have an effect on  $\text{Ca}^{2+}$  homeostasis at a range of concentrations of 1 to 20  $\mu\text{M}$  (Fig. 5 *a* and Table I).<sup>2</sup> The co-microperfusion of 20  $\mu\text{M}$   $\text{Ins}(1,3,4,5)\text{P}_4$  with 2  $\mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$  produced  $[\text{Ca}^{2+}]_i$  increases indistinguishable from those induced by microperfusion of 2  $\mu\text{M}$   $\text{Ins}(1,4,5)\text{P}_3$  alone (Fig. 5 *b* and *c*, Table I).

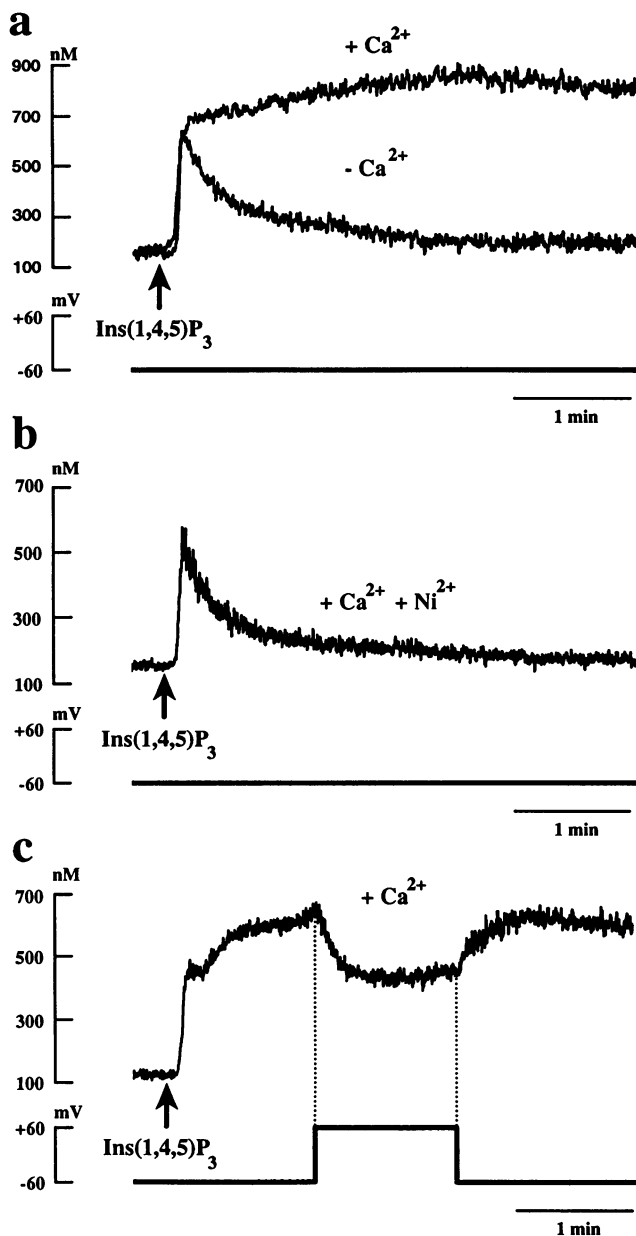
$[\text{Ca}^{2+}]_i$  elevations and  $\text{Ca}^{2+}$  influx. As both the physiological agonist FMLP and the intracellular messenger  $\text{Ins}(1,4,5)\text{P}_3$  induced an initial  $[\text{Ca}^{2+}]_i$  rise due to  $\text{Ca}^{2+}$  release from intracellular stores, they might induce  $\text{Ca}^{2+}$  influx via activation of plasma membrane  $\text{Ca}^{2+}$  channels which are sensitive to  $[\text{Ca}^{2+}]_i$  increases (8). To test this hypothesis, we pro-

2. In 3 out of 39 cells,  $\text{Ins}(1,3,4,5)\text{P}_4$  (8–20  $\mu\text{M}$ ) caused delayed and minor  $[\text{Ca}^{2+}]_i$  elevations. These elevations were independent of the extracellular  $\text{Ca}^{2+}$  concentration and were observed in a similar percentage of cells microperfused with control intracellular solution (Table I).

Table I.  $[\text{Ca}^{2+}]_i$  Changes Induced by FMLP and Inositol Phosphates in Voltage-Clamped HL-60 Cells

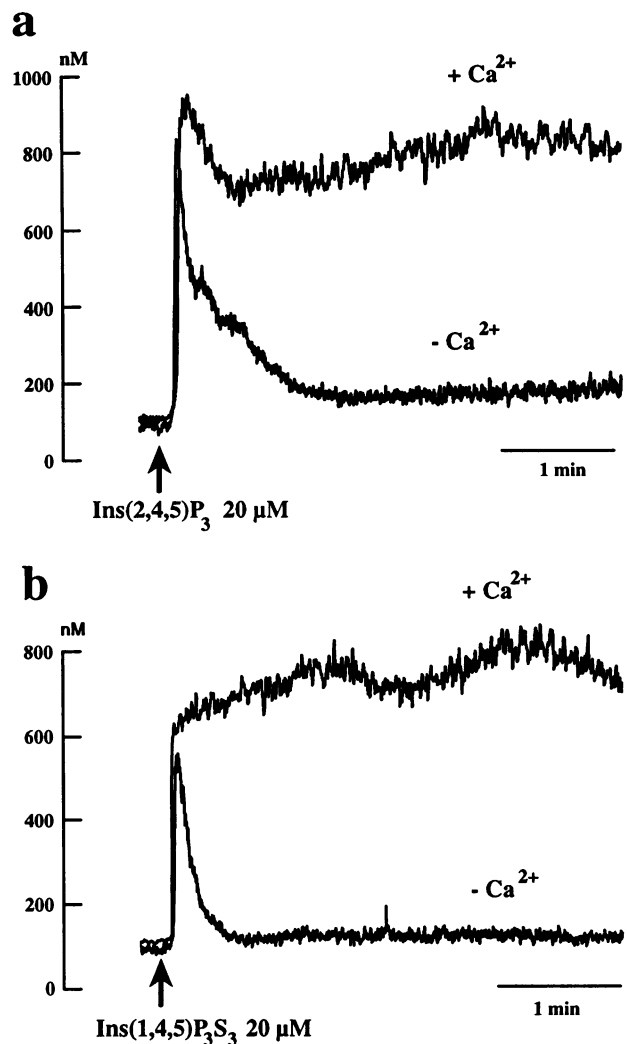
Stimulus	Concentration	$\text{Ca}^{2+}_{\text{ext}}$	Basal $[\text{Ca}^{2+}]_i$	Peak $[\text{Ca}^{2+}]_i$	120-s $[\text{Ca}^{2+}]_i$	Lag time	Initial slope	Responding cells	Tested cells
	$\mu\text{M}$			nM		s	nM/s	%	n
$\text{Ins}(1,4,5)\text{P}_3$	1	+	100±20	110±20	110±20	>30	<1	0	4
$\text{Ins}(1,4,5)\text{P}_3$	2	+	120±15	390±60	260±50	11±1	30±10	78	18
$\text{Ins}(1,4,5)\text{P}_3$	5	+	100±20	460±40	240±10	7±2	80±20	100	4
$\text{Ins}(1,4,5)\text{P}_3$	10	+	130±10	680±60	570±70	4±1	220±20	100	24
$\text{Ins}(1,4,5)\text{P}_3$	10	–	130±30	610±140	190±50	5±1	210±40	100	5
$\text{Ins}(2,4,5)\text{P}_3$	20	+	120±20	640±140	630±130	9±2	210±30	100	5
$\text{Ins}(2,4,5)\text{P}_3$	20	–	140±20	520±70	220±40	15±4	200±40	100	7
$\text{Ins}(1,4,5)\text{P}_3\text{S}_3$	20	+	130±20	610±170	390±60	8±1	230±10	80	5
$\text{Ins}(1,4,5)\text{P}_3\text{S}_3$	20	–	110±10	530±70	180±20	14±2	210±20	100	7
$\text{Ins}(1,3,4,5)\text{P}_4$	8–20	+	110±10	220±40	170±10	>30	<1	11	19
$\text{Ins}(1,3,4,5)\text{P}_4$	8–20	–	90±10	130±40	130±40	>30	<1	10	10
$\text{Ins}(1,4,5)\text{P}_3 + \text{Ins}(1,3,4,5)\text{P}_4$	2 + 20	+	110±10	370±50	270±30	7±1	40±10	76	17
$\text{Ins}(3,4,5,6)\text{P}_4$	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  $\text{Ca}^{2+}$ -containing or  $\text{Ca}^{2+}$ -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  $[\text{Ca}^{2+}]_i$ , measured before application of the stimulus; (b) the peak  $[\text{Ca}^{2+}]_i$ , measured during the 30-s interval, after the application of the stimulus; (c) the delayed phase of the  $[\text{Ca}^{2+}]_i$  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  $[\text{Ca}^{2+}]_i$  increase; (e) the slope of the initial 10 s of the  $[\text{Ca}^{2+}]_i$  increase; (f) the number of responding cells (defined as cells showing an increase in  $[\text{Ca}^{2+}]_i > 100$  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.



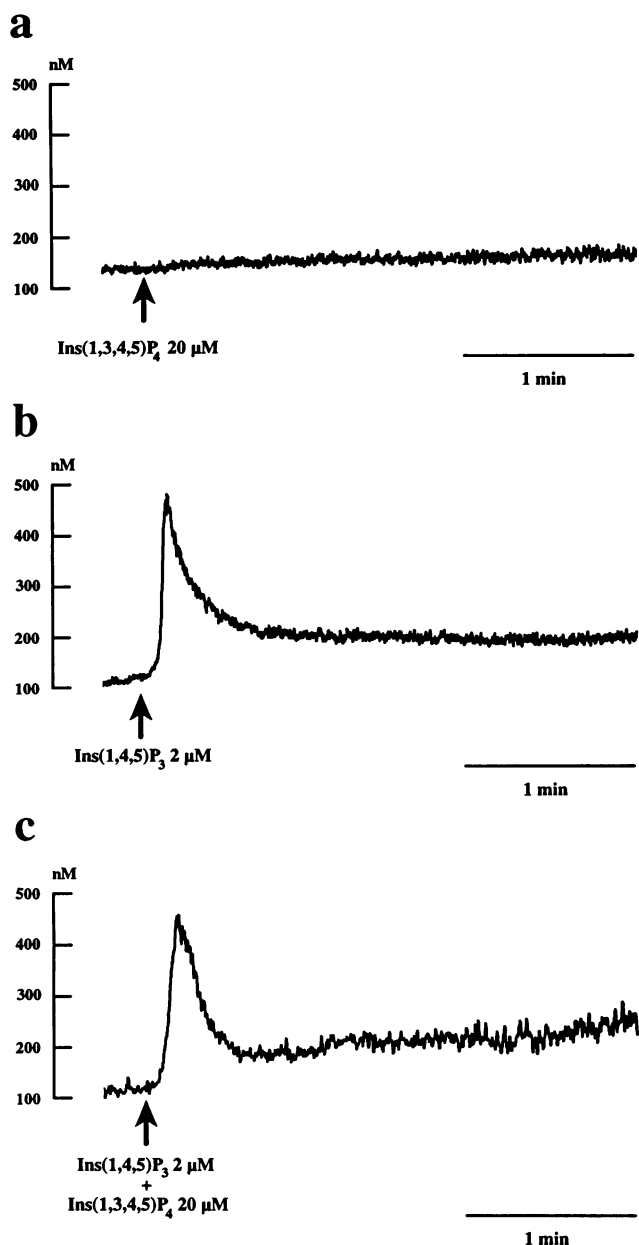
**Figure 3.** Effects of extracellular  $Ca^{2+}$ ,  $Ni^{2+}$ , and plasma membrane depolarization on the  $[Ca^{2+}]_i$  changes induced by  $Ins(1,4,5)P_3$ . Indo-1-loaded HL-60 cells were voltage-clamped in the whole cell configuration. The pipette contained  $10 \mu M$   $Ins(1,4,5)P_3$ ; arrows 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  $Ca^{2+}$ .

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



**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  $20 \mu M$   $Ins(2,4,5)P_3$  in the presence (upper trace) and absence (lower trace) of extracellular  $Ca^{2+}$ . (b)  $[Ca^{2+}]_i$  response to  $20 \mu M$   $Ins(1,4,5)P_3S_3$  in the presence (upper trace) and absence (lower trace) of extracellular  $Ca^{2+}$ . Traces have been superimposed for comparison.

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  $10 \mu M$   $Ins(1,4,5)P_3$ . 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  $\mu M$  Ins(1,3,4,5) $P_4$ . (b)  $[Ca^{2+}]_i$  response to 2  $\mu M$  Ins(1,4,5) $P_3$ . (c)  $[Ca^{2+}]_i$  response to the combination of 2  $\mu M$  Ins(1,4,5) $P_3$  and 20  $\mu M$  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<sup>3</sup> 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.

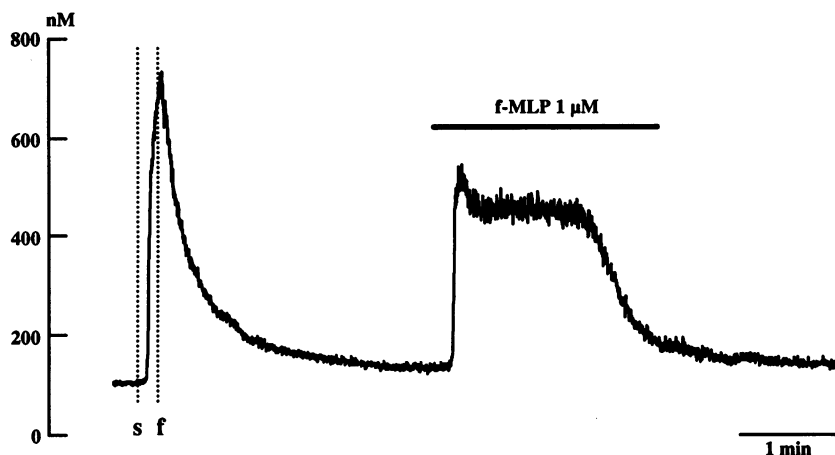


Figure 6. Effects of an imposed rapid  $[Ca^{2+}]_i$  transient on  $[Ca^{2+}]_i$  in an adherent HL-60 cell. An Indo-1-loaded HL-60 cell was allowed to adhere on a glass coverslip in the presence of extracellular  $Ca^{2+}$ . The cell was transiently impaled with a micropipette ( $s$  = start,  $f$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

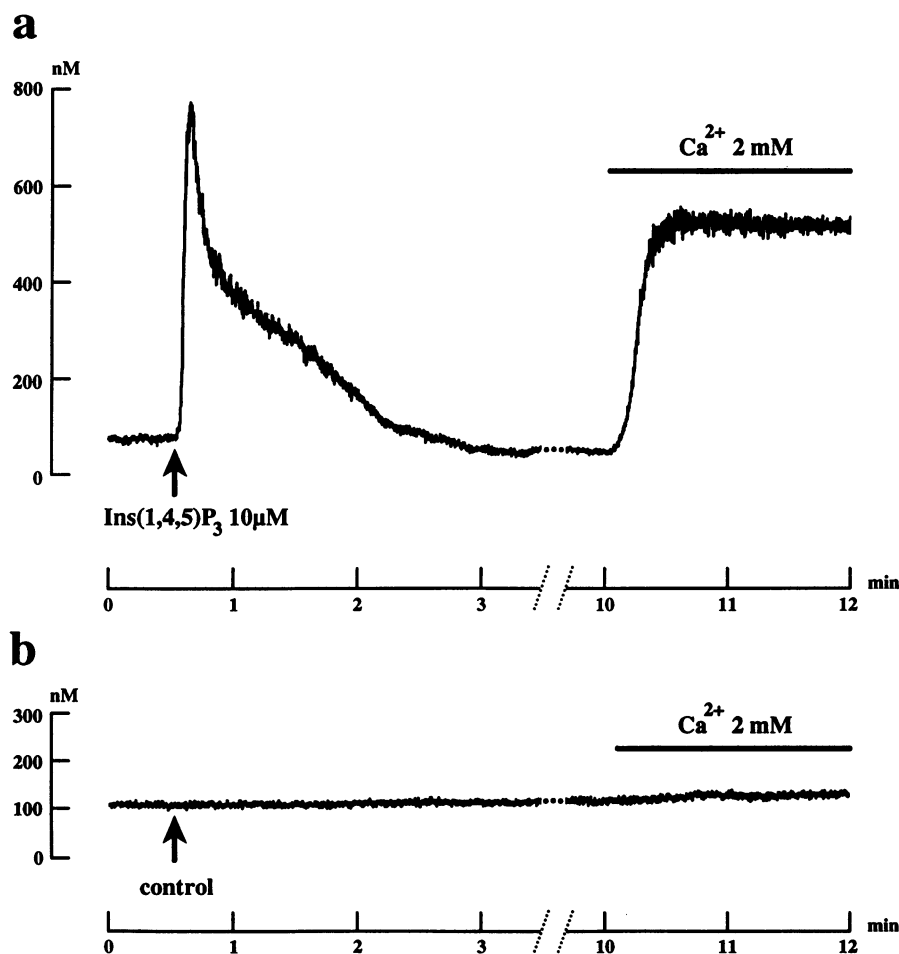
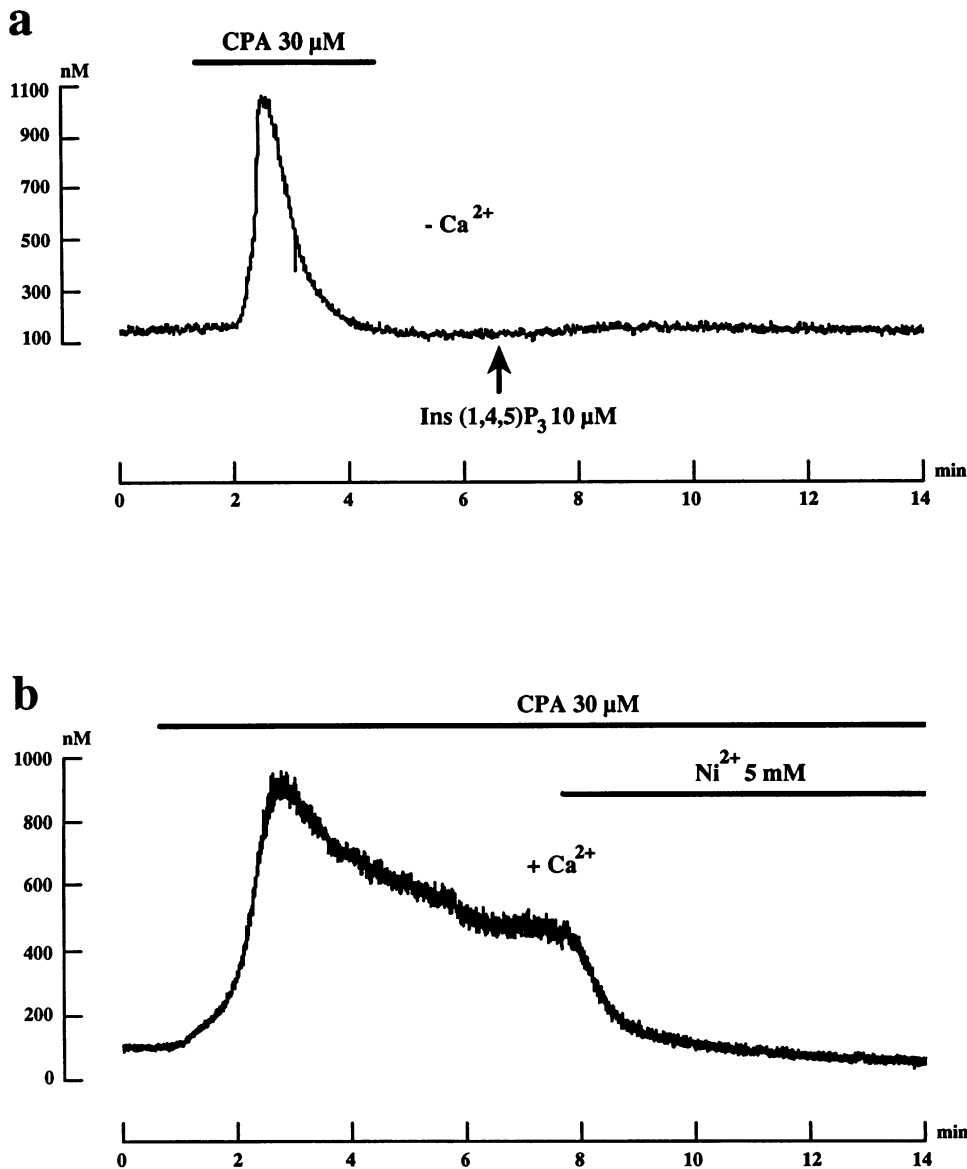


Figure 7. Effects on  $[Ca^{2+}]_i$  of extracellular  $Ca^{2+}$  addition to cells microperfused with Ins(1,4,5) $P_3$  in the absence of extracellular  $Ca^{2+}$ . Indo-1-loaded HL-60 cells were voltage-clamped at  $-60$  mV in the absence of extracellular  $Ca^{2+}$ . The pipette contained either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

strates 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  $\sim 1 \mu$ M and the half-maximal concentration  $\sim 5 \mu$ M. 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  $0.3 \mu$ M in unstimulated HL-60 cells, and  $2.2 \mu$ M 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 ( $1$ – $10 \mu$ M) are within a biologically

relevant range. Similar to results from studies performed in other cell types (21, 27, 28), the  $\text{Ins}(1,4,5)\text{P}_3$  concentrations necessary in the patch pipette for maximal effects on  $[\text{Ca}^{2+}]_i$  were around 10  $\mu\text{M}$ , i.e.,  $\sim 5$ –10 times higher than half-maximal concentrations for  $\text{Ca}^{2+}$  release in permeabilized cells or in homogenates (4). Two explanations seem to be possible for these differing results. Cells might be less sensitive to  $\text{Ins}(1,4,5)\text{P}_3$  under the whole-cell patch clamp condition. This is conceivable, as the  $\text{Ins}(1,4,5)\text{P}_3$  binding to its receptor depends on many cofactors, such as pH, and concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and ATP (29). Alternatively, however, the effective  $\text{Ins}(1,4,5)\text{P}_3$  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  $\text{Ins}(1,4,5)\text{P}_3$  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  $\text{Ins}(1,4,5)\text{P}_3$  analogs are only double of the respective concentrations of  $\text{Ins}(1,4,5)\text{P}_3$ , while in permeabilized cells or in homogenates these compounds are usually 5–10 times less potent than  $\text{Ins}(1,4,5)\text{P}_3$ . This observation indirectly suggests that  $\text{Ins}(1,4,5)\text{P}_3$  metabolism may play a role for the relatively low  $\text{Ins}(1,4,5)\text{P}_3$  sensitivity of whole cell patch-clamped cells.

How might  $\text{Ins}(1,4,5)\text{P}_3$  induce  $\text{Ca}^{2+}$  influx? So far, four major hypotheses have received most attention: First, inositol tetrakisphosphate-activated  $\text{Ca}^{2+}$  influx: According to this hypothesis,  $\text{Ca}^{2+}$  influx is due to the combined action of  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ins}(1,3,4,5)\text{P}_4$  (9, 16, 17). Indeed, in HL-60 cells, there is an excellent temporal correlation between  $\text{Ins}(1,3,4,5)\text{P}_4$  generation and  $\text{Ca}^{2+}$  influx (3). Here we show that microperfusion of  $\text{Ins}(1,3,4,5)\text{P}_4$  is neither necessary nor sufficient for the induction of  $\text{Ca}^{2+}$  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  $\text{Ins}(1,3,4,5)\text{P}_4$  on submaximal concentrations of  $\text{Ins}(1,4,5)\text{P}_3$  could be found. Thus, our results are not compatible with the inositol tetrakisphosphate hypothesis. Second,  $[\text{Ca}^{2+}]_i$ -activated  $\text{Ca}^{2+}$  influx: In this hypothesis,  $\text{Ca}^{2+}$  influx is activated by cytosolic  $\text{Ca}^{2+}$  ions. Neutrophils possess non-selective  $\text{Ca}^{2+}$ -activated cation channels that can conduct  $\text{Ca}^{2+}$ . The activation of these channels by the initial  $\text{Ca}^{2+}$  release from internal stores has been proposed to mediate  $\text{Ca}^{2+}$  influx (8). In this study, we could not detect, in HL-60 cells, a sustained  $\text{Ca}^{2+}$  influx in response to  $[\text{Ca}^{2+}]_i$  increases that were induced independently from emptying of intracellular  $\text{Ca}^{2+}$  stores. In addition,  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  influx could be temporarily dissociated (Fig. 7). Thus, our results argue against a central role of a  $[\text{Ca}^{2+}]_i$  elevation in the mediation of  $\text{Ca}^{2+}$  influx. Third, directly  $\text{Ins}(1,4,5)\text{P}_3$ -mediated  $\text{Ca}^{2+}$  influx and fourth, capacitative  $\text{Ca}^{2+}$  influx: Having excluded a role for  $\text{Ins}(1,3,4,5)\text{P}_4$  and  $[\text{Ca}^{2+}]_i$ , the most likely explanations for the induction of  $\text{Ca}^{2+}$  influx by  $\text{Ins}(1,4,5)\text{P}_3$  are either a direct effect of  $\text{Ins}(1,4,5)\text{P}_3$  on the  $\text{Ca}^{2+}$  influx pathway in the plasma membrane, or mediation of  $\text{Ca}^{2+}$  influx by the filling state of the intracellular  $\text{Ca}^{2+}$  store. As the  $\text{Ca}^{2+}$ -ATPase inhibitor, cyclopiazonic acid, induces  $\text{Ca}^{2+}$  influx without raising levels of  $\text{Ins}(1,4,5)\text{P}_3$  (20), our results strongly suggest that the emptying of intracellular  $\text{Ca}^{2+}$  pools by  $\text{Ins}(1,4,5)\text{P}_3$  is a factor that

regulates the  $\text{Ca}^{2+}$  permeability of the plasma membrane (10, 11). However, we cannot exclude an additional direct effect of  $\text{Ins}(1,4,5)\text{P}_3$  on the  $\text{Ca}^{2+}$  influx pathway. Recent studies in hepatocytes support such a concept of a dual action of  $\text{Ins}(1,4,5)\text{P}_3$  (27). Thus, further studies are necessary to determine whether the capacitative mechanism of  $\text{Ca}^{2+}$  influx is the only, or one of several mechanisms that regulate receptor-mediated  $\text{Ca}^{2+}$  influx in myeloid cells.

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