Cyclopiazonic Acid Depletes Intracellular Ca\(^{2+}\) Stores and Activates an Influx Pathway for Divalent Cations in HL-60 Cells*

(Received for publication, June 17, 1991)

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The filling state of intracellular Ca\(^{2+}\) stores has been proposed to regulate Ca\(^{2+}\) influx across the plasma membrane in a variety of tissues. To test this hypothesis, we have used three structurally unrelated inhibitors of the Ca\(^{2+}\)-ATPase of intracellular Ca\(^{2+}\) stores and investigated their effect on Ca\(^{2+}\) homeostasis in HL-60 cells. Without increasing cellular inositol (1,4,5)trisphosphate levels, all three inhibitors (cyclopiazonic acid, thapsigargin, and 2,5-Di-tert-butylhydroquinone) released Ca\(^{2+}\) from intracellular stores, resulting in total depletion of agonist-sensitive Ca\(^{2+}\) stores. The Ca\(^{2+}\) release was relatively slow with a lag time of 5 s and a time to peak of 60 s. After a lag time of approximately 15 s, all three Ca\(^{2+}\)-ATPase inhibitors activated a pathway for divalent cation influx across the plasma membrane. At a given concentration of an inhibitor, the plasma membrane permeability for divalent cations closely correlated with the extent of depletion of Ca\(^{2+}\) stores. The influx pathway activated by Ca\(^{2+}\)-ATPase inhibitors conducted Ca\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), and Ba\(^{2+}\) and was blocked, at similar concentrations, by La\(^{3+}\), Ni\(^{2+}\), and Cd\(^{2+}\), as well as by the imidazole derivate SK&F 96365. The divalent cation influx in response to the chemotactic peptide fMLP had the same characteristics, suggesting a common pathway for Ca\(^{2+}\) entry. Our results support the idea that the filling state of intracellular Ca\(^{2+}\) stores regulates Ca\(^{2+}\) influx in HL-60 cells.

 Activation of myeloid cells by cell surface agonists causes Ca\(^{2+}\) release from internal stores and Ca\(^{2+}\) influx across the plasma membrane (1-3). It has been clearly demonstrated that the release of Ca\(^{2+}\) from internal stores is mediated by inositol (1,4,5)trisphosphate (Ins(1,4,5)P\(_3\)) (4-7). In contrast, the mechanism underlying receptor-mediated Ca\(^{2+}\) entry is poorly understood. In myeloid cells, it has been proposed that

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\text{Ins(1,3,4,5)P}_2 \text{ might play a role in the regulation of Ca}^{2+} \text{ entry (3) or that the initial [Ca}^{2+}] \text{, increase, due to Ca}^{2+} \text{ release from intracellular stores, activates a Ca}^{2+} \text{-conductive plasma membrane channel (8). However, subsequently it has been shown that [Ca}^{2+}] \text{, increases are neither necessary nor sufficient to induce Ca}^{2+} \text{ influx (9). Studies in various other cell types have suggested a correlation between depletion of intracellular Ca}^{2+} \text{ pools and Ca}^{2+} \text{ entry across the plasma membrane (10-13). Thus, according to the so-called capacitative model of Ca}^{2+} \text{ entry, the filling state of the intracellular Ca}^{2+} \text{ stores determines the Ca}^{2+} \text{ permeability of the plasma membrane. Recently, three inhibitors of Ca}^{2+}\text{-ATPases of intracellular Ca}^{2+} \text{ pools have been described, thapsigargin (TG), cyclopiazonic acid (CPA), and 2,5-Di-tert-butylhydroquinone}^2 \text{(DBHQ) (14-16). All three, structurally unrelated, compounds inhibit Ca}^{2+} \text{ uptake in the Ins(1,4,5)P}_3\text{-sensitive Ca}^{2+} \text{ pool in HL-60 homogenates.}^3 \]

In accordance with the capacitative model of Ca\(^{2+}\) entry, thapsigargin added to intact cells not only depletes Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) stores, but also induces Ca\(^{2+}\) influx in a variety of cellular systems (for synopsis see Table 2 of Ref. 14). In contrast, studies in hepatocytes did not observe Ca\(^{2+}\) influx in response to DBHQ, despite depletion of Ins(1,4,5)P\(_3\)-sensitive intracellular Ca\(^{2+}\) stores by the compound (17-19). The Ca\(^{2+}\)-ATPase inhibitor CPA has so far not been tested for its effects on Ca\(^{2+}\) release or Ca\(^{2+}\) influx in intact cells.

If, in HL-60 cells, the capacitative model of Ca\(^{2+}\) entry reflects indeed the mechanism of receptor-mediated Ca\(^{2+}\) influx, experimental analysis should confirm the following theoretical predictions. 1) All Ca\(^{2+}\)-ATPase inhibitors that efficiently empty intracellular Ca\(^{2+}\) stores should induce Ca\(^{2+}\) influx with dose responses similar to Ca\(^{2+}\) release from stores. 2) Emptying of the Ca\(^{2+}\) stores should precede Ca\(^{2+}\) influx. 3) Various cations should, in a similar manner, either permeate or block the Ca\(^{2+}\) influx pathway activated by fMLP and the three inhibitors.

In this study we demonstrate that all three Ca\(^{2+}\)-ATPase inhibitors induced Ca\(^{2+}\) release from Ins(1,4,5)P\(_3\)-sensitive Ca\(^{2+}\) stores and promoted Ca\(^{2+}\) influx across the plasma membrane in HL-60 cells. Emptying of the stores preceded Ca\(^{2+}\) influx. The influx pathway activated by the receptor agonist fMLP and by the Ca\(^{2+}\)-ATPase inhibitors conducted Ca\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), and Ba\(^{2+}\). The sensitivity of the influx pathway to block by La\(^{3+}\), Ni\(^{2+}\), and Cd\(^{2+}\) was identical,

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*This research was supported by Grant 32-30161.90 from the Swiss National Foundation for Scientific Research and by a grant from the Swiss Ligue against Rheumatism. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: Ins(1,4,5)P\(_3\), inositol(1,4,5)trisphosphate; TG, thapsigargin; DBHQ, 2,5-Di-tert-butylhydroquinone; CPA, cyclopiazonic acid; fMLP, N-formyl-l-methionyl-l-leucyl-l-phenylalanine; DTPA, diethylenetriamine pentaacetic acid; EGTa, [ethylenebis(oxyethylenenitrito)]etraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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2 The compound referred to in the publications of Orrenius and coworkers (16) as 2,5-Di(tert-butyl)-1,4-benzoquinone is commercialized under the name 2,5-Di-tert-butylhydroquinone. We prefer to use the latter designation as it, albeit shorter, unequivocally describes the structure of the compound.

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whether it was activated by fMLP or by the Ca\textsuperscript{2+}-ATPase inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**—CPA, fMLP, and DTPA were purchased from Sigma, DBHQ from Fluka (Buchs, Switzerland), TG and fetal calf serum from Gibco (Paisley, Scotland), fura-2 acetoxy-methylene (fura-2/AM) and fura-2 free acid from Molecular Probes (Eugene, OR). SK&F 96365 was provided by Dr. J. Merritt, Smithkline Beecham (Rahway, NJ). All other chemicals were of analytical grade and were obtained from Sigma, Merck, and Fluka. The medium referred to as "Ca\textsuperscript{2+}" medium contained (in mM): NaCl 138; KCl 6; CaCl\textsubscript{2} 1.1; MgCl\textsubscript{2} 1; EGTA 0.1; glucose 20; HEPES 20. The "Ca\textsuperscript{2+}"-free medium had the same ionic composition; however, it contained 1 mM EGTA, and CaCl\textsubscript{2} was omitted. For experiments with La\textsuperscript{3+}, it was necessary to reduce the concentration of HEPES to 10 mM in order to avoid precipitation.

**Culture of HL-60 Cells**—Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (5 units/ml) and streptomycin (50 \mu g/ml). The cells were passaged twice every week and differentiated by adding Me\textsubscript{2}SO (final concentration 1.3% v/v) to the cell suspension 7 days before experiments.

**Ca\textsuperscript{2+}** Measurements—[Ca\textsuperscript{2+}], was measured with the fluorescent Ca\textsuperscript{2+} indicator fura-2 as described previously (3). Cells (2 x 10\textsuperscript{5} cells/suspension) were loaded for 45 min at 37 °C with 2 \mu M fura-2/AM, then diluted to 10\textsuperscript{5} cells/ml and kept on ice. Just before use, 1 ml of a sample of 5 x 10\textsuperscript{5} cells and 0.1% bovine serum albumin was centrifuged and resuspended in the indicated medium. Fluorescence measurements were performed on a Perkin-Elmer fluorimeter (LS3, Perkin-Elmer Cetus), thermostated at 37 °C. Excitation and emission wavelengths were 340 and 505 nm, respectively. Calibration of fura-2 fluorescence was performed as described previously (1). In additional experiments, similar results were obtained with excitation ratio measurements at 340/380 nm.

**Assessment of Mn\textsuperscript{2+} Influx**—Entry of Mn\textsuperscript{2+} into cells was measured using the fura-2 fluorescence quenching technique. Cells were loaded as for [Ca\textsuperscript{2+}], and fluorescence monitored in a Ca\textsuperscript{2+}-containing medium at the Ca\textsuperscript{2+}-induced excitation wavelength 360 nm. Then, Mn\textsuperscript{2+} (0.5 mM) was added and its entry into cells measured as rate of fluorescence decrease during the first 2 min following its addition. We routinely added the heavy metal chelator DTPA (2 mM) at the end of an experiment to measure the percentage of fluorescence decrease due to extracellular fura-2 quenching. It accounted for less than 5% of the total fluorescence quenched by Mn\textsuperscript{2+} and was subtracted before calculation of the rate of Mn\textsuperscript{2+} entry. No increase in extracellular fura-2 was observed after exposure of cells to the Ca\textsuperscript{2+}-ATPase inhibitors, demonstrating that these compounds were not cytotoxic.

**Assessment of Plasma Membrane Permeability for Other Divalent Cations**—Activation of the influx pathway for divalent cations was assessed using a procedure analogous to the Mn\textsuperscript{2+} quenching technique. For the measures of cations/fura-2 free acid fluorescence, fura-2 free acid (10 \mu M) was dissolved in a Ca\textsuperscript{2+}-containing medium and the fluorescence measured at the Ca\textsuperscript{2+}-induced excitation wavelength of 360 nm. Similar to Mn\textsuperscript{2+}, the divalent cation Co\textsuperscript{2+} quenched the fluorescence of fura-2, while Zn\textsuperscript{2+} and Ba\textsuperscript{2+} increased fluorescence. To study the entry of the respective ions into intact cells, fluorescence of fura-2 loaded cells was measured at 360 nm. Co\textsuperscript{2+} (0.1 mM), Zn\textsuperscript{2+} (0.1 mM), or Ba\textsuperscript{2+} (5 mM) were added, and divalent cation entry was detected as fluorescence decrease (Co\textsuperscript{2+}) or increase (Zn\textsuperscript{2+}, Ba\textsuperscript{2+}). DTPA (2-20 mM) was added at the end of the experiment to estimate the percentage of the fluorescence changes due to extracellular fura-2. Sensitivity of the Co\textsuperscript{2+}, Zn\textsuperscript{2+}, and Ba\textsuperscript{2+} entry pathway to inorganic Ca\textsuperscript{2+} channel blockers was assessed by preincubating the cells with Ni\textsuperscript{2+} (10 mM), Cd\textsuperscript{2+} (10 mM), or La\textsuperscript{3+} (100 \mu M).

**Inositol Phosphates Measurements**—Inositol phosphate concentrations were measured as described previously (20).

**Data Recording and Analysis**—Fluorescence values are given as percent of total Ca\textsuperscript{2+}-dependent fura-2 fluorescence (for calibration, see Ref. 1). Fluorescence traces were digitized using a 12-bit Multi-1\textsuperscript{1/2} A/D converter (Acqiris, SICMU, Geneva, Switzerland). Data were recorded at a rate of 50 Hz, filtered with a moving average procedure, and stored on an AT computer. Statistical analysis and fitting procedures were done with SigmaPlot software (Jandel Scientific, Corte Madera, CA). Values are given as mean (range) of 4 to 25 different experiments performed on at least 2 different days.

**RESULTS**

Effects of CPA on [Ca\textsuperscript{2+}], in Ca\textsuperscript{2+}-containing and Ca\textsuperscript{2+}-free Medium—When added to fura-2-loaded HL-60 cells in a Ca\textsuperscript{2+}-containing medium, CPA caused a concentration-dependent increase in [Ca\textsuperscript{2+}], (Fig. 1A), as assessed by an increase in fura-2 fluorescence. The Ca\textsuperscript{2+}-ATPase inhibitors TG and DBHQ caused a similar concentration-dependent increase in [Ca\textsuperscript{2+}]. Half-maximal [Ca\textsuperscript{2+}] elevations were obtained with 3.2 (2-10) \mu M CPA, 4.2 (3-8) nM TG, and 2.4 (1-5) \mu M DBHQ (n = 6-9, mean (range)). The maximal effect was obtained with 30 \mu M CPA, 30 nM TG, and 30 \mu M DBHQ (Fig. 1B). The maximal fluorescence increase elicited by any of the three Ca\textsuperscript{2+}-ATPase inhibitors was approximately 1.5-fold higher than the peak observed in response to the Ca\textsuperscript{2+}-mobilizing agonist fMLP (47.3 (37-58)%), n = 25 versus 33.2 (21-40)%), n = 15, mean (range).

In cells suspended in a Ca\textsuperscript{2+}-containing medium, addition of CPA, TG, or DBHQ 1 min after administration of fMLP caused an additional increase in [Ca\textsuperscript{2+}], while addition of fMLP after a maximal concentration of any of the inhibitors did not further increase [Ca\textsuperscript{2+}], (data not shown).

The fMLP-induced [Ca\textsuperscript{2+}], increase was transient, while the [Ca\textsuperscript{2+}], increases caused by the three inhibitors were sustained for the time of observation (up to 20 min). The time to plateau of the [Ca\textsuperscript{2+}] increase in response to a maximally effective concentration of CPA was 260 (120-360) s (n = 25, mean (range)) and thus markedly longer than the time to peak observed after stimulation with fMLP (19 (15-30) s, n = 15, mean (range)).

CPA induced [Ca\textsuperscript{2+}], elevations when cells were suspended in a Ca\textsuperscript{2+}-free medium, demonstrating that it releases Ca\textsuperscript{2+} from intracellular stores (Fig. 2A). Ca\textsuperscript{2+} release due to CPA was slow compared to fMLP-induced Ca\textsuperscript{2+} release (time to peak 55 (40-80) s, n = 12 versus 13 (12-16) s, n = 15, mean (range)).

**FIG. 1.** The Ca\textsuperscript{2+}-ATPase inhibitor CPA causes a sustained [Ca\textsuperscript{2+}], increase in the presence of extracellular Ca\textsuperscript{2+}. A. fura-2-loaded cells were suspended in a Ca\textsuperscript{2+}-containing buffer and exposed either to a maximal concentration of the agonist fMLP (left panel) or to various concentrations of CPA (right panel). The traces on the right panel have been superimposed for comparison. Addition of fMLP caused a transient [Ca\textsuperscript{2+}], increase, while addition of the Ca\textsuperscript{2+}-ATPase inhibitor caused a sustained [Ca\textsuperscript{2+}], increase. B. Ca\textsuperscript{2+} rises observed with increasing concentrations of CPA and of the structurally unrelated Ca\textsuperscript{2+}-ATPase inhibitors thapsigargin and 2,5-Di-tert-butydroquinone. The changes in fluorescence are expressed as % of total Ca\textsuperscript{2+}-dependent fura-2 fluorescence. Open triangles, thapsigargin; filled squares, cyclopiazonic acid; filled triangles, 2,5-Di-tert-butyldroquinone. Data are from a single representative experiment out of six.
but was comparable to the Ca²⁺ release produced by TG and DBHQ (not shown). The dose-response curves obtained in Ca²⁺-free medium were similar to those obtained in Ca²⁺ medium (Figs. 2B and 1B, respectively). However the [Ca²⁺]i increase in Ca²⁺-free medium was only transient, raising the possibility that the prolonged Ca²⁺ increase observed in Ca²⁺ medium was due to Ca²⁺ influx across the plasma membrane.

Source of the Ca²⁺ Mobilized by the Inhibitors—Addition of maximally effective concentrations of CPA before exposure to fMLP in Ca²⁺-free medium, entirely abolished fMLP-induced Ca²⁺ release (Fig. 3A, right trace), presumably by depleting the Ins(1,4,5)P³-sensitive Ca²⁺ store. In contrast, after addition of a maximally effective concentrations of fMLP, CPA was still able to release Ca²⁺ (Fig. 3A, left trace). The dose-inhibition curves of fMLP-induced Ca²⁺ release by the three inhibitors (Fig. 3B) were similar to the dose responses for induction of [Ca²⁺]i increase; half-maximal inhibition was obtained with 30 μM CPA, 30 nM TG, and 2.6 (1.5-2.8) μM DBHQ (n = 4-6, mean (range)). 100% inhibition was achieved with 30 μM CPA, 30 nM TG, and 30 μM DBHQ. To estimate which percentage of total intracellular Ca²⁺ stores was depleted by the Ca²⁺-ATPase inhibitors, we added, in the absence of extracellular Ca²⁺, the Ca²⁺ ionophore ionomycin to cells preincubated with or without the Ca²⁺-ATPase inhibitors. After exposure to a maximal dose of CPA, TG, or DBHQ, the amount of ionomycin releasable Ca²⁺ was less than 10% of the amount of ionomycin-releasable Ca²⁺ in the absence of inhibitors (data not shown). These results show that most of the intracellular Ca²⁺ stores can be depleted by any of the three Ca²⁺-ATPase inhibitors while the pool that is released by fMLP is only a part of the total pool that is loaded by CPA-, TG-, and DBHQ-sensitive Ca²⁺-ATPase.

Induction of Ca²⁺ Influx by CPA, TG, and DBHQ—As the sustained increase in [Ca²⁺]i upon exposure to CPA, TG, and DBHQ was not observed in the absence of extracellular Ca²⁺, we next investigated whether it was due to Ca²⁺ influx. The divalent cation Mn²⁺ has been shown to permeate through the neutrophil Ca²⁺ influx pathway activated by chemotactic peptides (2, 3, 9). Its intracellular presence can be easily detected as it binds with a high affinity to Ca²⁺-sensitive fluorescent dyes and thereby quenches their fluorescence. If the Ca²⁺ influx pathway activated by the Ca²⁺-ATPase inhibitors is identical to the pathway activated by chemotactic peptides, it should also conduct Mn²⁺. We therefore compared the rate at which Mn²⁺ quenched the fura-2 fluorescence in cells preincubated with either CPA, TG, DBHQ, or the agonist fMLP. The experiments were performed at an excitation wavelength of 360 nm, a wavelength at which the fluorescence of fura-2 is independent of [Ca²⁺]. When added to control cells, Mn²⁺ caused a slow and continuous decrease in fluorescence. After exposure of cells to a maximal dose of fMLP for 1 min, this basal quenching rate was 2-fold increased (1.9 (1.8-2.1), n = 25, mean (range)) (see also Ref. 3). CPA (0.3-30 μM) caused a concentration-dependent increase of the rate of fura-2 quenching (Fig. 4A). The dose-response curves for CPA, TG, and DBHQ were similar to the dose-response curves for [Ca²⁺], mobilization (Fig. 4B). The increase in fura-2 quenching by Mn²⁺ with maximal concentrations of Ca²⁺-ATPase inhibitors was 2.5- (2.2-2.7), 3.2- (2.8-3.5), and 2.4- (2.1-2.8) fold of basal for CPA, TG, and DBHQ, respectively (n = 11-14, mean (range)). Thus, maximal concentrations of CPA, TG, and DBHQ caused more Mn²⁺ influx than 10⁻⁶ M fMLP. The fluorescence quenching by Mn²⁺ was not reversed by addition of the non-permeant heavy metal chelator DTPA (2 mM) and was therefore due to Mn²⁺ influx and not to quenching of extracellular fura-2. In a different set of experiments, Ca²⁺ instead of Mn²⁺ was added to cells after incubation with the Ca²⁺-ATPase inhibitors in Ca²⁺-free medium. Immediate increases in fura-2 fluorescence with dose-response curves similar to those for Mn²⁺ were observed (data not shown).

Permeation and Block by Divalent Cations of the Influx Pathway Activated by CPA and fMLP—We next investigated the sensitivity to inorganic channel blockers of the influx pathway activated by the Ca²⁺-ATPase inhibitors and fMLP. Cells were preincubated with Ni²⁺, Cd²⁺, or La³⁺ for 1 min...
Ca\textsuperscript{2+}-ATPase Inhibitors and Ca\textsuperscript{2+} Influx

**Sensitivity of the Influx Pathway to SK&F 96365**—The imidazole derivative SK&F 96365 has been recently described as blocking receptor-mediated Ca\textsuperscript{2+} influx in several cellular systems, including neutrophils (21). We have therefore studied the effect of this compound on the CPA-induced Mn\textsuperscript{2+} influx. 10 \mu M SK&F 96365 inhibited CPA-induced Mn\textsuperscript{2+} influx by 82 (75–93)% and fMLP-induced Mn\textsuperscript{2+} influx by 80 (72–89)% (n = 4, mean (range)). Similar results were obtained with TG and DBHQ (not shown).

**Time Course of Activation of the Influx Pathway for Divalent Cations**—If depletion of intracellular stores is a signal for Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+} release should precede influx. To determine the activation kinetics of the influx pathway for divalent cations induced by the Ca\textsuperscript{2+}-ATPase inhibitors, we determined fura-2 fluorescence quenching by Mn\textsuperscript{2+} at different times after exposure of cells to a maximal dose of CPA (Fig. 6A). The time course of Mn\textsuperscript{2+} influx induced by CPA differed from that of Mn\textsuperscript{2+} influx triggered by fMLP (see also Ref. 3). In cells exposed to fMLP, the rate of fura-2 quenching increased after 10 s, was maximal after 30 s, and returned to basal levels after 6 min. In cells preincubated with CPA, the rate of fura-2 quenching started to increase after a lag period of approximately 15 s, reached its maximum at 1 min, and remained elevated for the period of observation (Fig. 6B). Thus, the Ca\textsuperscript{2+}-ATPase inhibitors caused a continuous increase in plasma membrane permeability for divalent cations, while fMLP produced only a transient increase. In contrast to the delayed onset of Mn\textsuperscript{2+} influx (>15 s, n = 3), the onset of Ca\textsuperscript{2+} release from internal pools was more rapid. The Ca\textsuperscript{2+} rise in response to CPA started after 4.3 (3–5) s (n = 7, mean (range)) when assessed in a Ca\textsuperscript{2+}-free medium (see also Fig. 6B) and 3.8 (2.5–5) s (n = 6, mean (range)) when assessed in a Ca\textsuperscript{2+}-containing medium. While at 15 s no Mn\textsuperscript{2+} influx was observed, Ca\textsuperscript{2+} release, i.e. the increase of fura-2 fluorescence in Ca\textsuperscript{2+}-free medium, was already 11.8 (8–15)% (n = 7, mean (range)) i.e. more than half of total, at this time point. The increase of fura-2 fluorescence in Ca\textsuperscript{2+}-containing medium was 12.6 (7–19)% (n = 6, mean (range)) at 15 s, i.e. identical to the one observed in Ca\textsuperscript{2+}-free medium. Similar results were obtained with the two other inhibitors (data not shown). Thus, by two criteria Ca\textsuperscript{2+} release preceded Ca\textsuperscript{2+} influx. (i) Lag time and initial rate of [Ca\textsuperscript{2+}]\textsuperscript{i} rise were identical in Ca\textsuperscript{2+}-containing and Ca\textsuperscript{2+}-free medium, suggesting that there is no early Ca\textsuperscript{2+} influx component and (ii) Mn\textsuperscript{2+} influx could be observed only with a delay of >10 s after the beginning of Ca\textsuperscript{2+} release. Thus, compatible with the concept of regulation of Ca\textsuperscript{2+} influx by the filling state of intracellular Ca\textsuperscript{2+} stores, Ca\textsuperscript{2+}-ATPase inhibitors first induce Ca\textsuperscript{2+} release and then Ca\textsuperscript{2+} influx.

**Effect of the Inhibitors on Inositol Phosphate Concentration**—Preincubation of cells with CPA, TG, or DBHQ did not increase Ins(1,4,5)P\textsubscript{3} levels, and caused a modest increase in intracellular Ins(1,3,4,5)P\textsubscript{4} and Ins(1,3,4)P\textsubscript{3} concentration, approximately to one-tenth of the Ins(1,3,4,5)P\textsubscript{4} and Ins(1,3,4)P\textsubscript{3} levels reached after stimulation with fMLP (not shown). Thus, although the Ca\textsuperscript{2+}-ATPase inhibitors are more potent than fMLP in the stimulation of Ca\textsuperscript{2+} influx, they do not produce significant changes in inositol phosphate concentrations.

**DISCUSSION**

This study is, to the best of our knowledge, the first demonstration that the Ca\textsuperscript{2+}-ATPase inhibitor CPA depletes intracellular Ca\textsuperscript{2+} stores and activates an influx pathway for divalent cations. The action of CPA is, therefore, in HL-60 cells, indistinguishable from the effects of two other, struc-
Ca²⁺-ATPase inhibitors and Ca²⁺ influx

**FIG. 5.** Ni²⁺, Cd²⁺, and La³⁺ block the CPA-induced Mn²⁺ influx in a concentration-dependent manner. A, fura-2-loaded cells were preincubated with either Cd²⁺, Ni²⁺, or La³⁺ for 1 min and subsequently exposed to CPA (30 μM) and, 1 min later, to Mn²⁺ (0.5 mM). The traces are superimposed for comparison. Increasing the doses of Ni²⁺ or Cd²⁺ reduced the rate of Mn²⁺ quenching. B, fura-2 quenching rate of cells exposed to CPA, fMLP, TG, and DBHQ was plotted against Ni²⁺, Cd²⁺, or La³⁺ concentration. The IC₅₀ of the different cations (in mM for Ni²⁺ and Cd²⁺, in μM for La³⁺) for inhibition of fura-2 quenching by Mn²⁺ are listed below (mean range):

| Cations | CPA | f-MLP | TG | DBHQ |
|---------|-----|-------|----|------|
| Ni²⁺    | 1.1 (0.9-1.2) | 1.1 (0.9-1.3) | 1.5 (0.9-2.0) | 1.2 (1.1-1.3) |
| Cd²⁺    | 2.4 (1.8-3.2) | 2.4 (1.9-2.7) | 2.4 (1.9-3.0) | 1.7 (1.5-1.9) |
| La³⁺    | 53 (24-82)    | 49 (40-62)    | 43 (30-55)     | 44 (30-56)     |

Values are from four to five determinations performed on at least 2 different days. Fitting was performed on the averaged values.

Curiously unrelated inhibitors of the microsomal Ca²⁺-ATPase, DBHQ, and TG. This identical cellular response to three structurally unrelated compounds provides new and strong evidence for the concept of regulation of Ca²⁺ influx by the filling state of the intracellular Ca²⁺ stores.

CPA, TG, and DBHQ have been shown to inhibit ATP-dependent Ca²⁺ uptake in microsomal fractions of HL-60 cells, but the mechanism by which they mobilize Ca²⁺ from Ins(1,4,5)P₃-sensitive stores in intact cells is not understood. It might be due to a relatively high Ca²⁺ permeability of the Ins(1,4,5)P₃-sensitive pool in resting cells which would necessitate a permanent Ca²⁺-ATPase activity to maintain resting [Ca²⁺]. Compatible with this hypothesis, studies in unstimulated HL-60 cells found a basal Ins(1,4,5)P₃ concentration of approximately 300 nM (22). As the half-maximal concentration of Ins(1,4,5)P₃ necessary to release Ca²⁺ from intracellular stores in permeabilized neutrophils is 750 nM (4), these concentrations would be expected to increase moderately the Ca²⁺ permeability of intracellular Ca²⁺ stores. Alternatively, one might consider that the Ca²⁺-ATPase inhibitors alter the Ca²⁺-ATPase structure in a manner that allows reversal of the Ca²⁺ flux through the pump. The latter event may occur at least under certain experimental conditions (for review see Ref. 23).

Ca²⁺ release occurred approximately 4 s, Ca²⁺ influx approximately 15 s after addition of Ca²⁺-ATPase inhibitors. Thus, release of Ca²⁺ from intracellular Ca²⁺ stores preceded Ca²⁺ influx by more than 10 s. This temporal sequence of initial Ca²⁺ release and delayed Ca²⁺ influx is also seen with the chemotactic peptide fMLP (3). However, due to the slower kinetics of the [Ca²⁺], increase, this phenomenon can be more clearly demonstrated with the Ca²⁺-ATPase inhibitors (Fig. 6).

While in HL-60 cells all three Ca²⁺-ATPase inhibitors...
Ca2+-ATPase Inhibitors and Ca2+ Influx

3) None of the compounds released fura-2 from the cytosol of fura-2-loaded HL-60 cells (see "Experimental Procedures"), i.e., they were not cytotoxic.

Although there is now convincing experimental documentation of the induction of Ca2+ influx by TG in all cell types (14), and at least one report of such an effect of DBHQ (24), it is presently not known if the influx pathways activated by physiological agonist and by Ca2+-ATPase inhibitors is the same. In this study we have extensively characterized permeation and block by various cations, as a mean to search for putative differences between the fMLP- and Ca2+-ATPase inhibitor-induced Ca2+ influx pathways. Previous research has shown that both permeation and block by cations differ markedly among various Ca2+ entry pathways and that this type of analysis is therefore a sensitive tool to search for heterogeneity of Ca2+ influx pathways. For example, the Zn2+ impermeability of the influx pathway in a mast cell line (26) distinguishes it from the one found in HL-60 cells. In hepatocytes, a Ni2+ and Cd2+ permeable agonist-induced Ca2+ influx pathway has been described (27), whereas in HL-60 cells both cations block Ca2+ influx (Fig. 5). In the case of voltage-dependent Ca2+ channels, the sensitivity to inorganic channel blockers allows to discriminate T-type, N-type, and L-type Ca2+ channels (19, 28-30). The N-type channel, for example, has been described to be approximately 50-fold more sensitive to Cd2+ than the T-type channel (19, 28-30). The Ca2+ influx pathway activated by both fMLP and Ca2+-ATPase inhibitors is permeant for Mn2+, Co2+, Ba2+, and Zn2+ and blocked by Cd2+, La3+, and Ni2+ at the same concentrations. In addition, the influx pathway activated by fMLP and Ca2+-ATPase inhibitors is equally sensitive to the imidazole derivative SK&F 96365. The Ca2+ influx pathway activated by CPA, fMLP, TG, or DBHQ thus exhibits an indistinguishable profile of block and permeation. These results strongly suggest that the Ca2+-ATPase inhibitors and the chemotactic peptide activate an identical Ca2+ influx pathway in HL-60 cells.

How might Ca2+-ATPase inhibitors stimulate Ca2+ influx? TG, CPA, and DBHQ inhibit Ca2+ uptake into intracellular Ca2+ stores and thereby deplete them. Thus, the filling state of intracellular Ca2+ stores is an obvious candidate for the mediation of the Ca2+-ATPase inhibitor-induced Ca2+ influx. However, during the depletion of the stores by the inhibitors, a [Ca2+]i rise due to Ca2+ release can be observed. For the following reasons it seems unlikely that this Ca2+ rise mediates Ca2+ influx in myeloid cells. (i) A [Ca2+]i rise is not a necessary stimulus for Ca2+ influx: as demonstrated previously (9), Mn2+ influx may occur despite virtual complete buffering of the agonist-induced Ca2+ release. (ii) The amplitude and the time course of the [Ca2+]i rise are not correlated with the magnitude and the time course of Ca2+ influx: fMLP induces a Ca2+ release with a larger amplitude than the Ca2+-ATPase inhibitors, but induces a quantitatively minor and shorter lasting Ca2+ influx. Ca2+-ATPase inhibitors cause, in Ca2+-free medium, a [Ca2+]i rise that lasts for approximately 2 min; however, the influx pathway for divalent cations remains active for the period of observation (up to 20 min). Thus, although, at this point, we cannot exclude some modulatory role of a [Ca2+]i rise, it does not appear to be the crucial messenger for the mediation of Ca2+ influx in myeloid cells. In contrast, our results are compatible with a key role of the filling state of the intracellular Ca2+ pool in the regulation of Ca2+ influx.

The mechanism of regulation of Ca2+-influx by the filling
state of intracellular Ca\textsuperscript{2+} stores must comprise at least three components: (i) an intravesicular Ca\textsuperscript{2+} sensor; (ii) a transmembrane signaling step from the inside to the outside of the Ca\textsuperscript{2+} pool; and (iii) a signaling step from the Ca\textsuperscript{2+} pool to the plasma membrane. The molecular basis of these steps is not yet known. The Ca\textsuperscript{2+}-ATPase inhibitors will be powerful tools to further elucidate the mechanism of Ca\textsuperscript{2+} influx in myeloid cells and other nonexcitable tissues.

Acknowledgments—We would like to thank Dr. Robert A. Clark and Dr. Werner Schlegel for helpful discussions, and Dr. P. Varnai for skilful help.

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