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Effect of Membrane Potential on Divalent Cation Transport Catalyzed by the "Electroneutral" Ionophores A23187 and Ionomycin*

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Depolarization of plasma membrane potential has a potent inhibitory effect on divalent cation influx catalyzed by the carboxylic ionophores ionomycin and A23187. This effect is observed in different cell models and does not depend on either inhibition of Ca^{2+}-activated cation channels or activation of Ca^{2+} extrusion mechanisms as suggested previously. A dependence of divalent cation influx on the magnitude of membrane potential is observed also in artificial liposomes. The inhibition of ionophore-dependent divalent cation transport by membrane potential depolarization can be modified varying the ionophore concentration and the external pH. These findings suggest that both neutral and positively charged ionophore-cation complexes can cross the plasma membrane and that their contribution to the overall transport process can be varied according to the experimental conditions.

Ionophores have been divided into two main groups on the basis of their physicochemical properties: (*a*) neutral ionophores, such as valinomycin, that form charged complexes with cations and catalyze their electrogenic transport down the electrochemical gradient; (*b*) carboxylic ionophores, such as nigericin, that form electroneutral zwitterionic complexes with cations, and promote an electroneutral exchange diffusion, governed by the cation chemical gradient. This latter type of transport is independent of membrane potential (1).

Among carboxylic ionophores, A23187 (2, 3) and ionomycin (4, 5) have been used extensively in biological studies because of their selective ability to transport Ca^{2+} and other divalent cations across natural and artificial membranes. Both ionophores increase cytosolic Ca^{2+} activity, either by catalyzing Ca^{2+} influx across the plasma membrane and/or by mobilizing Ca^{2+} from internal stores.

In eukaryotic cells, rise in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, and alterations of membrane conductance are often coupled, either directly, through Ca^{2+} modulation of ionic currents, or indirectly by concomitant activation of different effector systems. The crosstalk between these two pathways has been often studied by using carboxylic ionophores to raise $[Ca^{2+}]_i$, by a mechanism supposedly independent of membrane potential (6–10). Surprisingly, two groups (6, 7) have recently demonstrated that Ca^{2+} influx catalyzed by both ionomycin and A23187 is inhibited by membrane potential depolarization ($\Delta\psi$). These authors interpreted their results as due to effects of $\Delta\psi$ on either Ca^{2+} channels or the plasma membrane Ca^{2+}-ATPase (6, 7). Here we show that in the neurosecretory cell line PC12 the ionophore-induced divalent cation transport can be severely inhibited by membrane potential depolarization. Similar results have been obtained in two other different cell lines, Jurkatt and J774. The effect of membrane potential could be observed also in an artificial membrane system, suggesting that the phenomenon does not depend on the biological properties of the cells, but rather on the ability of the carboxylic ionophores to form membrane-permeable charged complexes with divalent cations. A model which accounts for the effect of membrane potential, ionophore concentration, and pH on carboxylic ionophore-dependent divalent cation transport is discussed.

EXPERIMENTAL PROCEDURES

Cells and Chemicals—PC12 cells (originated from a rat pheochromocytoma) were obtained from Dr. P. Calissano (University of Rome), Jurkatt human lymphoma cells were a gift of Dr. F. Malavasi (University of Torino), and the mouse macrophage line J774 was obtained from Dr. S. C. Silverstein (Columbia University, New York). The first two lines were subcultured in Falcon plastic flasks (Falcon Labware, Division of Becton Dickinson & Co., Oxnard, CA). PC12 were grown in RPMI 1640 medium (Flow Laboratories, Milan, Italy) supplemented with 2 mM glutamine, 10% (v/v) horse serum, 5% fetal calf serum, and gentamycin (30 μg/ml) (11). Jurkatt cells were grown in the same medium, without horse serum but with 10% fetal calf serum. The J774 mouse macrophage cell line was grown in spinner cultures in Dulbecco's modified Eagle's medium (Flow Laboratories, Milan, Italy), containing 2 mM glutamine, 10% heat-inactivated horse serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) (12). Fura-2-AM, fura-2 free acid, bis-oxonol,[1] and BCECF-AM were obtained from Molecular Probes (Molecular Probes Inc., Eugene, OR). Quin2-AM, A23187, and ionomycin were obtained from Calbiochem. All other reagents were analytical or highest available grade.

Fura-2 Loading and Measurement of $[Ca^{2+}]_i$—Cells were centrifuged and resuspended in standard saline at a concentration of 5×10^6/ml and loaded with fura-2-AM (13) as described previously (14). At the end of the loading period, 30 min, the cells were harvested, washed, and resuspended at a concentration of 5×10^6/ml in RPMI 1640 medium containing 1% fetal calf serum and kept at room temperature until used. Immediately before use, an aliquot of the loaded cell suspension was centrifuged and resuspended in saline medium containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5.5 mM glucose, 1 mM $CaCl_2$, 20 mM Hepes/NaOH buffer, pH 7.4, at 37 °C. This medium was used throughout this work and is

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[1] The abbreviations used are: bis-oxonol, bis-(1,3-diethylthiobarbiturate)trimethineoxonol; AM, acetoxymethyl ester; BCECF, 2′,7′-bis-(2-carboxylethyl)-5- (and -6-) carboxyfluorescein; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TPEN, *N,N,N′,N′*-tetrakis(2-pyridylmethyl)ethylenetriaminepentaacetic acid; DTPA, diethylenetriaminepentaacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

referred to as standard medium. The medium for fluorescence measurements was routinely supplemented with 250 μM sulfinpyrazone to prevent fura-2 leakage (15, 16). With J774 cells, 250 μM sulfinpyrazone was present also during loading with fura-2-AM (15). The medium for PC12 cells also contained 20 μM verapamil in order to block voltage-activated Ca^{2+} channels. In some experiments KCl or choline-HCl isosmotically substituted NaCl. Where indicated, $CaCl_2$ was omitted (Ca^{2+}-free media).

Fura-2 fluorescence was recorded in a Perkin-Elmer LS5 spectrofluorimeter connected to a Perkin-Elmer Data Station. The excitation and emission wavelengths were 340 and 505 nm, respectively. Calibration of $[Ca^{2+}]_i$ was performed as described previously (14). The initial rate of Mn^{2+} influx catalyzed by the ionophores was calculated from the initial rate of fura-2 fluorescence quenching, corrected for the rate of fura-2 quenching in the absence of the ionophores. Values of fura-2 quenching were expressed as percent of the total fura-2 signal. All experiments were performed at 37 °C and each experiment is representative of at least three independent trials.

Membrane Potential—The measurement of plasma membrane potential with bis-oxonol was performed as described in detail by Di Virgilio *et al.* (17).

Measurement of Intracellular pH (pH_i)—pH_i was measured with the fluorescent probe BCECF, as described previously (18).

Black Lipid Film—Planar phospholipid bilayer membranes were formed at room temperature across a 0.15-mm diameter hole in a 6-μm thick Teflon septum separating two buffered salt solutions according to the Montal technique (19). Monolayers were spread from an 8 mg/ml solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids) in *n*-hexane:ethanol, 100:1. The aqueous solutions, 4 ml on each side, were Ca^{2+}-free standard saline, pH 7.0, at room temperature. Ionic currents flowing through the membrane were recorded as described by Menestrina (20). The membrane conductance was 10 picosiemens, and the leaky current was around 0.5 pA. Ionophores, at concentrations ranging from 10^{-7} to 5×10^{-5} M, were added in some experiments on only one side of the membrane and in others on both sides. Similarly, Ca^{2+} concentration was increased from Ca^{2+}-free up to 10 mM, and the pH was made acidic or alkaline (range 6–9) by adding a small volume of 1 N HCl or NaOH until the desired pH was achieved.

Preparation of Liposomes—Liposomes were prepared essentially as described by Olson *et al.* (21). Briefly, egg phosphatidylcholine (15 mg/ml chloroform) was dried by a stream of N_2 on the bottom of a glass tube in the presence of several glass beads. The dry film was gently resuspended in a solution containing 125 mM KCl, 5 mM EGTA, 100 μM fura-2, 20 mM Hepes/KOH buffer. The suspension was left at room temperature for 30–40 min and then mixed by vortexing for 10 periods of 20 s at 30-s intervals. The multilamellar vesicles thus obtained were kept at room temperature until used. Before use, aliquots (0.6 μmol of lipids) were centrifuged (12,000 × *g*/10 min at 4 °C) and resuspended in 2 ml of either Na^+- or K^+-based media.

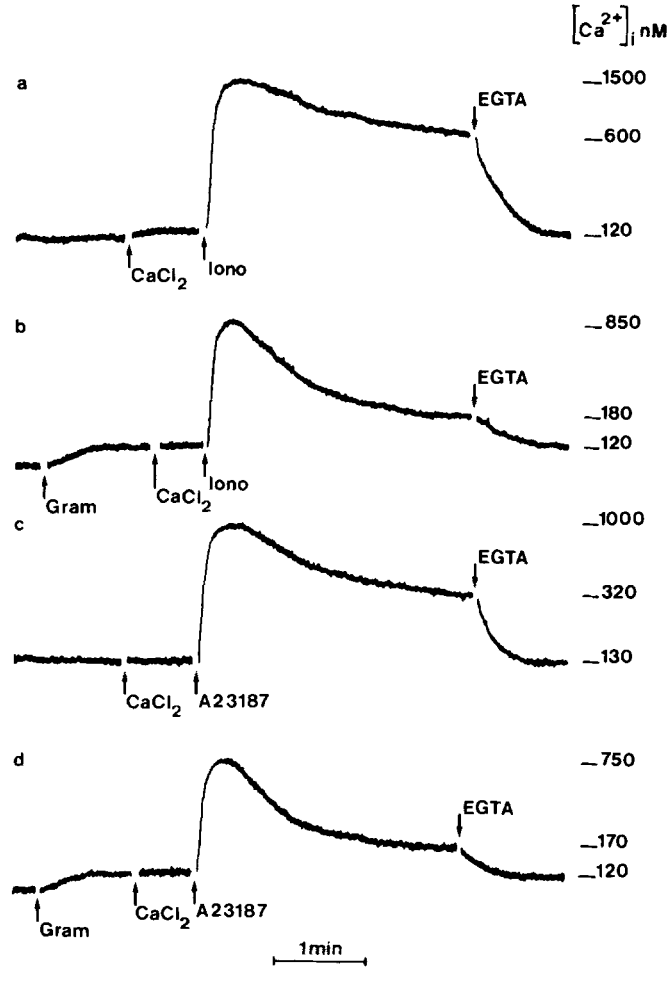

FIG. 1. **Effect of gramicidin D on $[Ca^{2+}]_i$ increases induced by ionomycin and A23187.** *a–d*, aliquots ($1–2 \times 10^6$ cells) of fura-2-loaded PC12 cells were resuspended in 2 ml of Ca^{2+}-free standard medium (pH 7.4 at 37 °C, containing 20 μM verapamil). Where indicated: gramicidin D (*Gram*) (0.5 μM), $CaCl_2$ (1 mM), ionomycin (*Iono*) (0.1 μM), A23187 (0.1 μM), and EGTA (1 mM) were added. In this and the following figures the scale on the *right* represents the calibration of fura-2 signal in terms of $[Ca^{2+}]_i$ (nanomolar).

RESULTS

Fig. 1 shows that in a suspension of PC12 cells loaded with fura-2, the steady-state $[Ca^{2+}]_i$ increase caused by 100 nM ionomycin or A23187 was higher in cells incubated in a standard medium, compared to cells predepolarized by the pore-forming ionophore gramicidin D. In order to distinguish whether plasma membrane potential, $\Delta\psi$, affected Ca^{2+} release from intracellular stores, Ca^{2+} influx, or both, the cells were resuspended in Ca^{2+}-free medium, challenged with ionomycin or A23187, and when $[Ca^{2+}]_i$ was back to resting level, $CaCl_2$ (0.3 mM) was added to the medium. The first rise of $[Ca^{2+}]_i$ represents Ca^{2+} release from stores, thereafter referred to as Ca^{2+} redistribution, whereas the $[Ca^{2+}]_i$ rise following addition of $CaCl_2$ is due to Ca^{2+} influx from the medium. Fig. 2 shows that although Ca^{2+} redistribution was unaffected by depolarization, Ca^{2+} influx was severely curtailed. The inhibitory effect of depolarization on $[Ca^{2+}]_i$ rises was observed (*cf.* Figs. 2, *b–d*, and 3, *a–b*), whether gramicidin D was added before or after the Ca^{2+} ionophores. A direct interaction between the carboxylic Ca^{2+} ionophores and gramicidin D can be excluded on the basis of the following evidence: (*a*) no effect of gramicidin D was observed when PC12 cells were incubated in a choline-based medium rather than in the standard (Na^+) medium (*cf.* Fig. 3, *a* and *c*). Under the former conditions, gramicidin D hyperpolarized the cells (*inset*, Fig. 3). In choline medium, addition of 60 mM NaCl after gramicidin D caused both depolarization and reduction of $[Ca^{2+}]_i$ (*cf.* Fig. 3*c* and *inset*). (*b*) Increasing the extracellular concentrations of KCl mimicked the effect of gramicidin D on both depolarization and Ca^{2+} influx (not shown and see Fig. 7). Observations similar to those reported in Figs. 1–3 have been made by Mohr and Fewtrell (6) in a basophilic leukemia cell line, and by Ishida and Chused (7) in human lymphocytes. Mohr and Fewtrell interpreted their unexpected findings as evidence for the existence in the basophils of Ca^{2+}-activated Ca^{2+} channels (sensitive to the Ca^{2+} electrochemical gradient) similar to those described by von Tscharner *et al.* (22) in human neutrophils. Ishida and Chused (7), instead, suggested that $\Delta\psi$ affected the rate of Ca^{2+} extrusion by the plasma membrane Ca^{2+} pump.

The experiments presented in Figs. 4 and 5 were designed to test experimentally the predictions of the two models.

Fig. 4 shows that in PC12 cells, extending the time interval between the initial $[Ca^{2+}]_i$ peak and $CaCl_2$ addition did not modify the initial rate of Ca^{2+} influx. Negligible reduction of

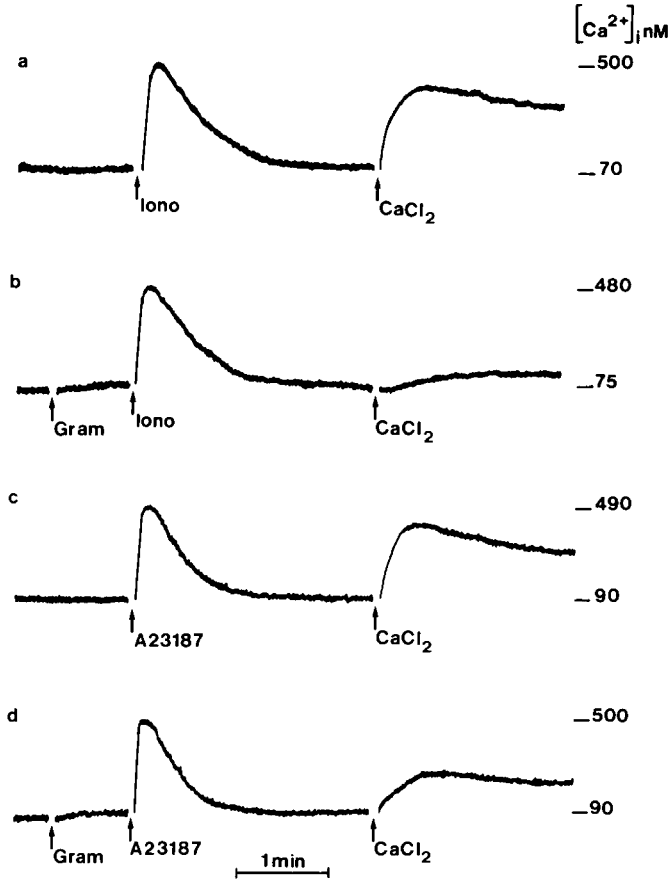

FIG. 2. **Effect of membrane potential on Ca^{2+} redistribution and influx stimulated by ionomycin and A23187.** *a–d*, conditions as in Fig. 1. Where indicated, gramicidin D (*Gram*) (0.5 μM), ionomycin (*Iono*) (0.1 μM), A23187 (0.1 μM), and $CaCl_2$ (0.3 mM) were added.

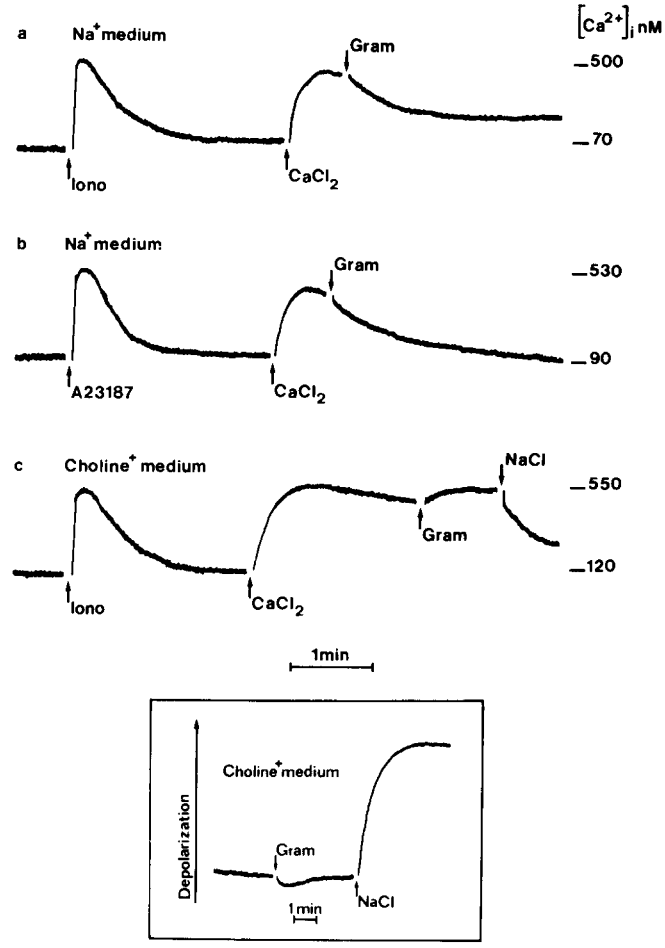

FIG. 3. **Effects of gramicidin D on membrane potential and Ca^{2+} influx induced by carboxylic ionophores.** *a* and *b*, Ca^{2+}-free, Na^+ medium. *c*, Ca^{2+}-free, choline$^+$ medium. Other conditions as in Fig. 1. *Inset*, membrane potential was measured with bis-oxonol. Medium as in *c*. Where indicated, ionomycin (*Iono*) (0.1 μM), A23187 (0.1 μM), $CaCl_2$ (0.3 mM), gramicidin D (*Gram*) (0.5 μM), and NaCl (60 mM) were added.

Ca^{2+} influx was detected even if Ca^{2+} was added 30 min after ionomycin, *i.e.* when $[Ca^{2+}]_i$ was well below resting level (80 *versus* 120 nM). To our knowledge all ion channels inactivate very rapidly upon removal of the stimulus.

The experiment presented in Fig. 5 argues against the hypothesis (7) that the inhibition of Ca^{2+} influx caused by depolarization is only apparent and due to accelerated Ca^{2+} extrusion: Mn^{2+} influx, caused by ionomycin (as revealed by quenching of fura-2 fluorescence) was severely reduced by depolarization. Unlike Ca^{2+}, Mn^{2+} is not (or extremely slowly) transported by either the Ca^{2+}-ATPase of the plasma membrane or that of intracellular stores (23). Chelation of extracellular Mn^{2+} with the membrane-impermeant heavy metal chelator, DTPA, immediately blocked quenching of fura-2 fluorescence, but the increase of the signal observed prolonging the incubation was negligible. If the cells were able to extrude or sequester Mn^{2+} efficiently, this would result in a time-dependent increase of the fluorescent signal. A rapid increase in fura-2 fluorescence was observed only upon addition of the membrane-permeant heavy metal chelator, TPEN (not shown).

Taken together the above mentioned data suggest that the effect of Δψ does not depend on the intrinsic properties of the biological system, but rather is related to the physicochemical characteristics of the Ca^{2+} ionophores themselves. If this is correct, an obvious prediction is that Δψ should affect the transport of divalent cations catalyzed by the carboxylic ionophores also in artificial membranes. We have been unable to detect any significant increase in black lipid film conductance using either A23187 or ionomycin.[2] However, the current carried by the ionophores could be too small to be appreciated in this experimental system. Liposomes containing the Ca^{2+} indicator fura-2 were thus used as an alternative approach. Multilamellar vescicles were obtained from a suspension of egg lecithin in a KCl-EGTA buffer containing 100 μM fura-2-free acid. These liposomes with fura-2 entrapped were resuspended in isosmotic Na^+ medium without KCl. The rate of Ca^{2+} influx, induced by ionomycin or A23187, was enhanced by addition of the electrogenic K^+ ionophore valinomycin (Fig. 6, *a* and *c*). In the presence of the large K^+ gradient between the inside and outside of the liposomes, valinomycin is expected to cause the formation of a large K^+ diffusion potential, negative inside. Valinomycin does not induce Ca^{2+} influx in the absence of ionophores (Fig. 6*a'*) and is uneffective on ionophore catalyzed Ca^{2+} influx if added in a K^+ medium (Fig. 6, *b* and *d*). The effect of valinomycin in K^+-free media is lower and more transient than expected (theoretically there is a very large K^+ gradient). The most likely explanation is the multilamellar nature of the liposomes, in which valinomycin would mainly hyperpolarize the outermost bilayer, but would have little effect on the membrane potential

[2] C. Fasolato, unpublished data.

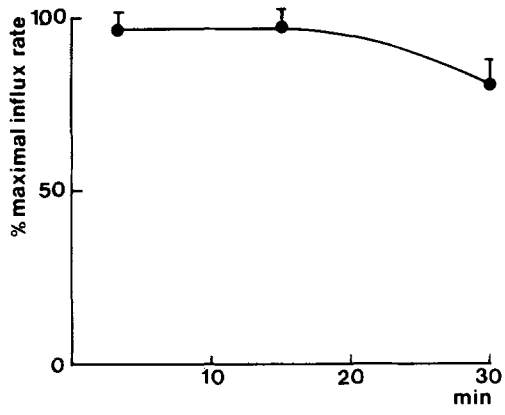

FIG. 4. **Effect of incubation time on Ca^{2+} influx induced by ionomycin.** Conditions as in Fig. 1. Fura-2 loaded PC12 cells were challenged with ionomycin (0.1 μM) in Ca^{2+}-free standard medium. $CaCl_2$ (1 mM) was added at different time after ionomycin addition (t = 0). The values are expressed as percent of the maximal influx rate. Mean ± range of two independent experiments.

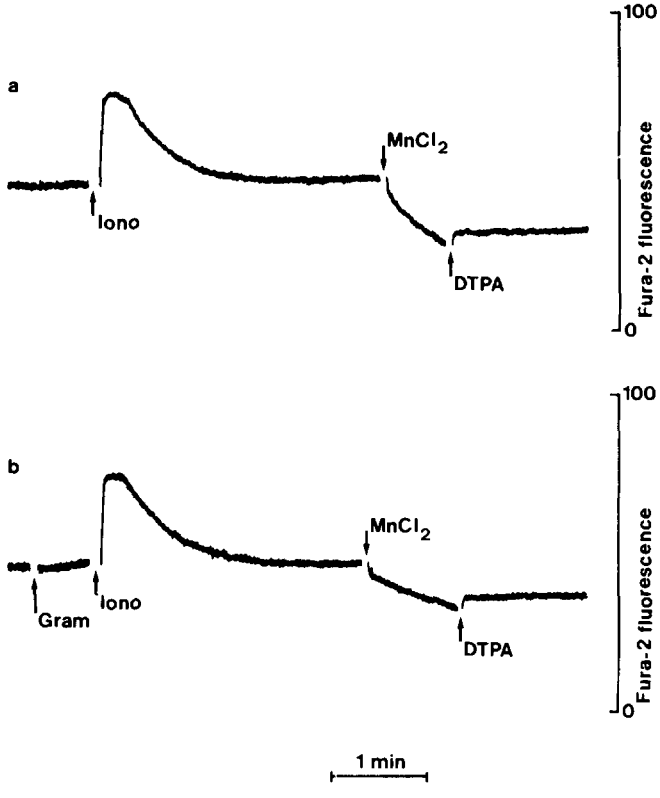

FIG. 5. **Depolarization inhibits Mn^{2+} influx induced by ionomycin.** Conditions as in Fig. 1. Where indicated, gramicidin D (*Gram*) (0.5 μM), ionomycin (*Iono*) (0.1 μM), $MnCl_2$ (0.1 mM), and DTPA (0.4 mM) were added. The scale on the *right* represents the total, Mn^{2+}-quenchable, fura-2 fluorescence. The first very rapid decrease (2–5% of total signal) represents quenching of external fura-2 and was neglected for the calculations of Mn^{2+} influx rate.

of the rest of the layers which encapsulate most of the fura-2.

The observation that even in liposomes the presence of a $\Delta\psi$ (generated by the valinomycin-induced K^+ efflux) affects the rate of Ca^{2+} influx appears a strong argument against a major role of Ca^{2+} channels or Ca^{2+} pumps in the effects observed in the cells. Nonetheless, the quantitative interpretation of changes of $\Delta\psi$ and other parameters (divalent cation and ionophore concentrations, pH, etc.) on ionophore-dependent Ca^{2+} influx rate is complicated by the existence of cellular Ca^{2+} extrusion mechanisms. On the other hand, the absence of efficient Mn^{2+} extrusion sequestration systems makes this divalent cation more convenient than Ca^{2+} for quantitative interpretation of net influx data.

Fig. 7 shows that in PC12 cells, incubated in isosmotic media at increasing KCl concentrations, the rate of Mn^{2+} influx catalyzed by 100 nM ionomycin showed an inverse correlation with the KCl concentration. Maximal inhibition of Mn^{2+} influx, *i.e.* similar to that observed with gramicidin D in normal Na^+ medium, was observed above 60 mM KCl. The inhibition of Mn^{2+} transport does not appear so drastic as that of Ca^{2+}, probably because of the higher Mn^{2+} affinity for the carboxylate ionophores ($K_{F(Ca^{2+})} = 3.7 \times 10^{-7}$ and $K_{F(Mn^{2+})} = 1.3 \times 10^{-4}$ for A23187, see Ref. 33).

The rate of divalent cation influx catalyzed by carboxylic ionophores depends on the concentration of the ionophore-cation complex. This in turn can be varied by changing the cation or the ionophore concentration and the pH of the external medium (pH_o). Fig. 8 shows that with both ionophores, increasing pH_o increased the influx rate of Mn^{2+} (Fig. 8*a*) and decreased the inhibitory effect of depolarization (Fig. 8*b*). The pH_o titration curve was different for the two ionophores, the inhibition of A23187 being already minimal at pH_o 7.8, whereas that of ionomycin continued to decrease up to pH_o 8.6 (Fig. 8*b*). It is worth mentioning that the carboxylic group of A23187 is completely dissociated at pH > 7.8 (24), whereas complete dissociation of the second acidic group of ionomycin (a cisoid-enolized β diketone group) is complete only at pH > 10 (5). Of interest is the observation (Fig. 8*c*) that pH_o has opposite effects on Mn^{2+} influx and Ca^{2+} redistribution. At acidic pH_o, redistribution was maximal and influx minimal, whereas the opposite was true at alkaline pH_o. We also tested the effect of gramicidin D on intracellular pH (pH_i) using the fluorescent indicator, BCECF. At pH_o 6.6, gramicidin D caused a very small acidification of pH_i (0.05 ΔpH), at pH_o 7.4 it had no effect, whereas a small alkalinization of pH_i was observed at pH_o 8.6 (not shown). Thus, the effect of gramicidin D on pH_i not only is negligible but should also favor influx at acidic pH_o and counteract it at alkaline pH_o. Reduction of the inhibitory effect of depolarization was also observed at constant pH_o by increasing the ionophore concentration (Fig. 9). In a number of experiments under different incubation conditions, the maximal inhibition of Mn^{2+} influx never exceeded 50–60% (see "Discussion"). It must be noted that in the experiment in Fig. 8*b* the concentration of the ionophores was chosen so that no inhibition of Mn^{2+} influx at alkaline pH_o occurred. At lower ionophore concentration some inhibition was observed also at the most alkaline pH_o.

DISCUSSION

The inhibitory effect of membrane potential depolarization upon divalent cation influx catalyzed by Ca^{2+} ionophores is a general and possibly ubiquitous phenomenon. In fact it was observed not only in PC12 cells, basophils (6), and lymphocytes (7), but we have obtained identical results in two other cell lines, the mouse macrophage line J774 and the human leukemia line Jurkatt.[3] Gelfand *et al.* (10), on the other hand, observed no effect of $[K^+]_o$ on $[Ca^{2+}]_i$ rises induced by ionomycin in T lymphocytes. The reason for this is not clear, but we observed that in T lymphocytes $[Ca^{2+}]_i$ rises, after an initial hyperpolarization, cause a persistent depolarization of $\Delta\psi$ (25). Mohr and Fewtrell (6) and Ishida and Chused (7)

[3] C. Fasolato and T. Pozzan, unpublished data.

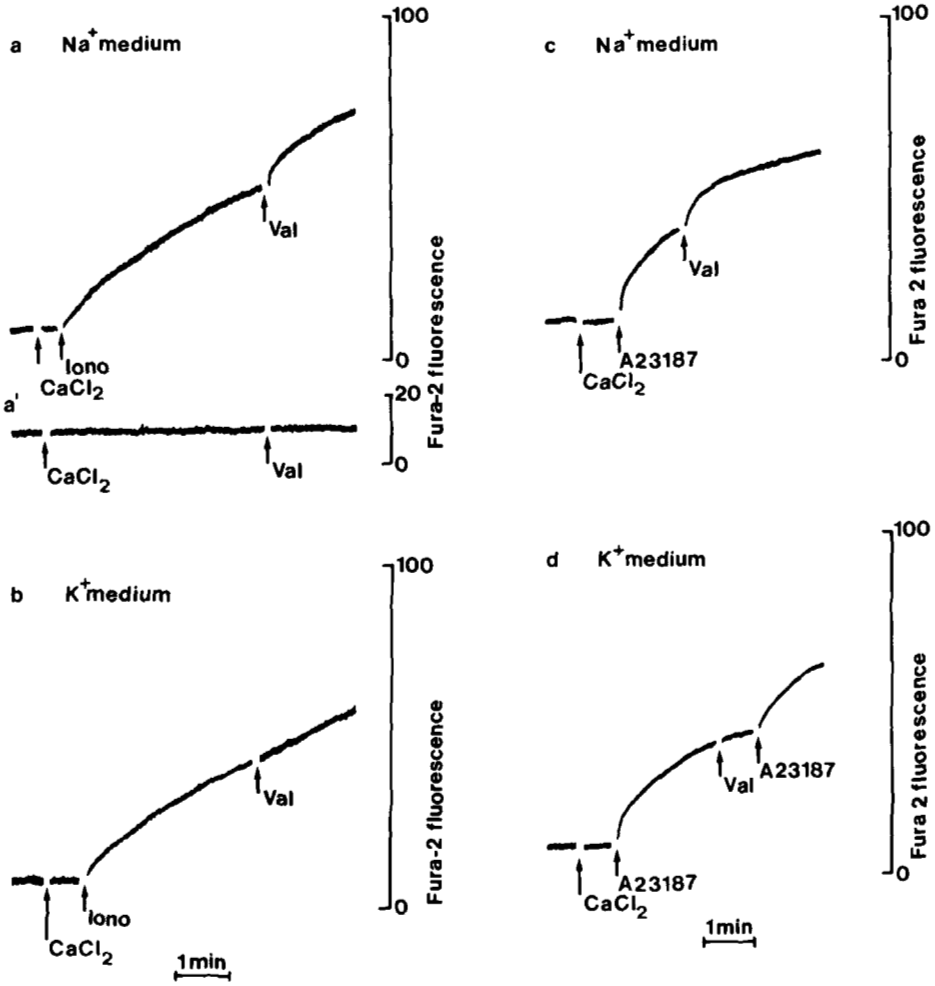

FIG. 6. **Effect of membrane potential on Ca^{2+} transport induced by carboxylic ionophores in liposomes.** Multilamellar vesicles (*MLV*) were prepared, as described under "Experimental Procedures," from egg lecithin resuspended in KCl-EGTA-based medium containing fura-2-free acid (0.1 mM). Internal and external pH were 7.4 and 6.8 for experiments with ionomycin (*Iono*) or A23187, respectively. *a*, *a'*, and *c*, Ca^{2+}-free, Na^+ medium; *b*, and *d*, Ca^{2+}-free, K^+ medium. Where indicated, $CaCl_2$ (0.1 mM), ionomycin (25 nM), A23187 (5 nM), and valinomycin (*Val*) (4 μM) were added. The scale on the *right* represents the Ca^{2+}-sensitive fura-2 signal trapped by the liposomes.

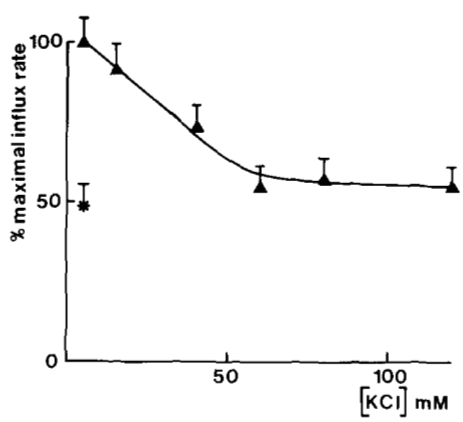

FIG. 7. **Dependence of Mn^{2+} influx rate induced by ionomycin on KCl concentration.** Conditions as in Fig. 5. Fura-2-loaded PC12 cells were incubated at 37 °C in a Ca^{2+}-free isosmotic media with different KCl concentrations. $MnCl_2$ (0.1 mM) was added 3 min after ionophore addition, *i.e.* when $[Ca^{2+}]_i$ was back to basal levels. Values are expressed as percent of maximal influx rate, *i.e.* that obtained with 5 mM KCl. Basal Mn^{2+} influx at each KCl concentration (*i.e.* without Ca^{2+} ionophore) was subtracted before calculating the percent inhibition. The level of Mn^{2+} influx rate obtained in standard medium in the presence of 0.5 μM gramicidin D is also shown (*).

proposed two alternative explanations for the inhibitory effect of depolarization on carboxylic ionophore-induced Ca^{2+} influx, *i.e.* Ca^{2+}-activated Ca^{2+} channels and membrane potential-sensitive Ca^{2+} extrusion. Although both phenomena have been described in a variety of experimental systems, our data argue against a biological property of the cells as the main determinant of the effects described in our models and probably also in the other cell types (6, 7).

A number of evidence argues against a major role of $[Ca^{2+}]_i$ activated cation channels in the influx of Ca^{2+} (or Mn^{2+}) following ionophore induced Ca^{2+} redistribution: (*a*) ion channels rapidly (milliseconds) inactivate upon removal of the stimulus. Whereas in the experiments described in Figs. 2–4 not only Ca^{2+} (or Mn^{2+}) influx was observed when adding $CaCl_2$ (or $MnCl_2$) to ionophore-treated cells at, or below, resting $[Ca^{2+}]_i$, but no, or minimal, inhibitory effect could be detected increasing the interval between ionophore and $CaCl_2$ (or $MnCl_2$) addition.

(*b*) The rate of cation influx is independent of the amplitude of the $[Ca^{2+}]_i$ rise due to redistribution from intracellular stores. In particular, varying pH_o has opposite effects on Ca^{2+} redistribution and Mn^{2+} influx (Fig. 8*c*). These effects are readily explained on the basis of the dissociation of the ionophore acidic group(s). At acidic pH_o the ionophore protonated form predominates. This in turn should favor the penetration of the ionophore into the cells and reduce the effective ionophore concentration available for catalyzing divalent cation transport. The opposite is true at alkaline pH_o.

(*c*) When a receptor agonist is used as a stimulus it causes a rise in $[Ca^{2+}]_i$ similar to that induced by a Ca^{2+} ionophore, but influx of Ca^{2+}, upon Ca^{2+} readdition, can be completely prevented by a specific receptor antagonist (26). If a $[Ca^{2+}]_i$-activated cation channel with long (min) memory existed,

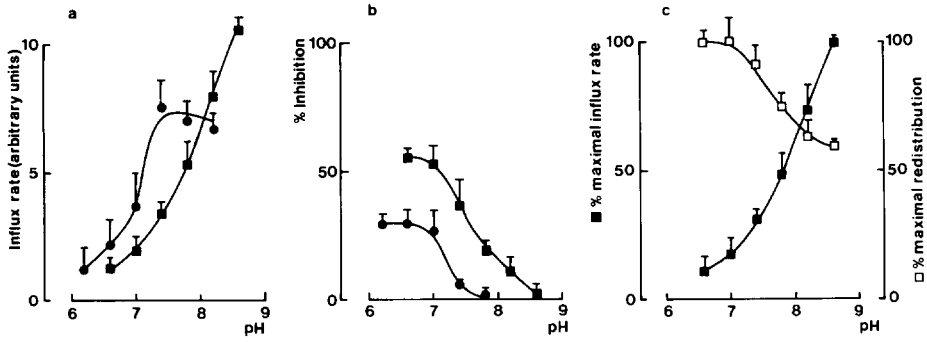

FIG. 8. **pH dependence of divalent cation transport induced by carboxylic ionophores.** *a–c*, Ca^{2+}-free standard medium buffered at different pH with 20 mM Hepes-Tris (37 °C). *a*, pH dependence of Mn^{2+} influx rate induced by (●) A23187 (50 nM) and (■) ionomycin (100 nM). Conditions as in Fig. 5. Values are expressed as arbitrary units of fura-2 fluorescence quenching, subtracted of basal Mn^{2+} influx (*i.e.* without Ca^{2+} ionophore) at each pH_o value. *b*, pH_o dependence of gramicidin D inhibition. Conditions as in *a*. Values are expressed as percent inhibition by gramicidin D (0.5 μM) compared with untreated controls at the same pH_o. ●, A23187; ■, ionomycin. *c*, pH_o dependence of Ca^{2+} redistribution and Mn^{2+} influx induced by ionomycin. Conditions as in *a* and *b*. Values are expressed as percent of maximal (□) extent of redistribution or influx rate (■).

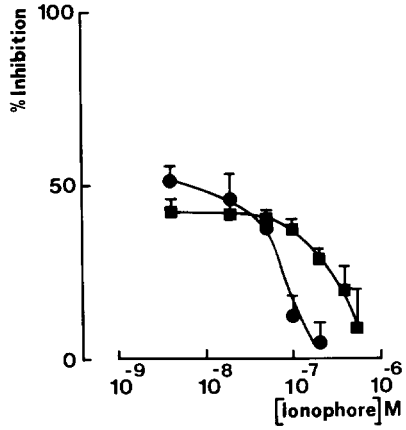

FIG. 9. **Dependence of gramicidin D inhibition on ionophore concentrations.** Conditions as in Fig. 5. Values are expressed as percent inhibition by gramicidin D on Mn^{2+} influx rate compared with untreated controls. ●, A23187; ■, ionomycin.

Ca^{2+} (or Mn^{2+}) influx should survive receptor inactivation.

A number of data also argues against a major stimulation of plasma membrane depolarization on Ca^{2+} extrusion mechanisms: (*a*) the Na^{2+}/Ca^{2+} exchange, known to be present in a variety of excitable cells, is inhibited, or reversed, by plasma membrane depolarization (27, 28). A decrease rather than an increase of Ca^{2+} efflux by this system is expected upon depolarization of the cells.

(*b*) Mn^{2+} is extruded poorly (or not at all) from the cytoplasm (23). The experiment presented in Fig. 5 shows that under our experimental conditions, PC12 cells cannot (or do so very inefficiently) extrude or sequester Mn^{2+} from the cytoplasm. Furthermore, drastic reduction in the cellular level of ATP does not reduce the inhibitory effect of $\Delta\psi$ on ionophore induced Ca^{2+} influx (not shown).

(*c*) The last conclusive evidence is that membrane potential can influence Ca^{2+} influx catalyzed by carboxylic ionophores in liposomes as well, *i.e.* in artificial membranes where neither pumps or channels exist.

Therefore, the only conclusion is that contrary to general belief, one or more step(s) in ionophore-catalyzed cation transport is sensitive to $\Delta\psi$. One possible explanation would be a direct effect of $\Delta\psi$ on ionophore distribution. This is not only unlikely on the basis of theoretical considerations (the protonated forms of the ionophores are much more rapidly diffusible across membranes than the cation-ionophore complexes (24)), but it is unsupported by experimental observations, at least with A23187. Using either the increase in fluorescence of A23187 which occurs upon interaction with cells (29), or by centrifugation and measurement of A23187 concentration in the pellet and in the supernatant, we have been unable to detect any effect of membrane potential on A23187 distribution.[3] An alternative explanation would be an effect of $\Delta\psi$ on local concentrations of charged species. This possibility cannot be ruled out completely at present, although it does not satisfactorily explain the effect of external pH and ionophore concentrations on the inhibitory effect of depolarization.

The explanation that we favor is that both neutral and positively charged ionophore-cation complexes are involved in cation transport across the membranes.

Let us consider the possible equilibria at the membrane-water interphase, either for the monobasic carboxylic ionophore A23187, AH (a,b,c), and the dibasic one ionomycin, IH_2 (a′,b′,c′,d′,e′), in the presence of divalent cations (M^{2+}).

$$AH \rightleftharpoons A^- + H^+ \quad (a)$$

$$A^- + M^{2+} \rightleftharpoons (AM)^+ \quad (b)$$

$$(AM)^+ + A^- \rightleftharpoons A_2M \quad (c)$$

$$IH_2 \rightleftharpoons IH^- + H^+ \quad (a')$$

$$IH^- + M^{2+} \rightleftharpoons (IHM)^+ \quad (b')$$

$$(IHM)^+ + IH^- \rightleftharpoons [(IH^-)_2M] \quad (c')$$

$$IH^- \rightleftharpoons I^{2-} + H^+ \quad (d')$$

$$I^{2-} + M^{2+} \rightleftharpoons IM \quad (e')$$

In the case of A23187, the existence of the positively charged form, $(AM)^+$, has been demonstrated experimentally (30), but its contribution to cation transport has been considered negligible. Wulf and Pohl (31) concluded from $^{45}Ca^{2+}$ flux and membrane conductance experiments that Ca^{2+} transport by A23187 is "mainly" electroneutral. They were in fact unable (and we confirm their results) to detect either significant changes in membrane conductance in the presence of Ca^{2+} + A23187 or effects of imposed membrane potential on A23187 catalyzed Ca^{2+} transport in artificial membranes. It appears, however, that these experiments were performed under conditions (high A23187 concentration and alkaline pH_o) that we have shown are the least affected by changes in $\Delta\psi$. In the

case of A23187, a dependence of inhibition on pH_o and ionophore concentration is expected, since the formation of the A_2M complex should have a quadratic dependence on the absolute A^- concentration (32). The shape of the pH_o dependence of inhibition by $\Delta\psi$ is also consistent with this hypothesis. Wulf and Pohl (31) themselves noticed a deviation from the predicted quadratic dependence on A23187 when the ionophore concentration was reduced below 10^{-7} M.

In the case of ionomycin, only the IM complex was thought to be membrane permeable. Theoretically, on the other hand, complete deprotonation of ionomycin is not required to form the divalent cation complex. In fact the highest alkaline pka of ionomycin is >8 (34), and thus the IH^- should be the most abundant species at physiological pH_o.

The dependence of inhibition by $\Delta\psi$ on the absolute ionomycin concentration suggests, in analogy to A23187, the formation of another neutral complex, $[(IH^-)_2M]$. The $[(IH^-)_2M]$ complex may have escaped detection in experiments performed when determining the Ca^{2+}-ionomycin stoichiometry because they have been performed at pH 10 (5).

CONCLUSIONS

We have shown that divalent cation influx catalyzed by the carboxylic ionophores A23187 and ionomycin is decreased by a depolarization of plasma membrane potential. We have also shown that the effect of $\Delta\psi$ on ionophore-induced Ca^{2+} influx can be observed in artificial liposomes. Theoretical and experimental evidence suggests that both charged and neutral cation-ionophore complexes are permeable across the lipid bilayer.

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