THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 265, No. 10, Issue of April 5, pp. 5635-5639, 1990
Printed in U.S.A.

Chelation of Cytoplasmic Ca^{2+} Increases Plasma Membrane Permeability in Murine Macrophages*

(Received for publication, November 29, 1989)

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Cytoplasmic free Ca^{2+} (Ca^{2+}_i) was chelated to 10–20 nM in the macrophage cell line J774 either by incubation with quin2 acetoxymethyl ester in the absence of external Ca^{2+} (Di Virgilio, F., Lew, P. D., and Pozzan, T. (1984) *Nature* 310, 691–693) or by loading [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) into the cytoplasm via reversible permeabilization of the plasma membrane with extracellular ATP (Steinberg, T. H., Newman, A. S., Swanson, J. A., and Silverstein, S. C. (1987) *J. Biol. Chem.* 262, 8884–8888; Di Virgilio, F., Meyer, B. C., Greenberg, S., and Silverstein, S. C. (1988) *J. Cell Biol.* 106, 657–666). After removal of ATP from the incubation medium, ATP-permeabilized Ca^{2+}_i-depleted macrophages recovered a near-normal plasma membrane potential which slowly depolarized over a 2–4 h incubation at low $[Ca^{2+}]_i$. In both ATP-treated and quin2-loaded cells, depolarization of plasma membrane potential was paralleled by an increase in plasma membrane permeability to low molecular weight aqueous solutes such as eosin yellowish (M_r 692), ethidium bromide (M_r 394), and lucifer yellow (M_r 463). This increased plasma membrane permeability was not accompanied by release of the cytoplasmic marker lactic dehydrogenase for incubations up to 4 h and was likely a specific effect of Ca^{2+}_i depletion since it was not caused by: (i) the mere incubation of macrophages with extracellular EGTA, *i.e.* at near-normal $[Ca^{2+}]_i$; and (ii) loading into the cytoplasm of diethylenetriaminepentaacetic acid, a specific chelator of heavy metals with low affinity for Ca^{2+}. Treatment of Ca^{2+}_i-depleted cells with direct (phorbol 12-myristate 13-acetate) or indirect (platelet-activating factor) activators of protein kinase C prevented the increase in plasma membrane permeability. Down-regulation of protein kinase C rendered Ca^{2+}_i-depleted macrophages refractory to the protective effect of phorbol 12-myristate 13-acetate. This report suggests a role for Ca^{2+}_i and possibly protein kinase C in the regulation of plasma membrane permeability to low molecular weight aqueous solutes.

Over the last few years, a number of techniques for manipulating $[Ca^{2+}]_i$ [1] in small mammalian cells have become available. The most well known takes advantage of the Ca^{2+}-buffering power of the fluorescent indicator quin2, which can be loaded into the cytoplasm of small mammalian cells by a nondisruptive technique (1, 2). Ca^{2+}_i can also be chelated by EGTA loading via either reversible permeabilization of the plasma membrane with extracellular ATP or scrape loading (3, 4). These techniques allow the investigation of physiological processes at exceedingly low levels of Ca^{2+}_i. It has been shown in several cell types that many cellular responses, such as secretory exocytosis (2, 5), activation of membrane oxidases (2), phagocytosis (3, 6), and receptor-triggered actin polymerization (7), occur almost unperturbed in cells in which $[Ca^{2+}]_i$ is lowered 10–20 times below resting levels. The ultrastructure of such $[Ca^{2+}]_i$-depleted cells is also well preserved, but for a slight swelling of the Golgi apparatus (5). Several cellular enzymatic systems are believed to be dependent on Ca^{2+} for their activity, and a host of regulatory Ca^{2+}-binding proteins have been isolated, with calmodulin being the most well known. Therefore, the observation that so many cellular responses occur at a very low $[Ca^{2+}]_i$ is puzzling.

However, in these studies, cell responses were investigated after brief (1 h) incubations, and nothing is known about the effects of prolonged reduction of $[Ca^{2+}]_i$ on cell physiology. The macrophage cell line J774 is particularly well suited for the investigation of the effects of Ca^{2+}_i depletion on cell physiology because it can be loaded with fairly high quin2 concentrations on one hand, and on the other, it can be easily and reversibly permeabilized to extracellular low molecular weight water solutes by brief exposure to extracellular ATP (3, 8, 9). ATP permeabilization allows loading into the cytoplasm of molecules up to M_r 900 and therefore of EGTA (M_r 380). Chelation of Ca^{2+}_i with a well known Ca^{2+} chelator has some advantages over chelation of Ca^{2+}_i with quin2 because it avoids possible (and occasionally documented (10)) toxic effects due to the intracellular hydrolysis of the ester. Some metabolic responses in this cell type after short depletion of Ca^{2+}_i have been investigated already (3). We observed with surprise that Ca^{2+}_i-depleted J774 macrophages spread normally on glass coverslips and phagocytosed via the Fc receptor (3). In this study, we further characterize the effects of Ca^{2+}_i deprivation in macrophages and provide evidence for a crucial role of Ca^{2+}_i in the preservation of the physiological selective permeability of the plasma membrane.

* This work was supported by grants from the Italian Ministry of Education (40% and 60%), the Italian Association for Cancer Research (AIRC), and the National Research Council of Italy (Special Projects Oncology and Biotechnology and Bioinstrumentation). 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.

[1] The abbreviations used are: $[Ca^{2+}]_i$, cytosolic free calcium concentration; quin2/AM, quin2 acetoxymethyl ester; fura-2/AM, fura-2 acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; PAF, platelet-activating factor; DTPA, diethylenetriaminepentaacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MATERIALS AND METHODS

Cells—J774 macrophages were cultured in suspension at 37 °C in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum (DM10H). They were harvested, centrifuged, suspended at a concentration of 5×10^5/ml, and plated on glass coverslips until used. J774 monolayers were used 12–18 h after plating. The experiment shown in Fig. 1 was performed with J774 macrophage suspensions.

All experiments were carried out, unless otherwise indicated, in saline medium containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM HEPES, pH 7.4, at 37 °C. Sulfinpyrazone (250 μM) was also routinely added to the medium (11). This medium is referred to as standard saline throughout this work. Where indicated, standard saline was supplemented with 10 mM EGTA/Tris.

Quin2 Loading—Quin2 loading was performed by incubating macrophage monolayers for 45 min in standard saline containing 50 μM quin2/AM in the presence or absence of 10 mM EGTA as previously described (2, 3). At the end of this incubation time, the monolayers were rinsed and further incubated in standard saline containing 1 mM Ca^{2+} or 10 mM EGTA.

EGTA Loading—EGTA was loaded into the cytoplasm of J774 macrophages by reversible permeabilization of the plasma membrane with ATP^{4-} as previously described (3, 8, 9). Briefly, macrophage monolayers were washed and incubated for 10 min at pH 7.8 in standard saline containing 10 mM EGTA/Tris and 5 mM ATP at 37 °C. After this incubation time, 10 mM $MgSO_4$ was added to remove ATP^{4-} and to reseal the lesions in the plasma membrane. Cells were then rinsed with DM10H containing 10 mM EGTA/Tris and were left to recover in this medium for 15 min at 37 °C. This protocol allowed buffering of $Ca^{2+}{}_i$ to a level varying from 3 to 25 nM (average of 20 nM). Permeabilization of the plasma membrane was assessed by counting the number of adherent cells stained by eosin red (0.2%). Alternatively, the percentage of nuclei stained by ethidium bromide (20 μM) was evaluated.

Measurement of $[Ca^{2+}]_i$—For measurement of $[Ca^{2+}]_i$, macrophages were plated on rectangular coverslips and loaded with either quin2/AM or fura-2/AM. Coverslips were then placed in a fluorometer cuvette and kept in position by a Teflon coverslip holder, and $[Ca^{2+}]_i$ was measured as previously described (3). No correction was applied to allow for the effect of cytoplasmic microviscosity.

Fluorescence measurements were performed in a Perkin-Elmer LS5 spectrofluorometer equipped with a thermostatically controlled cuvette holder and magnetic stirrer.

Measurement of Plasma Membrane Potential—Macrophages were suspended in the fluorometer cuvette at a concentration of 2×10^5/ml in standard saline containing 10 mM EGTA/Tris. To this suspension, 200 nM bisoxonol was added, and fluorescence measurements were performed as described previously (12).

Measurement of Plasma Membrane Permeability—Permeability of the plasma membrane was assessed by measuring the uptake either of the DNA stain ethidium bromide (M_r 394) or of the extracellular marker eosin yellowish (M_r 692). Uptake of ethidium bromide was measured fluorometrically at the wavelength pair 360–580 nm. Briefly, J774 macrophages were plated on rectangular coverslips at a concentration of 5×10^5/ml as previously described (3). After various times, coverslips were rinsed and placed in a fluorometer cuvette containing 20 μM ethidium bromide. 100% fluorescence, which corresponded to 100% of cells permeable to ethidium bromide, was obtained by permeabilizing the macrophages with 10 μM digitonin. About 5% of plated macrophages were already permeable to ethidium bromide in the absence of any treatment. This percentage correlates well with estimates performed by eosin yellowish uptake. At the cell density used in this experiment, the increase in ethidium bromide fluorescence correlates linearly with the increase in the number of permeable cells.[2] Uptake of eosin yellowish was quantitated by counting stained cells under an inverted Olympus IMT-2 microscope at a magnification ×40. Both methods gave essentially similar results. Lactic dehydrogenase was measured according to standard methods (13).

Down-regulation of Protein Kinase C—Protein kinase C was down-regulated according to the method of Rodriguez-Pena and Rozengurt (14). Briefly, macrophage monolayers were incubated for 12 h in DM10H containing 500 nM phorbol 12,13-dibutyrate. At the end of this incubation time, monolayers were rinsed several times with DM10H without phorbol 12,13-dibutyrate and used for experiments.

Enzyme Release—Lactic dehydrogenase was measured according to standard methods (14).

Chemicals—Fura-2/AM and quin2/AM were purchased from Boehringer Mannheim. PMA, PAF, dibutyryl cAMP, and DTPA were from Sigma. All other chemicals were analytical grade.

RESULTS

Chelation of $Ca^{2+}{}_i$ Increases Permeability of Plasma Membrane to Low Molecular Weight Aqueous Solutes—We have recently shown (3) that reversible permeabilization of mouse macrophages with extracellular ATP in the presence of 10 mM EGTA allows trapping of this Ca^{2+} chelator into the cytoplasm and therefore chelation of $Ca^{2+}{}_i$. Fig. 1 shows an experiment in which changes in the plasma membrane potential of J774 macrophages, ATP-permeabilized in the presence (*trace b*) or absence (*trace a*) of 10 mM EGTA, were followed. In separate experiments, EGTA loading by this technique allowed reduction of $[Ca^{2+}]_i$ to 10–20 nM. $[Ca^{2+}]_i$ of control macrophages was about 120 nM after ATP permeabilization and resealing. The plasma membrane potential of EGTA-loaded, $Ca^{2+}{}_i$-depleted macrophages was restored to near-normal once ATP^{4-}, the ionic form of ATP responsible for permeabilization of the plasma membrane, was removed by adding excess Mg^{2+}. However, prolonged incubation of macrophages under conditions of deprivation of $Ca^{2+}{}_i$ caused a progressive depolarization of plasma membrane potential (data not shown) and an increased permeability to extracellular markers (eosin yellowish or ethidium bromide). After 4 h, about 50% of $Ca^{2+}{}_i$-depleted cells were freely permeable to eosin yellowish as opposed to only 10% of control macrophages incubated in the presence of external EGTA but not EGTA-loaded (Fig. 2). The increase in plasma membrane permeability was selective for low molecular weight solutes since release of lactic dehydrogenase, a cytoplasmic soluble enzyme, was only slightly above that of controls (Fig. 2). Besides eosin yellowish, ethidium bromide (M_r 394), lucifer yellow (M_r 463), and fura-2 (free acid) (M_r 831) also had free access to the cytoplasm of macrophages under these conditions. The prolonged incubation in the presence of external EGTA caused a gradual fall in $[Ca^{2+}]_i$ in control macrophage monolayers that were not $Ca^{2+}{}_i$-depleted. However, even after 4 h, $[Ca^{2+}]_i$ was still 3–4-fold higher than in EGTA-loaded, $Ca^{2+}{}_i$-depleted macrophages, as shown in Table I.

An alternative procedure for lowering $[Ca^{2+}]_i$ to very low levels (10–20 nM) in macrophage suspensions and monolayers is quin2 loading in the absence of extracellular Ca^{2+} and in the presence of EGTA (1–3). In Table II, it is shown that

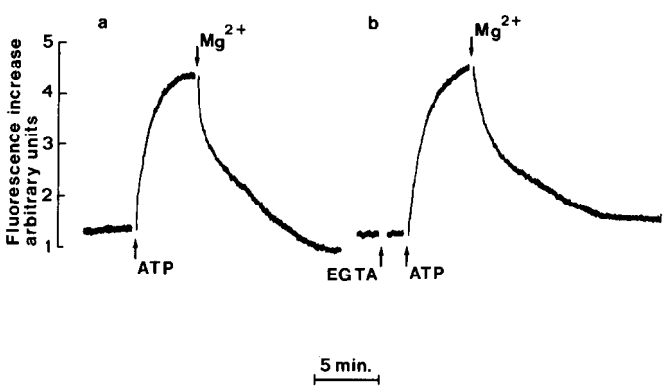

FIG. 1. **$Ca^{2+}{}_i$-depleted macrophages restore normal plasma membrane potential.** J774 macrophages were suspended at a concentration of 2×10^5/ml in standard saline containing 100 nM bisoxonol. 5 mM ATP, 10 mM $MgCl_2$, and 10 mM EGTA were added where indicated.

[2] F. Di Virgilio, unpublished data.

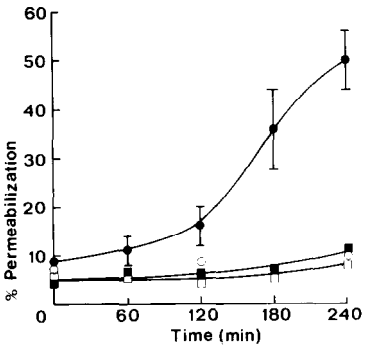

FIG. 2. **Chelation of $Ca^{2+}{}_i$ increases permeability of plasma membrane to eosin yellowish in macrophage monolayers.** J774 macrophage monolayers were reversibly permeabilized with ATP as described under "Materials and Methods." After 5 min of incubation in permeabilization buffer, extracellular ATP was removed, and the cells were allowed to recover for 15 min in DM10H containing 10 mM EGTA. Control macrophages were ATP-permeabilized in Ca^{2+}-free, EGTA-free saline. Both control and EGTA-loaded monolayers were then shifted to standard saline supplemented with 10 mM EGTA and further incubated for the indicated periods. ○ and ●, percent control and $Ca^{2+}{}_i$-depleted macrophages stained by eosin yellowish, respectively. □ and ■, release of lactate dehydrogenase from control and $Ca^{2+}{}_i$-depleted macrophages as percentage of total, respectively. Data are means ± S.D. of seven to nine separate determinations.

TABLE I

Resting $[Ca^{2+}]_i$ of control and $Ca^{2+}{}_i$-depleted macrophage monolayers

Macrophage monolayers were loaded with either 10 μM fura-2 (columns 1–3) or 50 μM quin2 (column 4) in the presence of 1 mM Ca^{2+} (columns 1 and 2) or 10 mM EGTA (columns 3 and 4). Macrophages in column 3 were also ATP-permeabilized to allow cytoplasmic loading of EGTA and therefore $Ca^{2+}{}_i$ depletion. Macrophages in column 4 were Ca^{2+}-depleted by quin2 loading in the presence of EGTA. After loading, the monolayers were further incubated for 4 h in standard saline containing 1 mM Ca^{2+} (column 1) or 10 mM EGTA (columns 2–4). The 4-h incubation was carried out at 22 °C to decrease leakage of the Ca^{2+} indicator out of the cells. Data are means ± S.D. of the number of determinations shown in parentheses.

Macrophages incubated in Ca^{2+}	Macrophages incubated in EGTA	Macrophages incubated in EGTA and $Ca^{2+}{}_i$-depleted	
nM	*nM*	*nM*	
100 ± 25 (9)	65 ± 20 (8)	20 ± 10 (9)	10 ± 10 (7)

TABLE II

Uptake of ethidium bromide by quin2-loaded, $Ca^{2+}{}_i$-depleted macrophage monolayers

Macrophages were plated on glass coverslips and incubated in standard saline containing 50 μM quin2/AM in the presence of 1 mM Ca^{2+} (second column) or in the absence of extracellular Ca^{2+} and in the presence of 10 mM EGTA (third column). After 45 min of incubation, cell monolayers were rinsed and further incubated for the indicated times in standard saline containing 1 mM Ca^{2+} (second column) or 10 mM EGTA (third column). Ethidium bromide (20 μM) fluorescence was measured in a Perkin-Elmer LS5 fluorometer. Data are expressed as percent averages ± S.D. of total fluorescence increase after addition of 10 μM digitonin. Values in parentheses indicate number of determinations.

Incubation time	Macrophages loaded in 1 mM Ca^{2+}	Macrophages loaded in Ca^{2+}-free 10 mM EGTA
h		
2	14 ± 7 (9)	26 ± 6 (9)
4	14 ± 3 (9)	40 ± 12 (9)

quin2-loaded, $Ca^{2+}{}_i$-depleted macrophages developed a significant increase in plasma membrane permeability to ethidium bromide over a 2–4-h incubation period (third column). On the contrary, control macrophages loaded with quin2 in the presence of 1 mM extracellular Ca^{2+} excluded ethidium bromide during the same incubation time (second column).

Loss of selective permeability of the plasma membrane to small extracellular hydrophilic molecules could be prevented by resupplementing macrophages with Ca^{2+} within 2.5 h from the beginning of the incubation at low $[Ca^{2+}]_i$ (data not shown) and was specifically dependent on the deprivation of $Ca^{2+}{}_i$ since loading DTPA into the cytoplasm did not cause any increase in plasma membrane permeability (Fig. 3). DTPA is a potent chelator of heavy metals with low affinity for Ca^{2+} (15). Thus, it mimics all the effects of EGTA on the homeostasis of intracellular metal ions but chelation of Ca^{2+}.

Increased Plasma Membrane Permeability Induced by Low $[Ca^{2+}]_i$ Can Be Prevented by Activators of Protein Kinase C— Several cellular functions dependent on $Ca^{2+}{}_i$ can be also activated via the protein kinase C pathway (2, 16). Therefore, we investigated whether activators of protein kinase C could substitute for Ca^{2+} in the preservation of the physiological plasma membrane barrier to small hydrophilic molecules. Incubation of $Ca^{2+}{}_i$-depleted macrophages with PMA at concentrations of 20–40 nM completely prevented eosin uptake (Fig. 4). Protein kinase C can also be activated by endogenous

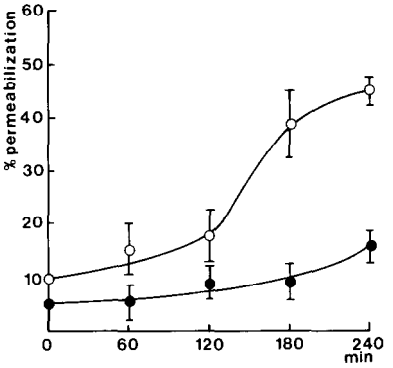

FIG. 3. **Chelation of intracellular heavy metals does not increase plasma membrane permeability to eosin yellowish in macrophage monolayers.** Macrophage monolayers were permeabilized in the presence of either 10 mM EGTA (○) or 500 μM DTPA (●). After resealing, both EGTA- and DTPA-loaded cells were kept in the presence of 1 mM external EGTA. Data are averages ± S.D. of three to five separate determinations.

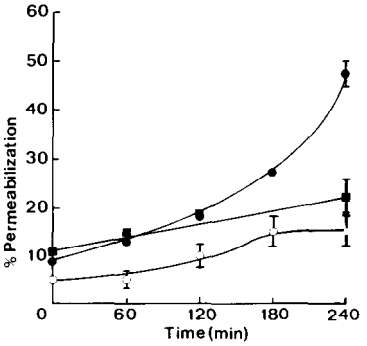

FIG. 4. **Activation of protein kinase C prevents increased plasma membrane permeability to eosin yellowish in $Ca^{2+}{}_i$-depleted macrophage monolayers.** Macrophage monolayers were permeabilized and $Ca^{2+}{}_i$-depleted as described for Fig. 2. 20 nM PMA (○), 100 nM PAF (■), or 1 mM dibutyryl cAMP (●) was present throughout the experiment. Data are averages ± S.D. of three to five separate determinations.

TABLE III

Pretreatment with PMA prevents increased uptake of ethidium bromide from $Ca^{2+}{}_i$-depleted macrophage monolayers

J774 macrophages plated on rectangular glass coverslips were permeabilized with ATP in the presence or absence of 10 mM EGTA. They were then resealed and incubated for the indicated times with or without PMA (40 nM). Ethidium bromide (20 μM) fluorescence was measured in Perkin-Elmer LS5 fluorometer. Data are expressed as percent averages ± S.D. of total fluorescence increase after addition of 10 μM digitonin. Values in parentheses indicate number of determinations.

Incubation time	Controls	+PMA
h		
2	15 ± 6 (11)	13 ± 6 (9)
4	27 ± 3 (17)	15 ± 4 (13)

TABLE IV

Release of lactic dehydrogenase from $Ca^{2+}{}_i$-depleted macrophage monolayers

Data, as percent of total lactate dehydrogenase content, are means ± S.D. of triplicate determinations. Macrophage monolayers were loaded with EGTA as described under "Materials and Methods" and incubated in standard saline supplemented with 10 mM EGTA with or without 40 nM PMA for the indicated times. Control macrophages were ATP-permeabilized in Ca^{2+}-free saline; therefore, they were not loaded with EGTA and were kept in the presence of 10 mM EGTA after resealing. Lactate dehydrogenase released in the supernatant of macrophage monolayers was measured according to Bergmeyer (13).

Incubation time	Controls	EGTA-loaded	EGTA-loaded, PMA-treated
h			
3	ND[a]	1.30 ± 0.5	2.60 ± 1.50
5	ND	9.5 ± 5	8.70 ± 2.4
8	7.5 ± 1.3	28 ± 2	27 ± 5

[a] ND, not determined.

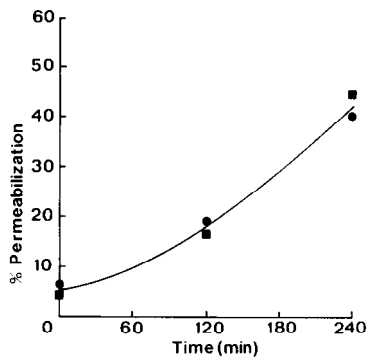

FIG. 5. **Down-regulation of protein kinase C abolishes protective effect of PMA on increased plasma membrane permeability to eosin yellowish in $Ca^{2+}{}_i$.** Macrophage monolayers were incubated for 12 h in DM10H supplemented with 500 nM phorbol 12,13-dibutyrate. At the end of this incubation time, they were washed, $Ca^{2+}{}_i$-depleted, and further incubated for the indicated times with 50 nM PMA (●). Control macrophages were ATP-permeabilized and $Ca^{2+}{}_i$-depleted, but were incubated in the absence of PMA (■). Permeabilization of the plasma membrane and depletion of $Ca^{2+}{}_i$ were carried out as described for Fig. 2. Data are averages of duplicate determinations from a single experiment performed on three separate occasions.

diacylglycerol generated by agonists directed against receptors linked to phosphoinositide turnover (16). One such agonists in macrophages is PAF (17). As shown in Fig. 4, incubation of J774 macrophages with PAF prevented, to a large extent, the permeabilizing effect of low $[Ca^{2+}]_i$ on the plasma membrane. Table III also shows that increased uptake of ethidium bromide can be prevented by pretreatment with PMA.

Release of lactate dehydrogenase from $Ca^{2+}{}_i$-depleted cells began 5–6 h after chelation of $Ca^{2+}{}_i$ and was not prevented by PMA. After 8 h, about 30% of the total cellular lactate dehydrogenase was released in the supernatant (Table IV).

Down-regulation of Protein Kinase C Abolishes Protective Effect of PMA on Increase of Plasma Membrane Permeability in $Ca^{2+}{}_i$-depleted Macrophages—To investigate further the role of protein kinase C, a specific inhibitor would be most helpful. Unfortunately, the compounds so far proposed as inhibitors of protein kinase C have either poor specificity or relevant side effects which make them ill-suited for the study of protein kinase C-dependent functions in intact cells (18). On the other hand, it was shown initially by Rodriguez-Pena and Rozengurt (14) and later confirmed by several other groups that incubation with phorbol esters for 12–24 h leads to the virtual disappearance of cellular protein kinase C activity and to unresponsiveness to protein kinase C-directed agonists. This protocol provides a reliable approach to down-regulation of protein kinase C and therefore to the investigation of cellular responses in the virtual absence of this enzyme. Fig. 5 shows that protein kinase C-down-regulated, $Ca^{2+}{}_i$-depleted macrophages became refractory to the protective effect of PMA. This is another important clue that the effects of PMA on the permeability of the plasma membrane are mediated by protein kinase C.

DISCUSSION

Since the initial observations by Sidney Ringer, calcium has been implicated in nearly all cell functions and responses. In recent years, however, it has become apparent that the mere fact that changes in $[Ca^{2+}]_i$ occur in parallel with many cellular responses does not indicate by necessity a trigger role for Ca^{2+}. In addition, it is also becoming clear that Ca^{2+} is only one of a host of intracellular mediators and that cellular responses can be activated via a Ca^{2+}-dependent or -independent pathway, depending on the contingent situation. This has been shown for secretory exocytosis (2), receptor-mediated endocytosis (19), phagocytosis (3, 6), and activation of membrane-bound enzymes such as the NADPH oxidase of phagocytic cells (2). These observations have questioned the universal role of Ca^{2+} as a mediator of cell responses on one hand and underlined the need for the investigation of the role of Ca^{2+} under more strict experimental conditions on the other. In particular, it is clear that the only correct approach to this problem will be the direct measurement and manipulation of $[Ca^{2+}]_i$ in intact cells. In general, a drastic deprivation of $Ca^{2+}{}_i$ for up to 30–60 min is relatively harmless to most cell types tested so far. Morphology is not grossly altered, but for a slight swelling of the Golgi apparatus; and plasma membrane permeability to monovalent ions is preserved, and a near-normal plasma membrane potential is kept. These observations suggest that, besides being unnecessary for several elicited responses, $Ca^{2+}{}_i$ is also not needed for the basic "housekeeping" routine on the short run. There are, however, circumstantial indications that permeability of the plasma membrane to Ca^{2+} itself is altered even after a brief incubation at low $[Ca^{2+}]_i$. In one of their early papers on quin2, Tsien *et al.* (1) reported that chelation of $Ca^{2+}{}_i$ with quin2 renders the plasma membrane of lymphocytes leaky to Ca^{2+} upon readdition of this cation to the extracellular medium. A similar effect, actually in macrophages, was also described by Kesteven (20). Since $Ca^{2+}{}_i$-depleted macrophages hold a normal plasma membrane potential and therefore a normal permeability to monovalent ions, the observation of Tsien *et al.* suggests that a specific pathway for the permeation of Ca^{2+}

across the plasma membrane is opened by low $[Ca^{2+}]_i$. The observations reported here show that prolonged chelation of $Ca^{2+}{}_i$ has additional and far more drastic effects on plasma membrane permeability. Macrophages depleted of $Ca^{2+}{}_i$ become leaky to low molecular weight aqueous solutes that are normally excluded by living cells. The effects of $Ca^{2+}{}_i$ depletion occur after 2–4 hour and require chelation of both intracellular and extracellular Ca^{2+} since the mere incubation in extracellular EGTA is unable to lower $[Ca^{2+}]_i$ to a level sufficient to trigger membrane permeabilization. The slow onset of the increase in plasma membrane permeability also suggests that it is not mediated directly by $Ca^{2+}{}_i$, but rather by the slow inactivation of a Ca^{2+}-dependent pathway. Moreover, plasma membrane permeabilization is prevented by treatment of macrophages with PMA or PAF, but not with dibutyryl cAMP; and since both PMA and PAF activate protein kinase C, the former directly and the second via generation of diacylglycerol, it is tempting to speculate that protein kinase C may also be involved in the regulation of this permeabilization process. In agreement with this hypothesis, down-regulation of protein kinase C abolishes the protective effect of PMA. The mechanism by which $Ca^{2+}{}_i$ and protein kinase C regulate the permeability of the plasma membrane is not known, but it exhibits intriguing similarities to the control of gap junctional permeability. Protein kinase C inhibits cell to cell communication (16, 21–23), probably as a consequence of inhibition of gap junctions. Treatment of mouse epidermal HEL-37 and 3T3 cells with either PMA or diacylglycerol blocks the transfer of microinjected fluorescein or [^{3}H]uridine between contacting cells (21, 22). Furthermore, gap junctions are kept open at resting physiological $[Ca^{2+}]_i$ and closed by increasing $[Ca^{2+}]_i$. Finally, gap junctions admit low molecular weight molecules comparable to those which permeate the plasma membrane of $Ca^{2+}{}_i$-depleted macrophages.

Lactate dehydrogenase was not significantly released from $Ca^{2+}{}_i$-depleted macrophages during the initial 3–4 h; however, eventually this cytoplasmic marker also leaked out regardless of the presence of PMA. This suggests that prolonged depletion of $Ca^{2+}{}_i$ causes irreversible alterations of the plasma membrane structure that cannot be fully compensated for by exogenous activation of protein kinase C.

Although the mechanism by which $Ca^{2+}{}_i$ regulates the permeability of the plasma membrane is currently only speculative, our results underscore the need for a careful control of the integrity of the plasma membrane in cells subjected to drastic manipulations of $[Ca^{2+}]_i$.

Acknowledgments—We wish to thank Prof. T. Pozzan for invaluable discussion and G. Ronconi for skillful technical support.

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