MOLECULAR PHARMACOLOGY, 23:78–85

Effect of Calmodulin and Calmodulin Antagonists on the Ca^{2+} Uptake by the Intracellular Ca^{2+}-Accumulating System of Guinea Pig Peritoneal Macrophages Treated with Saponin

MASATO HIRATA, EIICHI SUEMATSU,[1] AND TOSHITAKA KOGA

Department of Biochemistry, Faculty of Dentistry, and Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Received June 3, 1982; Accepted August 2, 1982

SUMMARY

Guinea pig peritoneal macrophages were treated with saponin, and the effects of calmodulin and "calmodulin antagonists" on the Ca^{2+} uptake of saponin-treated macrophages were examined. With this intracellular Ca^{2+}-accumulating system we found that intact macrophages contained 672 ng/4 × 10^6 cells (2874 ng/mg of protein), which was reduced to 64 ng/4 × 10^6 cells (799 ng/mg of protein) by treatment of the macrophages with saponin. Exogeneously added calmodulin affected neither the maximal capacity of the Ca^{2+} uptake nor the apparent affinity of Ca^{2+} of the saponin-treated macrophages. All of the calmodulin antagonists examined [chlorpromazine, trifluoperazine, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, and *N*-(6-aminohexyl)-1-naphthalenesulfonamide] inhibited the Ca^{2+} uptake of saponin-treated macrophages. However, the concentrations of these drugs required for half-maximal inhibition of the Ca^{2+} uptake were much higher than those described for the calmodulin stimulation of phosphodiesterase. Troponin I, which inhibited calmodulin-stimulated phosphodiesterase activity, did not inhibit Ca^{2+} uptake. Inhibition of Ca^{2+} uptake by chlorpromazine and trifluoperazine was not reduced by the addition of excess calmodulin and was not altered by changes in concentration of both Mg-ATP and free Ca^{2+}. The Ca^{2+} accumulated in saponin-treated macrophages was released by the addition of chlorpromazine and trifluoperazine, and, after removal of the drugs, Ca^{2+} accumulation was restored. This release of Ca^{2+} by chlorpromazine and trifluoperazine was concentration-dependent, and the concentration required for half-maximal release of Ca^{2+} was similar to that for half-maximal inhibition of Ca^{2+} uptake. From these findings, we conclude that the intracellular Ca^{2+}-accumulating system in guinea pig peritoneal macrophages was not stimulated by calmodulin. Although calmodulin antagonists inhibited Ca^{2+} uptake and released accumulated Ca^{2+} in saponin-treated macrophages, these effects may be unrelated to the specific effect of the drugs on calmodulin.

INTRODUCTION

CaM[2] seems to play a pivotal role in many cellular functions (1). In the Ca^{2+}-transport system, the (Ca^{2+} + Mg^{2+})-ATPase activity of erythrocytes is enhanced by CaM (2–4). In addition to erythrocytes, CaM stimulates the Ca^{2+}-transport system of the plasma membranes of most cell types, e.g., cardiac muscles (5), macrophages (6), and adipocytes (7), by a direct interaction with (Ca^{2+} + Mg^{2+})-ATPase (4, 5).

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

[1] Department of Pharmacology, Faculty of Medicine.

[2] The abbreviations used are: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TNI, troponin I, PDE, phosphodiesterase; CPZ, chlorpromazine; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, *N*-(6-aminohexyl)-1-naphthalenesulfonamide.

Katz and Remtulla (8) noted CaM stimulation of Ca^{2+} transport in sarcoplasmic reticulum-enriched cardiac microsomes. However, this stimulation was not due to the direct interaction of CaM with (Ca^{2+} + Mg^{2+})-ATPase, but rather to the CaM-dependent phosphorylation of phospholamban (9), which was originally discovered in the case of cyclic AMP-dependent phosphorylation. Thus, both direct and indirect stimulatory mechanisms of the Ca^{2+}-transport system by CaM have been documented.

Carafoli *et al.* (10) reported that CaM could be extracted from the endoplasmic reticulum-rich fractions of both lobster muscle and rat liver, suggesting some essential role of CaM in the Ca^{2+}-transport system of both types of tissues. On the other hand, Pershadsingh *et al.* (7) found that, although CaM stimulated Ca^{2+} transport in plasma membrane vesicles of adipocytes prepared

0026-895X/83/010078-08$02.00/0

from rat epididymal fat-pads, it did not stimulate Ca^{2+} transport in endoplasmic reticulum-enriched adipocyte microsomes. Therefore, they concluded that the CaM stimulation of Ca^{2+} transport occurs in plasma membranes but probably not in endoplasmic reticulum, and that the sarcoplasmic reticulum of cardiac muscle was an exception. However, the biochemical preparations of both plasma membrane and endoplasmic reticulum were often intercontaminated.

We reported that the plasma membrane of guinea pig peritoneal macrophages could be destroyed easily and selectively by treatment with saponin, and that in macrophages so treated two elements of the intracellular Ca^{2+}-accumulating system were observed in the presence of Mg-ATP, i.e., one in the absence of sodium azide and the other in the presence of it (11).

In the present study, we attempted to determine whether CaM could stimulate Ca^{2+} transport in the intracellular Ca^{2+}-accumulating system, which was observed in the presence of sodium azide, of guinea pig peritoneal macrophages.

MATERIALS AND METHODS

Preparation of macrophages and saponin treatment. The peritoneal macrophages of guinea pigs were prepared and treated with saponin by essentially the same method as described previously (11), except that 2 mM EGTA was included during the saponin treatment.

Preparation of various proteins, CaM, TNI, and CaM-deficient PDE. CaM was prepared from dog brain by the method of Yazawa *et al.* (12). Troponin was prepared from rabbit skeletal muscle, and TNI was separated from the troponin by the method of Ebashi (13). CaM-deficient PDE was prepared from dog brain by the method of Watterson *et al.* (14), except that it was eluted from a DEAE-cellulose column by an NaCl gradient from 0.1 M to 0.4 M at pH 7.5.

Assays of Ca^{2+} uptake and Ca^{2+} release of saponin-treated macrophages. The uptake and release of Ca^{2+} in saponin-treated macrophages were assayed by a filtration method using ^{45}Ca (11). Saponin-treated macrophages (4×10^6) were preincubated for 2 min at 37° in 1 ml of 0.13 M KCl, 20 mM Tris-maleate buffer (pH 6.80), 5 mM NaN_3, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ (containing 1 μCi of ^{45}Ca), and a specific concentration of EGTA to obtain a desired free Ca^{2+} concentration. The reaction was started by the addition of 5 mM ATP. The incubation was continued for various times at 37°, and the reaction was stopped by passing the mixture through a glass-fiber filter (Whatman GF/C, pore size 1.2 μm). The filter was washed twice with 2 ml of the above solution without ^{45}Ca, dried, and counted in 10 ml of 0.4% 2,5-diphenyloxazole and 0.02% 1,4-bis[2-(4-methyl-5-phenyloxazole)] benzene] in toluene. Almost all saponin-treated macrophages were trapped on the filter, because no cells were observed in the filtrates under a phase-contrast microscope. Furthermore, the same value of Ca^{2+} uptake was obtained when cells were trapped on the filter, which had a pore size of 0.45 μm. The apparent binding constant of EGTA for Ca^{2+} was assumed to be 1×10^6 M^{-1} at pH 6.80 (11). When the concentration of Mg-ATP was changed (Fig. 6), various concentrations of $MgCl_2$ and ATP were added to the reaction mixture under the assumption that the apparent affinity constant of ATP for Mg^{2+} was 4×10^3 M^{-1} (11).

The Ca^{2+} release in saponin-treated macrophages was assayed as follows. The Ca^{2+} uptake of the saponin-treated macrophages was determined as described above. At an appropriate time, a small volume of concentrated drugs, CPZ and TFP, was added to the reaction mixture for Ca^{2+} uptake. The mixture was incubated at 37°, and the reaction was stopped by passing the mixture through a filter as described above. As a control, the same volume of distilled water was added to the reaction mixture, so that the specific activity of ^{45}Ca was not changed between the experimental tube and the control tube.

Extraction of soluble fractions of both untreated macrophages and saponin-treated macrophages and assays of PDE activity. Cells (1×10^8) of both untreated macrophages and saponin-treated macrophages were homogenized in 2 ml of 10 mM Tris-HCl (pH 7.5) and 1 mM EGTA in a motor-driven homogenizer with a Teflon pestle, and centrifuged at $140,000 \times g$ for 60 min. The supernatant then was dialyzed against 10 mM Tris-HCl (pH 7.5). The dialyzed extracts were diluted with 10 mM Tris-HCl (pH 7.5) and boiled in water for 3 min to denature the endogenous PDE, and their CaM content was determined. PDE activity was assayed by the two-step method of Cheung (15).

Polyacrylamide gel electrophoresis and determination of protein concentration. Electrophoresis was carried out on 12% polyacrylamide gels by the method of Laemmli (16).

Protein concentration was determined by the biuret reaction and/or the method of Lowry *et al.* (17), using bovine serum albumin as a standard.

Materials. CPZ was obtained from Sigma Chemical Company (St. Louis, Mo.). TFP was a gift from Yoshitomi Pharmaceutical Science Company Ltd. (Japan) through the courtesy of Dr. Y. Kawano, Chief of Pharmacy, Kyushu University Hospital attached to the Faculty of Dentistry. W-7 and W-5 were obtained from Rikaken Company (Japan). These drugs were freshly dissolved just prior to addition to the incubation mixture and were protected from light.

RESULTS

Content of CaM in the soluble fractions of macrophages and saponin-treated macrophages. Jamieson and Vanaman (18) prepared and characterizted CaM from the murine macrophage cell line, $P388D_1$. Figure 1a shows that the soluble fractions of both untreated macrophages and saponin-treated macrophages could stimulate activity of PDE prepared from dog brain only in the presence of Ca^{2+}—like CaM prepared from dog brain—indicating that the peritoneal macrophages of the guinea pig contain CaM. From the protein concentration required for half-maximal activation of CaM-deficient PDE activity in comparison with the purified CaM, we calculated the CaM content in the soluble fractions of both untreated macrophages and saponin-treated macrophages. To check the validity of our method for determination of the CaM content, we examined the CaM content of the soluble fractions of rat brain cortex and

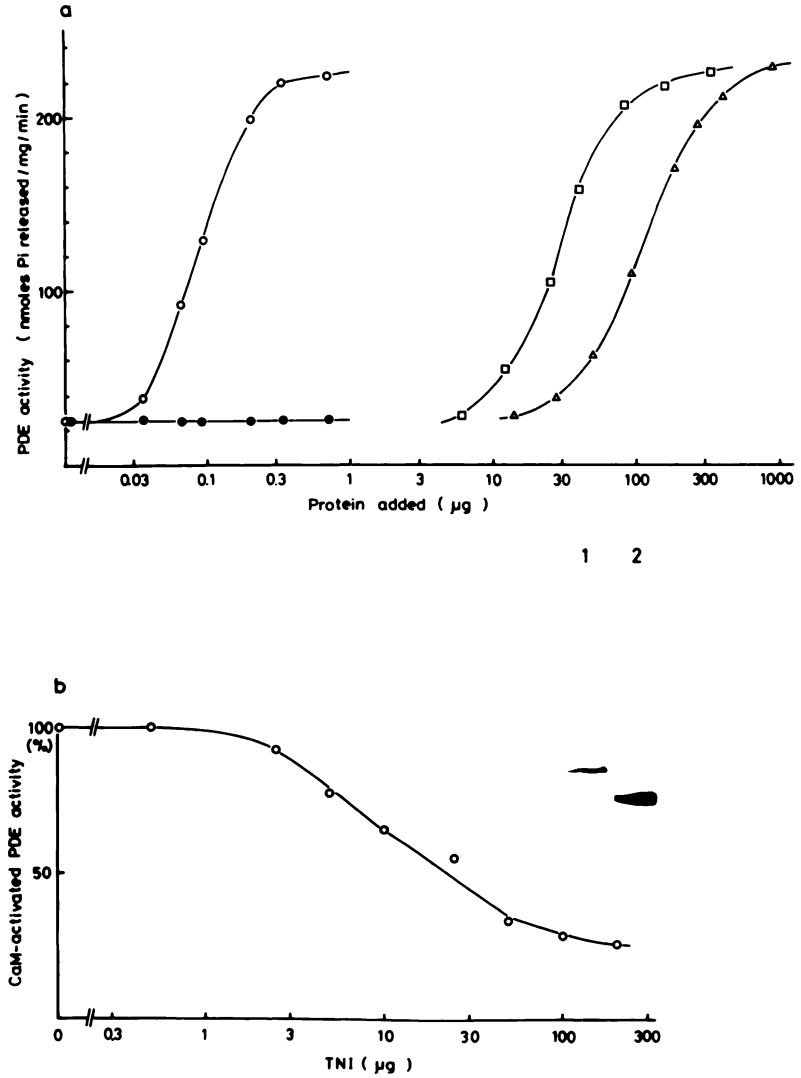

FIG. 1. *Stimulation of PDE activity by CaM and extracts of heat-treated and saponin-treated macrophages, and inhibition of CaM-activated PDE activity by TNI*

a. Stimulation of PDE activity by CaM and extracts of both heat-treated macrophages and saponin-treated macrophages. PDE activity was assayed by a two-step procedure in the presence of either 0.1 mM $CaCl_2$ (*open symbols*) or 1 mM EGTA (*closed symbols*). ○, CaM; □, heat-treated extracts of macrophages; △, heat-treated extracts of saponin-treated macrophages.

b. Inhibition of CaM-activated PDE activity by TNI. PDE activity was assayed in the presence of 0.1 mM $CaCl_2$, CaM (0.38 μg/ml), and various concentrations of TNI. *Inset*, Electrophoretic patterns of TNI (*1*) and CaM (*2*) which were used in the study.

lung, and obtained values of 377 and 54.3 μg/g (wet weight), respectively. Previously, Kakiuchi (19) reported that the CaM content of both rat brain cortex and lung were 379 and 75 μg/g (wet weight), respectively. The CaM content of the soluble fraction was reduced from 672 to 64 ng/4 × 10^6 cells, or from 2874 to 799 ng/mg of protein, by treatment of the macrophages with saponin (Table 1). When the saponin treatment of macrophages was carried out with high-ionic strength solution, e.g., above 0.3 M KCl, CaM was not detected in saponin-treated macrophages by the present method. However, macrophages so treated could not be used for assay of Ca^{2+} uptake because the cells aggregated.

Figure 1b shows the effect of TNI prepared from rabbit skeletal muscle on CaM-activated PDE activity. TNI inhibited CaM-activated PDE activity, probably because TNI could interact with CaM in the presence of Ca^{2+}. Shenolikar *et al.* (20) reported that TNI inhibited CaM-activated phosphorylase kinase from skeletal muscle.

Lack of effect of CaM on the Ca^{2+} uptake of saponin-treated macrophages. Figure 2 shows the effect of exog-

TABLE 1

Contents of CaM in soluble fractions of both macrophages and saponin-treated macrophages

The concentrations of CaM in soluble fractions of both untreated macrophages and saponin-treated macrophages were determined on the basis of the protein concentration required for the half-maximal activation of PDE activity in comparison with the purified CaM (Fig. 1a), and are expressed as means ± standard error of the mean.

Macrophages	CaM	
	ng/4 × 10^6 cells	ng/mg protein
Untreated (n = 6)	672 ± 29	2874 ± 320
Saponin-treated (n = 5)	64 ± 8	799 ± 91

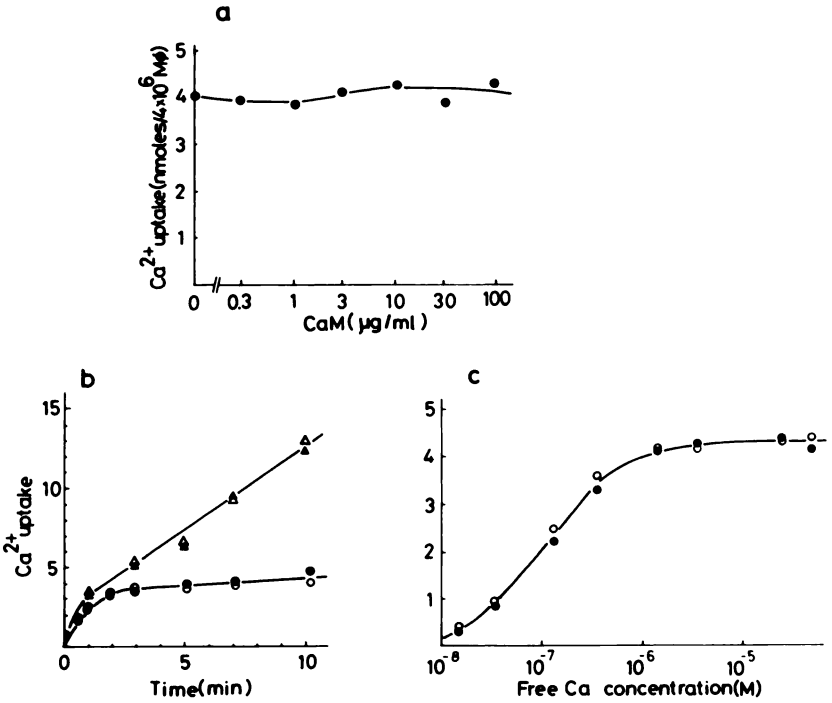

FIG. 2. *Effect of CaM on the Ca^{2+} uptake of saponin-treated macrophages (Mø).*

The Ca^{2+} uptake of saponin-treated macrophages was assayed as described under Materials and Methods. a, The CaM concentration added was varied; the free Ca^{2+} concentration was 3.5×10^{-6} M. b, The free Ca^{2+} concentration was 3.5×10^{-6} M. ○, ●, In the absence of oxalate; △, ▲, in the presence of oxalate (5 mM). *Open symbols* represent control experiments; *closed symbols* represent experiments carried out in the presence of CaM (10 μg/ml). c, The free Ca^{2+} concentration was varied. ○, control; ●, CaM (10 μg/ml). The energy-independent Ca^{2+} uptake was carried out in the absence of ATP and subtracted from the value of Ca^{2+} uptake in the presence of ATP. All other figures are the same.

enously added CaM on the Ca^{2+} uptake of saponin-treated macrophages. CaM at concentrations up to 100 μg/ml did not affect the maximal Ca^{2+} uptake of saponin-treated macrophages (Fig. 2a). Vincenzi *et al.* (21) reported that sufficient preincubation of the $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocytes with CaM in the presence of Ca^{2+} was critical for the observation of CaM activation of $(Ca^{2+} + Mg^{2+})$-ATPase. Under our experimental conditions, the preincubation time was 2 min and the incubation time was 10 min. Even when measuring the Ca^{2+} uptake of saponin-treated macrophages for 10 min after preincubation for 20 min at 37°, CaM did not stimulate the maximal Ca^{2+} uptake (data not shown). Furthermore, CaM (10 μg/ml) did not affect the time course of Ca^{2+} uptake of the saponin-treated macrophages, in either the presence or absence of the Ca^{2+}-precipitating anion, oxalate (Fig. 2b). Moreover, CaM (10 μg/ml) did not change the apparent affinity of saponin-treated macrophages for Ca^{2+} (Fig. 2c). The lack of effect of CaM on the Ca^{2+} uptake of saponin-treated macrophages apparently differed from the effects on $(Ca^{2+} + Mg^{2+})$-ATPase of erythrocytes (2–4), and the Ca^{2+} transport of the plasma membranes of macrophages (6), adipocytes (7), and the sarcoplasmic reticulum of cardiac muscle (8, 9).

Effect of CaM antagonists on the Ca^{2+} uptake of saponin-treated macrophages. Phenothiazines and naphthalenesulfonamides reportedly interact with CaM in the presence of Ca^{2+}, and inhibit the CaM activation of PDE activity and the $(Ca^{2+} + Mg^{2+})$-ATPase activity of erythrocyte (1, 22, 23). Furthermore, TNI from skeletal muscle interacts with CaM in the presence of Ca^{2+}, and thus inhibits the muscle phosphorylase kinase (20) and PDE activity (Fig. 1b). Phenothiazines, naphthalenesulfonamides, and TNI have been designated "CaM antagonists" and have been widely used.

Although the CaM content of the soluble fraction of saponin-treated macrophages was substantially reduced to 64 ng/4 × 10^6 cells (Table 1), the Ca^{2+} uptake or the $(Ca^{2+} + Mg^{2+})$-ATPase activity of the inside-out vesicles or the ghost of human erythrocytes was stimulated approximately 2 times by the addition of about 60 ng of CaM (3, 23). Thus, the role of endogenous CaM in the Ca^{2+} uptake of saponin-treated macrophages could not be completely excluded. Therefore, we examined the effect of CaM antagonists on the Ca^{2+} uptake of saponin-treated macrophages (Fig. 3). Both phenothiazines (CPZ and TFP) and naphthalenesulfonamides (W-7 and W-5) inhibited the Ca^{2+} uptake of saponin-treated macrophages in a dose-dependent manner (Fig. 3a). Naphthalenesulfonamides were less effective than phenothiazines. However, the half-maximal inhibitory concentrations (IC_{50}) of phenothiazines (CPZ and TFP) were about 100 μM, the IC_{50} of CPZ was slightly higher, and that of TFP was much higher than those described for CaM activation of PDE activity and of $(Ca^{2+} + Mg^{2+})$-ATPase activity; i.e., the IC_{50} values of CPZ and TFP were around 50 μM and 10 μM, respectively (22, 23). Moreover, TNI at concentrations up to 200 μg/ml did not inhibit the Ca^{2+} uptake of saponin-treated macrophages (Fig. 3b), in contrast with the CaM activation of PDE activity (Fig. 1b) and phosphorylase kinase (20). Therefore, it seems likely that the inhibition of Ca^{2+} uptake of saponin-treated macrophages by CaM antagonists might be due to nonspecific effects which are unrelated to interactions with

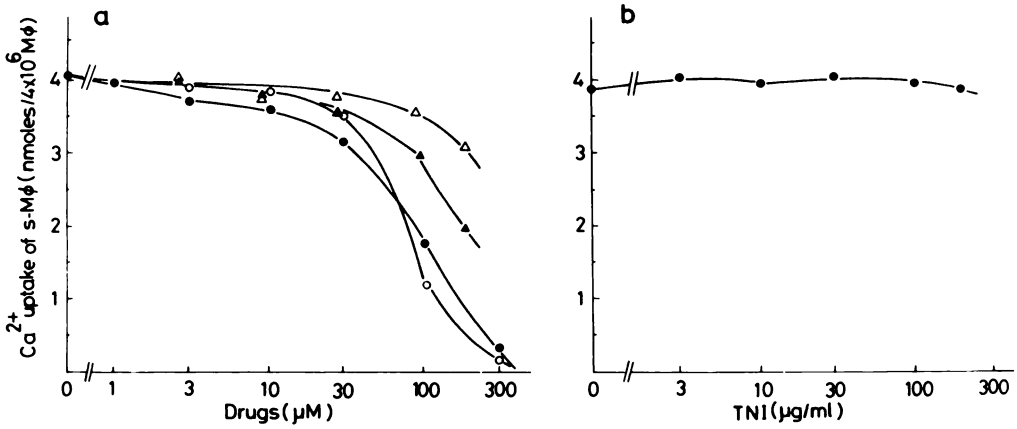

FIG. 3. *Effect of CaM antagonists on the Ca^{2+} uptake of saponin-treated macrophages (s-Mø).* a. ○, TFP; ●, CPZ; △, W-5; ▲, W-7. b. TNI. The free Ca^{2+} concentration was 3.5×10^{-6} M.

CaM of CaM antagonists, as previously noted by Caroni and Carafoli (5).

Characteristics of the inhibitory effect of CaM antagonists on Ca^{2+} uptake of saponin-treated macrophages. Figure 4 shows the effect of exogenously added CaM on the Ca^{2+} uptake of saponin-treated macrophages in the presence of CPZ. CaM at concentrations up to 100 μg/ml did not reduce the inhibition of Ca^{2+} uptake by 50 μM CPZ. Since CaM would interact with CPZ in the presence of Ca^{2+}, the inhibitory effect of CPZ on Ca^{2+} uptake was quite apparent even with a bound form of CPZ, i.e., CaM-CPZ complexes. Essentially the same result was obtained with TFP.

Figure 5 shows the inhibition of Ca^{2+} uptake of saponin-treated macrophages by CPZ at various concentrations of free Ca^{2+}. The inhibitory effect of CPZ on the Ca^{2+} uptake was appreciable with the free Ca^{2+} concentration over 10^{-7} M, and the degree of inhibition was not changed with an increase in the concentration of free Ca^{2+}. Furthermore, the apparent affinity constant of saponin-treated macrophages for Ca^{2+}, which is the reciprocal of the free Ca^{2+} concentration for half-maximal Ca^{2+} uptake, was not changed in either the presence or absence of CPZ, indicating that the inhibition of Ca^{2+} uptake by CPZ might not be due to the competition between CPZ and Ca^{2+} for the Ca^{2+} binding sites during the mechanism of Ca^{2+} uptake. Essentially the same result was obtained with TFP.

Figure 6 shows the inhibition of the Ca^{2+} uptake of saponin-treated macrophages by CPZ at various concentrations of Mg-ATP. Over 2 mM Mg-ATP was required to obtain the maximal Ca^{2+} uptake, as previously demonstrated (11). The Ca^{2+} uptake was invariably inhibited by CPZ at a concentration of Mg-ATP ranging from 0.5 mM to 5 mM. Thus, inhibition of Ca^{2+} uptake by CPZ may not be due to the competition between CPZ and Mg-ATP for binding sites of the substrate for Ca^{2+} uptake.

Release of Ca^{2+} in saponin-treated macrophages by CPZ. Kurebayashi and Ogawa (24) reported that CPZ released accumulated Ca^{2+} in the sarcoplasmic reticulum of skeletal muscle, and they assumed that this release was due to an enhancement of the Ca^{2+}-induced Ca^{2+}-release mechanism, as was proposed in the case of caffeine and halothane (25). Therefore, we next examined the release of Ca^{2+} in saponin-treated macrophages by CPZ. As shown in Fig. 7, Ca^{2+} taken up was rapidly released by the addition of 50 μM of CPZ, and the maximal release by CPZ was obtained within 3 min after the addition of CPZ. After the removal of CPZ, the Ca^{2+}

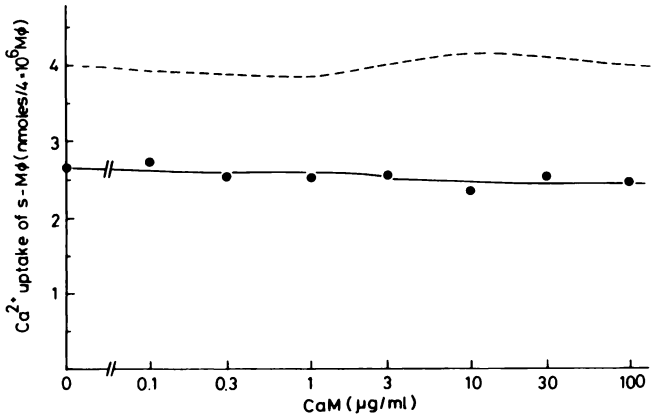

FIG. 4. *Effect of CaM on the Ca^{2+} uptake of saponin-treated macrophages (s-Mø) in the presence of CPZ.*

The Ca^{2+} uptake was carried out in the presence of 50 μM CPZ and various concentrations of CaM. The free Ca^{2+} concentration was 3.5×10^{-6} M. The *broken line* was drawn from Fig. 2a.

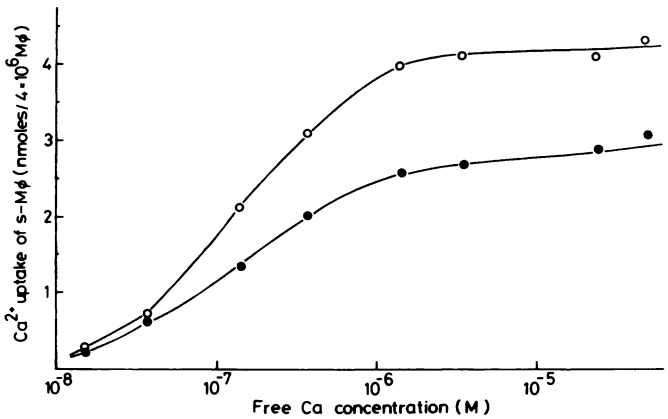

FIG. 5. *Ca^{2+} uptake of saponin-treated macrophages (s-Mø) at various concentrations of free Ca^{2+} and the inhibition by CPZ.*

○, Control; ●, 50 μM CPZ.

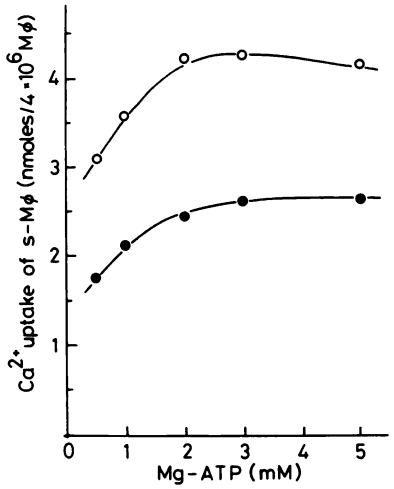

FIG. 6. *Ca^{2+} uptake of saponin-treated macrophages (s-Mø) at various concentrations of Mg-ATP, and inhibition by CPZ.*

Free Ca^{2+} and Mg^{2+} concentrations were 3.5×10^{-6} M and 1 mM, respectively. ○, Control; ●, 50 μM CPZ.

level was again restored to the original level within 5 min. In this experiment, we used a free Mg^{2+} concentration of 1 mM. When the free Mg^{2+} concentration was increased to 10 mM, the value of the maximal release of Ca^{2+} with CPZ was not affected (data not shown). The Ca^{2+}-induced Ca^{2+} release in the sarcoplasmic reticulum of skeletal muscle was inhibited with increase in the free Mg^{2+} concentration (24, 25). Figure 8 shows the Ca^{2+} remaining in saponin-treated macrophages after the maximal release by the addition of various concentrations of CPZ. The release of Ca^{2+} by CPZ was dose-dependent, and the half-maximal release of Ca^{2+} was obtained by the addition of about 100 μM CPZ, at free Ca^{2+} concentrations of both 3.5×10^{-6} M and 1.4×10^{-7} M. Although the

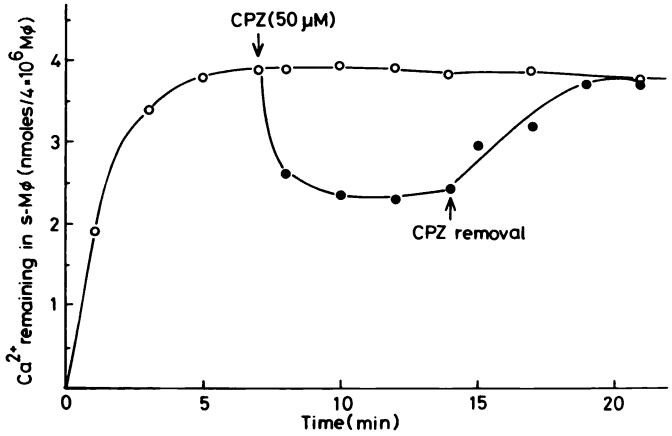

FIG. 7. *Ca^{2+} release by CPZ in saponin-treated macrophages (s-Mø)*

The Ca^{2+} uptake of s-Mø was carried out as described under Materials and Methods. Seven minutes after the start of the Ca^{2+} uptake, CPZ was added to give a final concentration of 50 μM (●); at the time indicated, an aliquot of the reaction mixture was passed through a glass-fiber filter, and the Ca^{2+} remaining in s-Mø was determined. As a control, the same volume of distilled water was added (○). At 7 min after the addition of CPZ, the cells were sedimented to remove CPZ, and fresh reaction solution containing the same specific activity of ^{45}Ca as the initial Ca^{2+} uptake solution was added. The free Ca^{2+} concentration was 3.5×10^{-6} M.

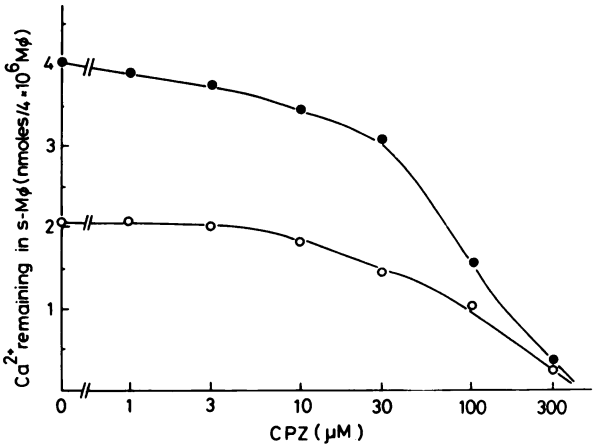

FIG. 8. *Ca^{2+} release in saponin-treated macrophages (s-Mø) by various concentrations of CPZ*

The Ca^{2+} release in s-Mø was carried out as described in Fig. 7. The Ca^{2+} remaining in s-Mø was determined at 5 min after the addition of various concentrations of CPZ. The free Ca^{2+} concentrations were 3.5×10^{-6} M (●) and 1.4×10^{-7} M (○).

Ca^{2+}-induced Ca^{2+} release in the sarcoplasmic reticulum of skeletal muscle was strongly dependent on the concentration of free Ca^{2+} (25), the release of Ca^{2+} in saponin-treated macrophages by CPZ was not. The dose-dependent release of Ca^{2+} in saponin-treated macrophages, as induced by CPZ, was much the same as the dose-dependent inhibition of Ca^{2+} uptake induced by CPZ (Fig. 5). Much the same dose-dependent release of Ca^{2+} was obtained with TFP.

DISCUSSION

We have previously shown that the treatment of macrophages with saponin results in the destruction of the plasma membrane (11), because cholesterol molecules are enriched in plasma membranes, but not in both endoplasmic reticulum and mitochondria; therefore, saponin selectively removes cholesterol molecules from the plasma membrane (26, 27). Electron microscopic observation of saponin-treated macrophages revealed that both endoplasmic reticulum and mitochondria preserve their intact structures, in spite of destruction of the plasma membrane.[3] Saponin-treated macrophages could accumulate Ca^{2+} in the presence of Mg-ATP in two ways: i.e., with high capacity (95 nmoles/4×10^6 cells) and low affinity for Ca^{2+} (3×10^5 M^{-1}) in the absence of the inhibitor of mitochondrial Ca^{2+} uptake, sodium azide, and with low capacity (4 nmoles/4×10^6 cells) and high affinity (6×10^6 M^{-1}) in the presence of sodium azide (11). The accumulation of Ca^{2+} in saponin-treated macrophages was substantially stimulated in the presence of oxalate, and the addition of Triton X-100 abolished the Ca^{2+} uptake of saponin-treated macrophages (11). Therefore, the accumulation of Ca^{2+} by Mg-ATP in saponin-treated macrophages was probably due to both endoplasmic reticulum and mitochondria. Since the Ca^{2+} uptake of saponin-treated macrophages was carried out throughout this study in the presence of sodium azide, the Ca^{2+} uptake would be due to endoplasmic reticulum, but not to mitochondria.

[3] M. Hirata, E. Suematsu, and T. Koga, unpublished data.

In the experiments of Ca^{2+} release by CPZ (Figs. 7 and 8), there is the possibility that the specific activity of ^{45}Ca was diluted and the Ca^{2+} remaining in saponin-treated macrophages was underestimated. This possibility may not apply, since the accumulated Ca^{2+} in the mitochondria of saponin-treated macrophages was released by the addition of EGTA and the saponin treatment of macrophages was carried out in the presence of 2 mM EGTA so that the mitochondria would not contain unlabeled Ca^{2+}. Therefore, the Ca^{2+} release would be due to endoplasmic reticulum, but not to mitochondria.

CaM, which was originally discovered in the activation of brain PDE activity (15, 28), is an important regulatory protein which binds Ca^{2+} and mediates Ca^{2+} stimulation of several enzyme activities, including all types of (Ca^{2+} + Mg^{2+})-ATPases of plasma membrane examined (1–7). On the other hand, with respect to the Ca^{2+}-transport system in the cytoplasm, CaM has been reported to stimulate Ca^{2+} transport in the sarcoplasmic reticulum of cardiac muscle (8, 9), and CaM has been extracted from endoplasmic reticulum of both lobster muscle and rat liver (10). However, Pershadsingh *et al.* (7) reported that CaM did not stimulate Ca^{2+} transport in the endoplasmic reticulum-rich fraction of the rat adipocytes. Thus, whether CaM would stimulate Ca^{2+} transport in the cytoplasm is controversial. We have now obtained evidence that CaM does not enhance either the maximal capacity for Ca^{2+} uptake or the apparent affinity for Ca^{2+} of Ca^{2+} accumulation by the endoplasmic reticulum of guinea pig peritoneal macrophages, under the condition of saponin treatment in the presence of an inhibitor of mitochondrial Ca^{2+} uptake (11). Therefore, it seems likely that even though stimulation of the Ca^{2+}-transport mechanism by CaM may be generally applicable to all types of plasma membranes, CaM stimulation of the Ca^{2+} transport of endoplasmic reticulum may be peculiar to cardiac muscle, probably due to the presence of a special protein, phospholamban, in cardiac sarcoplasmic reticulum (9).

CaM antagonists, both phenothiazines and naphthalenesulfonamides, inhibited the Ca^{2+} uptake of saponin-treated macrophages. However, inhibition of Ca^{2+} uptake by CaM antagonists required a higher concentration than did inhibition of CaM stimulation of PDE activity (22, 23), and the order of potency of inhibition of PDE activity by various CaM antagonists was not applicable to inhibition of Ca^{2+} uptake. Furthermore, inhibition of Ca^{2+} uptake by phenothiazines was not reduced by the addition of excess CaM, and TNI, which inhibited the CaM stimulation of PDE activity and phosphorylase kinase (20), did not inhibit Ca^{2+} uptake. Therefore, the inhibitory effect of Ca^{2+} uptake by CaM antagonists does not seem to be due to a specific effect on endogenous CaM in the saponin-treated macrophages. The inhibition of Ca^{2+} uptake by CaM antagonists was not modified by changes in the concentration of either Mg-ATP or free Ca^{2+}, both of which are important factors in Ca^{2+} uptake.

Kurebayashi and Ogawa (24) reported that CPZ as well as caffeine and halothane enhanced the Ca^{2+}-induced Ca^{2+} release mechanism in sarcoplasmic reticulum of skeletal muscle. Caffeine is a well-known stimulator of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum of skeletal, cardiac, and smooth muscle (25). However, Ca^{2+} which had accumulated in saponin-treated macrophages was not released by the addition of caffeine under conditions in which Ca^{2+} is readily released in the sarcoplasmic reticulum of muscles by the addition of caffeine.[4] Furthermore, the release of accumulated Ca^{2+} in saponin-treated macrophages by CPZ was not inhibited by an increase in free Mg^{2+} concentration. The Ca^{2+}-induced Ca^{2+} release in the sarcoplasmic reticulum of muscles was completely inhibited by an increase in free Mg^{2+} concentration (25). Thus, the release of Ca^{2+} in saponin-treated macrophages by CPZ might not be due to an enhancement of Ca^{2+}-induced Ca^{2+} release.

Phenothiazines are water-soluble cationic detergents and preferentially partition into phospholipid within the membrane, particularly to phosphatidylserine on the inner half of the lipid bilayer (29). In fact, Naccache *et al.* (30) reported that TFP induced the release of lactate dehydrogenase from rabbit peritoneal neutrophils, probably due to leakage of the plasma membrane. Naphthalenesulfonamide had the same effect on the plasma membrane of neutrophils, although much higher concentrations were required (30). Furthermore, TFP modified Ca^{2+} transport in the plasma membrane of cardiac muscle, apart from the specific effect on CaM (5). In addition, Kurebayashi and Ogawa (24) reported that CPZ released Ca^{2+} from the sarcoplasmic reticulum of skeletal muscle. Thus, inhibition of Ca^{2+} uptake and the release of accumulated Ca^{2+} in saponin-treated macrophages by CaM antagonists may not be related to a specific effect of the drugs on CaM, probably because of their detergent-like action.

In conclusion, the intracellular Ca^{2+}-accumulating system in guinea pig peritoneal macrophages was not enhanced by CaM. CaM antagonists, both phenothiazines and naphthalenesulfonamides, made the membrane leaky, inhibited Ca^{2+} uptake, and released accumulated Ca^{2+} in saponin-treated macrophages. Furthermore, these CaM antagonists strongly inhibited active Ca^{2+} uptake by mitochondria of guinea pig peritoneal macrophages, in low concentrations (31). Therefore, when one is using CaM antagonists in experiments on intact cells and/or membrane vesicles, great care should be taken in interpreting the results.

ACKNOWLEDGMENTS

We thank M. Ohara for critical reading of the manuscript, and K. Higuchi for secretarial services.

REFERENCES

1. Cheung, W. Y. Calmodulin plays a pivotal role in cellular regulation. *Science (Wash. D. C.)* **207**:19–27 (1980).
2. Jarrett, H. W., and J. T. Penniston. Partial purification of the (Ca^{2+} + Mg^{2+})-ATPase activator from human erythrocytes: its similarity to the activator of 3′:5′-cyclic nucleotide phosphodiesterase. *Biochem. Biophys. Res. Commun.* **77**:1210–1216 (1977).
3. Waisman, D. M., J. M. Gimble, D. B. P. Goodman, and H. Rasmussen. Studies of the Ca^{2+} transport mechanism of human erythrocyte inside-out plasma membrane vesicles. I. Regulation of the Ca^{2+} pump by calmodulin. *J. Biol. Chem.* **256**:409–414 (1981).
4. Niggli, V., J. T. Penniston, and E. Carafoli. Purification of the (Ca^{2+} + Mg^{2+})-ATPase from human erythrocyte membrane using a calmodulin affinity column. *J. Biol. Chem.* **254**:9955–9958 (1979).
5. Caroni, P., and E. Carafoli. The Ca^{2+} pumping ATPase of heart's sarcolemma:

[4] M. Hirata and T. Koga, unpublished observation.

characterization, calmodulin dependence, and partial purification. *J. Biol. Chem.* **256**:3263–3270 (1981).
6. Lew, D. P., and T. P. Stossel. Calcium transport by macrophage plasma membranes. *J. Biol. Chem.* **255**:5841–5846 (1980).
7. Pershadsingh, H. A., M. Landt, and J. M. McDonald. Calmodulin-sensitive ATP dependent Ca^{2+} transport across adipocyte plasma membranes. *J. Biol. Chem.* **255**:8983–8986 (1980).
8. Katz, S., and M. A. Remtulla. Phosphodiesterase protein activator stimulates calcium transport in cardiac microsomal preparations enriched in sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* **83**:1373–1379 (1978).
9. LePeuch, C. J., J. Haiech, and J. G. Demaille. Concerted regulation of cardiac sarcoplasmic reticulum transport by cyclic adenosine monophosphate-dependent and calcium-calmodulin-dependent phosphorylations. *Biochemistry* **18**:5150–5157 (1979).
10. Carafoli, E., V. Niggli, K. Malstrom, and P. Caroni. Calmodulin in natural and reconstituted calcium transporting system. *Ann. N. Y. Acad. Sci.* **356**:258–266 (1980).
11. Hirata, M., and T. Koga. ATP-dependent Ca^{2+} accumulation in intracellular membranes of guinea pig macrophages after saponin treatment. *Biochem. Biophys. Res. Commun.* **104**:1544–1549 (1982).
12. Yazawa, M., M. Sakuma, and K. Yagi. Calmodulins from muscles of marine invertebrates, scalop and sea anemone: comparison with calmodulins from rabbit skeletal muscle and pig brain. *J. Biochem.* **87**:1313–1320 (1980).
13. Ebashi, S. Separation of troponin into its three components. *J. Biochem.* **72**:787–790 (1972).
14. Watterson, D. M., W. G. Harrelson, P. M. Keller, F. Sharief, and T. C. Vanaman. Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase. *J. Biol. Chem.* **251**:4501–4513 (1976).
15. Cheung, W. Y. Cyclic 3′,5′-nucleotide phosphodiesterase: demonstration of an activator. *Biochem. Biophys. Res. Commun.* **38**:533–638 (1970).
16. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **277**:680–685 (1970).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
18. Jamieson, G. A., Jr., and T. C. Vanaman. Isolation and characterization of calmodulin from a murine macrophage-like cell line. *J. Immunol.* **125**:1171–1177 (1980).
19. Kakiuchi, S. Modulator protein (calmodulin; CDR) as a Ca^{2+}-receptive protein in the cell and its function. *Jpn. J. Pharmacol. [Suppl.]* **30**:47P (1980).
20. Shenolikar, S., P. T. W. Cohen, P. Cohen, A. C. Nairn, and S. V. Perry. The role of calmodulin in the structure and regulation of phosphorylase kinase from rabbit skeletal muscle. *Eur. J. Biochem.* **100**:329–337 (1979).
21. Vincenzi, F. F., T. R. Hinds, and B. U. Raess. Calmodulin and the plasma membrane calcium pump. *Ann. N. Y. Acad. Sci.* **356**:232–244 (1980).
22. Levin, R. M., and B. Weiss. Binding of trifluoperazine to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *Mol. Pharmacol.* **13**:690–697 (1977).
23. Raess, B. U., and F. F. Vincenzi. Calmodulin activation of red blood cell (Ca^{2+} + Mg^{2+})-ATPase and its antagonism by phenothiazines. *Mol. Pharmacol.* **18**:253–158 (1980).
24. Kurebayashi, N., and Y. Ogawa. Ca^{2+} releasing action of chlorpromazine on fragmented sarcoplasmic reticulum. *Jpn. J. Pharmacol. [Suppl.]* **31**:214P (1981).
25. Endo, M. Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**:71–108 (1977).
26. Ohtsuki, I., M. Manzi, G. E. Palade, and J. D. Jamieson. Entry of macromolecular tracers into cells fixed with low concentration of aldehydes. *J. Microsc. Biol. Cell.* **31**:199–126 (1978).
27. McMurray, W. C. Phospholipids in subcellular organelles and membranes, in *Form and Function of Phospholipids* (G. B. Ansell, R. M. C. Dawson, and J. N. Hawthorn, eds.). Elsevier, Amsterdam, 205–251 (1973).
28. Kakiuchi, S., R. Yamazaki, and H. Nakajima. Properties of a heat-stable phosphodiesterase activating factor isolated from brain extract. *Proc. Jpn. Acad.* **46**:587–592 (1970).
29. Carey, M. C., P. C. Horom, and D. M. Small. A study of the physiocochemical interactions between biliary lipids and chlorpromazine hydrochloride. *Biochem. J.* **153**:519–531 (1976).
30. Naccache, P. H., T. F. P. Molski, T. Alobaidi, E. L. Becker, H. J. Showell, and R. I. Sha'afi. Calmodulin inhibitors block neutrophil degranulation at a step distal from the mobilization of calcium. *Biochem. Biophys. Res. Commun.* **97**:62–68 (1980).
31. Hirata, M., E. Suematsu, and T. Koga. Calmodulin antagonists inhibit Ca^{2+} uptake of mitochondria of guinea pig peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **105**:1176–1181 (1982).

Send reprint requests to: Dr. Masato Hirata, Department of Biochemistry, Faculty of Dentistry, Kyushu University, Fukuoka 812, Japan.