Uncoupling of Ca^{2+} transport ATPase in muscle and blood platelets by diacylglycerol analogues and cyclosporin A antagonism

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The possibility that diacylglycerol analogues might have a wider spectrum of intracellular targets than the well-known protein kinase C was investigated with vesicles containing the Ca^{2+} -ATPase derived from the dense tubular system in platelets and from the sarcoplasmic reticulum of skeletal muscle. The diacylglycerol analogues PMA and 1-oleoyl-2-acetyl-*rac*-glycerol (OAG) inhibited Ca^{2+} accumulation by these vesicles, an effect that was antagonized by cyclosporin A. The inhibitory activity of PMA and OAG resulted from the uncoupling of the Ca^{2+} -ATPase, characterized by a pronounced inhibition of Ca^{2+} uptake accompanied by a discrete decrease in ATPase activity and by the inhibition of the enzyme's phosphorylation by P_i , leading to both a decrease in ATP synthesis and an enhancement of Ca^{2+}

efflux. The inhibition of Ca^{2+} uptake by PMA was found to decrease as the Ca^{2+} concentration in the medium was raised from 0.1 to 10.0 μM . This was observed with muscle, but not with platelet vesicles. In contrast, the ability of cyclosporin A to antagonize the inhibition of Ca^{2+} uptake by PMA also increased when the Ca^{2+} concentration in the medium was raised from 0.1 to 10.0 μM , but this was observed with both muscle and platelet vesicles. The fact that phospholipase C activity and products from the inositol metabolism have been described as localized in regions of the sarcoplasmic reticulum where Ca^{2+} -ATPase and Ca^{2+} channels are found suggests a possible physiological role for these products in the regulation of cytosolic Ca^{2+} levels.

INTRODUCTION

The activation of a cell-membrane receptor by an external signal can generate second-messenger systems capable of transducing this signal intracellularly. In several cell types the second-messenger cascade triggered involves the phosphoinositide cycle, with the hydrolysis of the membrane phospholipid $\text{PtdIns}(4,5)\text{P}_2$ by phospholipase C, leading to the generation of $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol. $\text{Ins}(1,4,5)\text{P}_3$ then mediates the release of Ca^{2+} from the endoplasmic reticulum, whereas diacylglycerol activates protein kinase C's turning on metabolic pathways under enzyme phosphorylation control [1,2]. Enzymes of the phosphoinositide cycle, phospholipase C activity and $\text{Ins}(1,4,5)\text{P}_3$ formation have also been found in the sarcoplasmic reticulum of skeletal muscle [3–6], in addition to the cell membrane.

Changes in cytosolic Ca^{2+} levels modulate many cellular responses, including muscle contraction, platelet activation and aggregation, and are therefore tightly controlled. Ca^{2+} storage in intracellular compartments is promoted by a membrane-bound protein, Ca^{2+} -ATPase. This enzyme translocates Ca^{2+} from the cytosol to the lumen of the sarcoplasmic/endoplasmic vesicles by using the energy derived from ATP hydrolysis [7–10]. The catalytic cycle of Ca^{2+} -ATPase can be reversed after a Ca^{2+} gradient has been formed across the vesicles' membrane. During this reversal Ca^{2+} leaves the vesicles through the ATPase in a process coupled with the synthesis of ATP from ADP and P_i [7,9,11,12]. It has been shown that arachidonic acid and several other hydrophobic drugs are able to uncouple the endoplasmic-reticulum Ca^{2+} -ATPases of different cell types; in this situation Ca^{2+} leaves the vesicles through the Ca^{2+} -ATPase without synthesizing ATP [10–18]. Thus, when uncoupled, the Ca^{2+} -ATPase behaves as a Ca^{2+} channel.

Increases in Ca^{2+} levels might activate many Ca^{2+} -dependent enzymes, including kinases such as protein kinase C and phos-

phatases such as calcineurin [19]. This latter enzyme has an important role in dephosphorylating key proteins of the signal-transduction pathways and is inhibited by the immunosuppressant drugs cyclosporin A (CsA) and FK506. The immunophilins are a family of soluble low-molecular-mass proteins that mediate the action of immunosuppressant drugs such as CsA and FK506. CsA binds to the cyclophilin class of immunophilins, and FK506 binds to the FK506-binding protein. Although the molecular mechanism whereby CsA and FK506 induce immunosuppression is not completely clear, it has been accepted that their mode of action depends on the conformation acquired after binding with their respective immunophilins [20,21]. Different authors have localized immunophilins to the sarcoplasmic reticulum, and the FK506-binding protein has been found to modulate both the $\text{Ins}(1,4,5)\text{P}_3$ and ryanodine Ca^{2+} channels [22–25].

In spite of the fact that both immunophilins and phospholipase C, which leads to the formation of diacylglycerol, have been identified in the sarcoplasmic reticulum, as far as we know a possible direct effect of these agents on the Ca^{2+} -ATPase has not been investigated before. In the present paper the effects of the diacylglycerol analogues PMA and 1-oleoyl-2-acetyl-*rac*-glycerol (OAG) and of the immunosuppressant drugs CsA and FK506 in Ca^{2+} transport were investigated by using vesicles derived from both the sarcoplasmic reticulum of skeletal muscle and endoplasmic reticulum of blood platelets.

METHODS

Outdated platelets were obtained from a blood bank in a concentrated form. Platelet vesicles derived from dense tubular system were prepared as described by Le Peuch et al. [26]. Sarcoplasmic-reticulum vesicles derived from the longitudinal

Abbreviations used: CsA, cyclosporin A; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol.

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tubules of rabbit skeletal muscle were prepared by the method of Eletr and Inesi [27]. This vesicle preparation does not contain significant amounts of ryanodine/cafeine-sensitive Ca^{2+} channels, nor does it exhibit the phenomenon of Ca^{2+} -induced Ca^{2+} release found in the heavy fraction of sarcoplasmic reticulum [28,29]. Protein concentration was estimated by the procedure of Lowry et al. [30].

Ca^{2+} uptake and efflux were measured by a filtration method with $^{45}\text{CaCl}_2$ and Millipore filters [31]. For the Ca^{2+} efflux experiments the vesicles were preloaded with ^{45}Ca in a medium containing 50 mM Mops/Tris (pH 7.0)/0.3 mM $^{45}\text{CaCl}_2$ /10 mM MgCl_2 /20 mM P_i /2 mM ATP containing 0.060 mg/ml vesicle protein. After 30–45 min of incubation at 35 °C, the loaded vesicles were sedimented by centrifugation at 100000 *g* for 40 min, the supernatant was discarded and the walls of the tubes were blotted to minimize contamination by the residual Ca^{2+} loading medium. The pellets were kept on ice and resuspended in ice-cold water immediately before use to a final concentration of 0.010 mg/ml protein.

ATP hydrolysis was determined by measuring the release of [^{32}P] P_i from [γ - ^{32}P]ATP. The reaction was quenched with 2 vol. of a suspension of activated charcoal in 0.1 M HCl [12]. After centrifugation, aliquots of the supernatant containing [^{32}P] P_i were counted in scintillation counter. Mg^{2+} -dependent ATPase activity was measured in the presence of 1 mM EGTA. The Ca^{2+} -stimulated ATPase activity was determined by subtracting the Mg^{2+} -dependent ATPase activity from the total ATPase activity measured in the presence of both Mg^{2+} and Ca^{2+} .

The free Ca^{2+} concentration was calculated as described by Fabiato and Fabiato [32] with the apparent association constants for Ca-EGTA determined by Schwartzenbach et al. [33].

[^{32}P] P_i was obtained from the Brazilian Institute of Atomic Energy and purified as described previously [12]. Phosphorylation of the Ca^{2+} -ATPase by [^{32}P] P_i was determined with Millipore filters as previously described and corrected for non-specific binding [12]. The reactions were quenched with 1 vol. of a mixture of 20% (w/v) trichloroacetic acid and 4 mM P_i . The filters were washed 15 times with 5 ml of an ice-cold solution containing 0.1 M HCl and 2 mM P_i and counted for radioactivity in a liquid-scintillation counter. Synthesis of ATP from ADP and [^{32}P] P_i was determined as previously described [12]. All experiments were performed at 35 °C.

PMA and OAG were purchased from Sigma; CsA and FK506 were gifts from Sandoz and Fujisawa Pharmaceutical Co. respectively. These four compounds were dissolved in DMSO. The amounts used were such that the DMSO concentration in the reaction mixture was never higher than 2% (v/v).

The results shown in the figures are typical experiments that were repeated at least three times with different membrane preparations.

RESULTS

Inhibition of Ca^{2+} uptake by PMA and OAG

The vesicles derived from skeletal muscle were able to accumulate a larger amount of Ca^{2+} than the vesicles derived from blood platelets [16,17]. The Ca^{2+} uptake of the two vesicles preparations was inhibited by the diacylglycerol analogues PMA and OAG (Figures 1–3). The addition of PMA to the medium after the vesicles were loaded promoted a rapid release of Ca^{2+} to the medium (Figure 2). For vesicles derived from platelets, the inhibitory activity of the diacylglycerol analogues did not depend on the Ca^{2+} concentration in the medium (Table 1), and the concentrations of OAG and PMA needed for 50% inhibition of Ca^{2+} uptake were found to be 0.8 and 2.0 $\mu\text{g/ml}$ respectively

Figure 1 Inhibition of Ca^{2+} uptake by PMA and antagonism by CsA in platelet (A) and muscle (B) vesicles

The assay medium composition was 50 mM Mops/Tris (pH 7.0)/50 μM $^{45}\text{CaCl}_2$ /2 mM MgCl_2 /10 mM P_i /2 mM ATP/0.010 mg/ml protein. Symbols: ●, control without drugs; ○, 10 $\mu\text{g/ml}$ PMA; ▲, 20 $\mu\text{g/ml}$ CsA; △, 10 $\mu\text{g/ml}$ PMA and 20 $\mu\text{g/ml}$ CsA.

(Figures 3A and 3B). For muscle vesicles, however, the inhibitory activity of PMA was found to vary with the Ca^{2+} concentration, being higher when the concentration of free Ca^{2+} in the medium was small (compare Figures 1B and 4). For platelet vesicles, the two drugs promoted an inhibition of both Ca^{2+} uptake and Ca^{2+} -dependent ATPase, but the decrease in the ATPase activity was less pronounced than that of uptake (Figure 3). For muscle vesicles an inhibition of the ATPase activity by PMA was noted only when the Ca^{2+} concentration in the medium was decreased from 10 to 0.4 μM (compare Figures 3B and 3C). The discrepancy between the inhibition of Ca^{2+} transport and that of ATPase activity suggests that the drugs uncouple the Ca^{2+} pump [7,14–18]. The cytosolic Ca^{2+} concentration of resting cells is in the range 0.2–0.4 μM , and it can increase to 10 μM during cell activation, leading to either muscle contraction or platelet aggregation [34]. The results shown in Figure 4(A) indicate that for skeletal muscle, PMA could trigger the release of Ca^{2+} when the cell is at rest, but the effect would decrease as the cytosolic Ca^{2+} concentration increased to a level sufficient to activate muscle contraction. This would not be observed in blood platelets, because the effect of the diacylglycerol analogues does not seem to be dependent on the Ca^{2+} concentration in the medium. This difference in Ca^{2+} dependence observed for the PMA effect is not related to different affinities for Ca^{2+} of the ATPase isoforms found in muscle and in platelet vesicles. In previous reports [35] it has been shown that the Ca^{2+} concentrations required for half-maximal activation of skeletal-muscle and blood platelet Ca^{2+} -ATPases are 0.14 and 0.25 μM respectively.

Another difference between the vesicles from platelets and muscle was noted when the Ca^{2+} uptake was measured in presence of different P_i concentrations. A large increase in the amount of Ca^{2+} accumulated by both the muscle and platelet vesicles was observed when the P_i concentration in the medium was raised from 1 to 10 mM. During transport this anion diffuses through the membrane and forms calcium phosphate crystals inside the vesicles, thus enhancing their loading capacity [7,9]. For the muscle vesicles, a 15–30% decrease in the inhibition promoted by PMA was detected when the P_i concentration was raised from 1 to 10 mM regardless of the Ca^{2+} concentration used (results not shown). In vesicles derived from blood platelets the inhibitory activity of PMA was practically the same in presence of either 1 or 10 mM P_i (Table 1).

Figure 2 Ca²⁺ release after the addition of PMA

The assay-medium composition was 50 mM Mops/Tris (pH 7.0)/2.5 mM ATP/2.5 mM MgCl₂/100 mM KCl/0.2 mM EGTA/0.185 mM ⁴⁵CaCl₂ containing either 10 mM (**A**, **B**) or 1 mM (**C**) P_i. The reaction was started by the addition of platelet (**A**) or muscle (**B**, **C**) vesicles to a final concentration of 0.010 mg/ml protein. The arrow indicates the addition of 10 µg/ml PMA. Symbols: ●, control without PMA; ○, after the addition of PMA.

Figure 3 Inhibition of Ca²⁺ uptake (●, ▲) and ATPase activity (○, △) by OAG and PMA

The assay medium composition was 50 mM Mops/Tris (pH 7.0)/2.5 mM MgCl₂/100 mM KCl/2 mM ATP/0.2 mM EGTA containing either 0.216 mM (**A**, **B**) or 0.128 mM (**C**) CaCl₂ and 10 mM P_i (**A**, **B**) or 1 mM (**C**) P_i. The free Ca²⁺ concentration in (**A**) and (**B**) was 10 µM and in (**C**) was 0.4 µM. The reaction was started by the addition of platelet (●, ○) or muscle (▲, △) vesicles to a final concentration of 0.010 mg/ml protein. In (**A**) and (**B**), 100% of Ca²⁺ uptake and ATPase activity for muscle vesicles (▲, △) were 3.47 µmol of Ca²⁺/30 min per mg and 5.06 µmol of P_i/30 min per mg respectively; for platelet vesicles (●, ○) these values were 0.11 µmol of Ca²⁺/60 min per mg and 3.14 µmol of P_i/60 min per mg respectively. In (**C**), 100% of Ca²⁺ uptake and ATPase activity for muscle vesicles were 1.02 µmol of Ca²⁺/30 min per mg and 34.8 µmol of P_i/30 min per mg respectively.

Table 1 Platelet vesicles: inhibition of Ca²⁺ uptake by PMA in the presence of different Ca²⁺ and P_i concentrations

The composition of the reaction medium was 50.0 mM Mops/Tris (pH 7.0)/2.0 mM MgCl₂/2.0 mM ATP/0.2 mM EGTA/0.010 mg/ml vesicle protein. The reaction time was 60 min. The values are means ± S.E.M. for three to six experiments.

Additions		[PMA] (µg/ml) ...	Ca ²⁺ uptake					
			(nmol/mg)			(%)		
[P _i] (mM)	[Ca ²⁺] (µM)		0	1	10	0	1	10
1	0.5		80 ± 14	60 ± 11	24 ± 10	100	75	30
	10.0		127 ± 5	77 ± 22	28 ± 13	100	61	22
10	0.5		518 ± 152	379 ± 6	155 ± 65	100	73	30
	10.0		433 ± 20	360 ± 56	100 ± 37	100	83	23

Figure 4 Correlation between Ca²⁺ concentration and PMA inhibitory activity in muscle vesicles

The assay medium composition in (A) was 50 mM Mops/Tris (pH 7.0)/2.5 mM MgCl₂/100 mM KCl/2 mM ATP/10 mM P_i/0.2 mM EGTA and different CaCl₂ concentrations to yield the free Ca²⁺ concentrations shown in the Figure. The assay medium in (B) was as in (A), except that the P_i and free Ca²⁺ concentrations were 1 mM and 0.5 μM respectively. Symbols: (A) ●, without PMA; ○, with 10 μg/ml PMA; (B) ●, without PMA; △, 0.4 μg/ml PMA; □, 1 μg/ml PMA; ○, 10 μg/ml PMA.

The effect of PMA does not seem to be mediated by protein kinase C, because the inhibition of Ca²⁺ uptake by increasing concentrations of PMA was exactly the same in the presence and the absence of 0.25 mM staurosporine, a protein kinase C inhibitor [36].

Ca²⁺ leakage and reversal of the Ca²⁺ pump

In earlier reports it was shown that Ca²⁺ leaks through the pump when vesicles previously loaded with Ca²⁺ are incubated in media containing EGTA and very low concentrations (0.1 mM) of both

P_i and Mg²⁺ [13]. The leakage decreases when the Ca²⁺-ATPase is phosphorylated by P_i, a condition attained when the concentrations of both P_i and Mg²⁺ are raised to 4 mM. PMA and OAG were found to both increase the leakage of Ca²⁺ and inhibit the phosphorylation of the muscle ATPase by P_i (Figure 5 and Table 2). The enhancement of Ca²⁺ leakage promoted by PMA could be detected even after the concentrations of P_i and Mg²⁺ were raised from 0.1 to 4.0 mM (Figures 5A and 5B). The reversal of the Ca²⁺ pump is observed when ADP is added to the medium after Ca²⁺-loaded vesicles have been phosphorylated by P_i [7,12,37]. Under this condition, the phosphoenzyme transfers its phosphate to ADP, leading to the synthesis of ATP and an increase in the Ca²⁺ efflux rate (Figures 5C and 5D). The enhancement of the Ca²⁺ efflux promoted by ADP is referred to in the literature as active Ca²⁺ efflux [11,12,37]. OAG and PMA inhibited both the active Ca²⁺ efflux and the synthesis of ATP observed after the addition of ADP (Figures 5C and 5D, and Table 2). The concentrations of PMA needed for half-maximal inhibition of ATP synthesis and of enzyme phosphorylation by P_i of the muscle vesicles were found to be practically the same (Figure 5D). This indicates that the inhibition of ATP synthesis was promoted by a decrease in the steady-state level of phosphoenzyme and not by an impediment in the phosphate transfer from the phosphoenzyme to ADP [7,12,37]. Similarly to muscle, in vesicles derived from blood platelets, PMA and OAG were found to increase the Ca²⁺ leakage measured in the absence of ADP and to inhibit the synthesis of ATP measured during reversal of the Ca²⁺ pump (Figure 6 and Table 2). We were not able to measure the phosphorylation by P_i of the platelet Ca²⁺-ATPase [8,17].

Effect of CsA

The inhibition of Ca²⁺ transport, Ca²⁺ efflux and ATP synthesis promoted by either PMA or OAG was antagonized by CsA (Figure 1 and Table 2). For the Ca²⁺-uptake measurements, the effect of CsA was found to be critically dependent on the Ca²⁺ concentration in the medium. In both vesicle preparations the antagonism of PMA by CsA decreased as the Ca²⁺ concentration

Figure 5 Effect of PMA on Ca²⁺ efflux (A–C), enzyme phosphorylation by P_i and ATP synthesis (D)

For the efflux experiments, vesicles previously loaded with ⁴⁵Ca were diluted to a final concentration of 0.010 mg/ml protein in a medium containing 50 mM Mops/Tris, pH 7.0, 2 mM EGTA and (A) 0.1 mM P_i and 0.1 mM MgCl₂, (B) 4 mM P_i and 4 mM MgCl₂ or (C) 4 mM P_i, 4 mM MgCl₂ and 0.1 mM ADP. The active Ca²⁺ efflux (▲, △) was calculated in (C) by subtracting the passive Ca²⁺ efflux measured in (B) from the total Ca²⁺ efflux measured in (C) (●, ○). Symbols in (A–C): ●, ▲, controls without PMA; ○, △, with 10 μg/ml PMA. In (D), for phosphoenzyme formation (○) the assay medium contained 50 mM Mops/Tris, pH 6.0, 2 mM EGTA, 1 mM [³²P]P_i, 10 mM MgCl₂, 2 mM EGTA and 0.020 mg/ml protein. The reaction time was 30 s. For ATP synthesis (●), the vesicles were preloaded with Ca²⁺ and diluted to a final concentration of 20 μg/ml in a medium containing 50 mM Mops/Tris, pH 7.0, 1 mM [³²P]P_i, 0.2 mM ADP, 1 mM MgCl₂ and 2 mM EGTA. In the Figure, 100% represents 1.24 μmol/g of phosphoenzyme and 0.12 μmol of ATP synthesized/min per mg.

Table 2 Antagonism by CsA of the PMA and OAG effects on the Ca²⁺-ATPase of muscle and platelets

In all experiments the concentrations of PMA and CsA were 10 and 20 $\mu\text{g/ml}$ respectively. The concentration of OAG was 10 $\mu\text{g/ml}$ for the experiments with muscle vesicles and 4 $\mu\text{g/ml}$ for those with platelet vesicles. The values in the Table are percentages of the control (without the addition of either OAG, PMA or CsA). Each value is the average of three to ten experiments. For the muscle vesicles the 100% values of Ca²⁺ uptake, ATPase activity, Ca²⁺ efflux and ATP synthesis were 107 ± 13 , 150 ± 49 , 244 ± 18 and 97 ± 8 nmol/min per mg of protein respectively, and for the platelet vesicles they were 4.1 ± 0.5 , 26.0 ± 5.2 , 5.9 ± 0.5 and 1.7 ± 0.4 nmol/min per mg of protein respectively. For phosphorylation of the muscle enzyme by P_i the steady-state level of the control (100%) was 2.28 ± 0.23 μmol of phosphoenzyme/g of protein. The assay medium and experimental conditions were as described in the legends to Figures 1, 3(A), 3(B), 5(A), 5(D) and 6.

Tissue	Additions	Ca ²⁺ uptake (%)	ATPase activity (%)	Ca ²⁺ efflux (%)	Phosphoenzyme (%)	ATP synthesis (%)
Muscle	None	100	100	100	100	100
	PMA	59	100	206	43	36
	PMA + CsA	102	100	104	90	73
	OAG	21	98	230	33	—
	OAG + CsA	74	100	99	62	—
Platelet	None	100	100	100	—	100
	PMA	13	58	191	—	12
	PMA + CsA	70	101	102	—	56
	OAG	27	35	—	—	36
	OAG + CsA	79	69	—	—	94

Figure 6 Platelet vesicles: increase in passive Ca²⁺ efflux and inhibition of ATP synthesis by PMA

Ca²⁺ efflux and ATP synthesis were measured as described for Figure 5. Symbols in (A): ●, without PMA; ○, with 10 $\mu\text{g/ml}$ PMA. In (B), 0% (without PMA) and 100% (10 $\mu\text{g/ml}$ PMA) enhancement of Ca²⁺ efflux corresponds to efflux rates of 0.055 and 0.110 μmol of Ca²⁺/10 min per mg respectively. For ATP synthesis, 100% corresponds to 0.017 μmol of ATP/10 min per mg.

of the medium went down from 10.0 to 0.5 μM (compare Figures 7B and 7C). Notice in Table 2 that CsA antagonized the effects of PMA on the Ca²⁺ efflux and ATP synthesis. These two features of the Ca²⁺ pump are measured in the absence of Ca²⁺ in the medium. At present we do not know why the Ca²⁺ dependence of the effect of CsA was apparent only when measuring the Ca²⁺ uptake and not when measuring the Ca²⁺ efflux and reversal of the Ca²⁺ pump.

The effects of PMA and OAG seem to be specifically antagonized by CsA, because FK506, another immunosuppressant drug, in concentrations from 0.1 to 40.0 $\mu\text{g/ml}$, did not antagonize the effects of either PMA or OAG measured in either platelet or muscle vesicles (results not shown). In earlier reports it was shown that the Ca²⁺-ATPase of both muscle and blood platelets is uncoupled by heparin, trifluoperazine and arachidonic acid [14–18]. Neither CsA nor FK506 were able to antagonize the

effects of these three drugs in muscle or platelet vesicles (results not shown).

DISCUSSION

The findings described indicate that, in addition to activating protein kinase C, diacylglycerol might also act directly on the sarcoplasmic/endoplasmic-reticulum Ca²⁺-ATPase, impairing Ca²⁺ accumulation. The inability, induced by PMA and OAG, to accumulate Ca²⁺ inside the vesicle preparations is explained by the increase in Ca²⁺ leakage from the vesicles, and to a smaller extent by Ca²⁺-pumping inhibition as assessed by ATPase activity. Furthermore the effects produced by the diacylglycerol analogues tested were antagonized by CsA.

It could be that, in addition to interacting with the Ca²⁺-ATPase, PMA and OAG also alter the structure of the lipid bilayer, thus enhancing the membrane permeability. The following results argue against this possibility: (1) the enhancement of Ca²⁺ efflux promoted by both PMA and OAG was abolished by CsA (Table 2); (ii) the inhibition of Ca²⁺ uptake greatly decreased when the Ca²⁺ concentration in the medium was raised from 0.1 to 10 μM (Figures 1B and 4); (3) the effect of PMA in Figure 7 was only antagonized by CsA when the Ca²⁺ concentration in the medium was increased from 0.1 to 10 μM . As far as we know, there is no published study to indicate that CsA can bind to the membrane lipids and alter the structure of the bilayer. Similarly, Ca²⁺ is only able to bind to the membrane when its concentration in the medium is raised to the range of 1–10 mM, i.e. to values two to three orders of magnitude higher than those used in this paper [7].

To rule out a possible involvement of protein kinase C, staurosporine, a potent protein kinase antagonist [36], was used, and did not antagonize the effect of PMA and OAG on Ca²⁺ transport. In spite of the fact that PMA was shown to be antagonized by CsA in various cell lines [38,39], the activation of protein kinase C by PMA is not antagonized by CsA [39], suggesting that, if this drug is capable of modifying the effects produced by the diacylglycerol analogues, this does not occur via the protein kinase C pathway.

In contrast, a number of pieces of evidence presented here indicate that the effect of PMA and OAG on Ca²⁺ accumulation is mediated by the Ca²⁺-ATPase itself and not by the Ins(1,4,5)P₃-

Figure 7 P_i and Ca^{2+} dependence of antagonism of PMA inhibition by CsA in platelet (●, ○) and muscle (▲, △) vesicles

The P_i and free Ca^{2+} concentrations in (A) and (B) were 10 mM and 10 μ M respectively and in (C) were 1 mM and 0.5 μ M respectively. Other additions to the assay medium were as in Figure 3. The reaction time was 60 min. Symbols: (A) ●, ▲, without PMA; ○, △, with 10 μ g/ml PMA; (B, C) ○, △, without CsA; ●, ▲, with 20 μ g/ml CsA.

sensitive or the ryanodine-sensitive Ca^{2+} channels. (1) The muscle vesicles used correspond to the light fraction derived from the longitudinal sarcoplasmic reticulum, which does not contain either the $Ins(1,4,5)P_3$ or the ryanodine Ca^{2+} channels [28,29]. (2) Both the $Ins(1,4,5)P_3$ and the ryanodine Ca^{2+} channels are modulated by immunophilins that bind to FK506 and not to CsA [20–25]. Despite the antagonism produced by CsA, the effects of PMA and OAG were not inhibited by FK506. (3) The Ca^{2+} -dependent ATPase activity of the vesicles was inhibited by PMA and OAG (Figure 3), contrary to what would be expected in a situation where the enhancement of Ca^{2+} permeability (i.e. Ca^{2+} ionophore) decreased the intravesicular concentration of this ion and therefore enhanced the rate of ATP hydrolysis [7]. (4) Phosphorylation by P_i , a specific reaction of the Ca^{2+} -ATPase, was inhibited by the analogues (Figure 5D and Table 2). It was shown that when this reaction is impaired the Ca^{2+} -ATPase becomes uncoupled and the Ca^{2+} already accumulated by the vesicles leaks to the medium through the ATPase [13,14,16,18,28].

Both muscle and blood platelets can be uncoupled by different hydrophobic drugs including heparin, arachidonic acid and trifluoperazine [14–18]. CsA regulates only the uncoupling promoted by PMA and OAG and not that induced by other hydrophobic drugs. Although it is not known whether regulation of this effect by CsA occurs via immunophilins, it is worth noting that immunophilins have already been shown to be associated with the membrane of the sarcoplasmic reticulum of skeletal muscle and the endoplasmic reticulum of different cells [22–24].

Two different Ca^{2+} dependences were observed, one related to the antagonism promoted by CsA and the other the inhibitory effect itself produced by PMA and OAG. CsA antagonism of the effect of PMA on Ca^{2+} uptake varied with the free Ca^{2+} concentration in both muscle and platelet vesicle preparations. The effect of PMA on the rate of Ca^{2+} uptake also varied with the free Ca^{2+} concentration, but this was observed only in muscle vesicles (Figure 4), not in vesicles derived from blood platelets (Table 1). This might be related to structural differences between the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase isoforms and could reflect differences between platelet and muscle cell requirements for cellular Ca^{2+} control [40–42].

The concentrations of CsA reported in published work to modify the properties of cells or cell organelles vary greatly, from

50–100 ng/ml to 10 μ g/ml, used for the inhibition of T-cell proliferation and to augment receptor-stimulated Ca^{2+} fluxes in isolated hepatocytes respectively [43–45]. The concentration range used for Ca^{2+} -flux experiments in hepatocytes is similar to that needed to antagonize the effects of diacylglycerol analogues on the Ca^{2+} -ATPase (Figure 7).

Finally, studies in rabbit skeletal muscle have reported phospholipase C activity both in the cytosol and bound to the membrane of the sarcoplasmic reticulum, particularly in the triad fraction of the sarcoplasmic reticulum [3–6]. This indicates that diacylglycerol can be made available in regions of the cell where the Ca^{2+} -ATPase is found. The major difference between the triad and the longitudinal tubules of the sarcoplasmic reticulum is the distribution of the $Ins(1,4,5)P_3$ -sensitive and ryanodine-sensitive Ca^{2+} -channels. Whereas the Ca^{2+} -ATPase is found in both regions, the $Ins(1,4,5)P_3$ and ryanodine channels are found only in the triads [7]. This raises the possibility that both channels and the Ca^{2+} -ATPase can be modulated by the products of phospholipase C, $Ins(1,4,5)P_3$ opening the Ca^{2+} channel and diacylglycerol uncoupling the Ca^{2+} -ATPase, both leading to Ca^{2+} release and accumulation in the region of the triad.

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