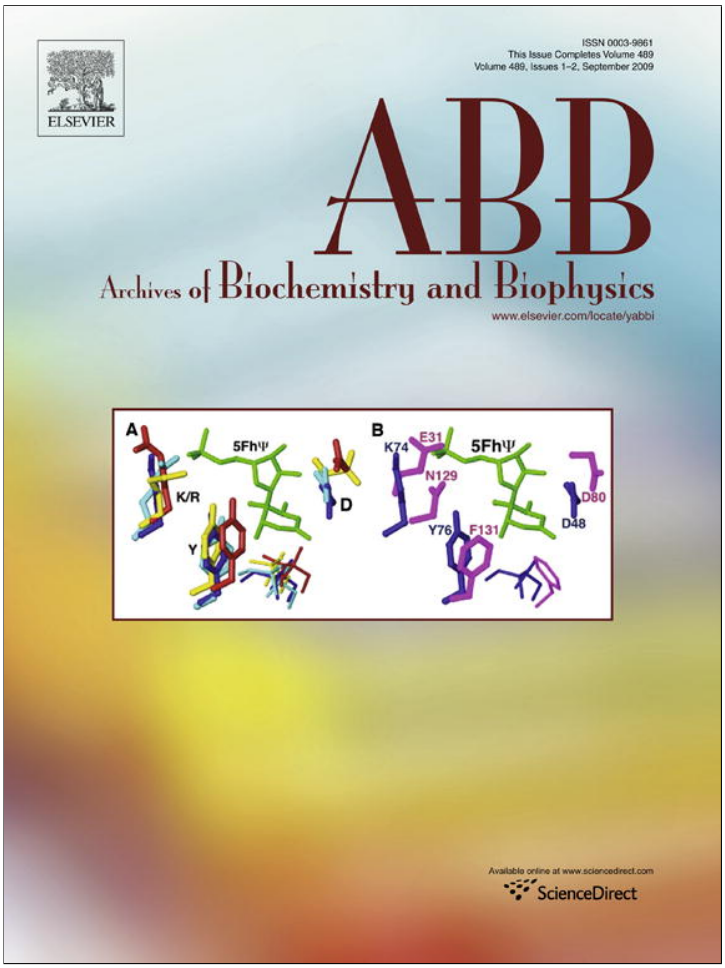

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Diacylglycerol regulates the plasma membrane calcium pump from human erythrocytes by direct interaction

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ABSTRACT

The plasma membrane Ca^{2+}-ATPase (PMCA) plays a key role in the regulation of the intracellular Ca^{2+} concentration. Ethanol stimulates this Ca^{2+} pump in an isoform-specific manner. On search for a physiological molecule that could mimic the effect of ethanol, we have previously demonstrated that some sphingolipids containing free "hydroxyl" groups, like ceramide, are able to stimulate the PMCA. Since diacylglycerol (DAG) structurally shares some characteristics with ceramide, we evaluate its effect on the PMCA. We demonstrated that DAG is a potent stimulator of this enzyme. The activation induced is additive to that produced by calmodulin, protein-kinase C and ethanol, which implies that DAG interacts with the PMCA through a different mechanism. Additionally, by different fluorescent approaches, we demonstrated a direct binding between PMCA and DAG. The results obtained in this work strongly suggest that DAG is a novel effector of the PMCA, acting by a direct interaction.

Ca^{2+} has been widely recognized as an intracellular signal, responsible for the control of a large number of cellular functions. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in resting cells is around 100 nM, a concentration far below equilibrium with that of the extracellular Ca^{2+} [1]. The plasma membrane calcium pump (PMCA)[1] [2], a member of the P-type ion-motive ATPase family [3] is one of the most important mechanisms for Ca^{2+} export present in all eukaryotic cells so far studied. This enzyme is responsible for regulating the resting levels of $[Ca^{2+}]_i$, and thus for ultimately controlling the activity of the numerous Ca^{2+}-sensitive enzymes [4]. The activity of this enzyme is highly regulated, being stimulated by calmodulin (CaM), [5,6], acidic phospholipids and polyunsaturated fatty acids [7], phosphorylation by cAMP-dependent protein-kinase [8] and by protein-kinase C (PKC) [9]. Beside, the PMCA can also be stimulated by controlled proteolysis, organic solvents and auto-aggregation of the enzyme [10–12]. We have shown that ethanol is able to stimulate this pump to a higher level than all the effectors previously mentioned [13,14]. Ethanol effect on the PMCA, besides being isoform-specific [14] is additive to that of CaM, which indicates that these two effectors stimulate the enzyme by different mechanisms. Because ethanol is not a physiological effector, we sought for an endogenous compound capable to induce similar effects on the calcium pump. In the search of such compounds we have studied the effect of amphiphilic lipids that, similar to ethanol, possesses free "hydroxyl" groups that evokes ethanol structure. This is the case of the some sphingolipids (ceramide and sphingosine), which act in many systems in combination with Ca^{2+} and even regulating the $[Ca^{2+}]_i$ [15,16]. Ceramide, which is a signal sphingolipid that induces apoptosis in many cancer cell lines [17] and regulates other enzyme processes [18], stimulates the PMCA from human erythrocytes [19] and from renal cells [20]. Ceramide affects positively both the affinity for Ca^{2+} and the V_{max} of the ATPase activity of PMCA. On the other hand, sphingosine, which has been reported to act in many systems antagonistically with ceramide, showed an inhibitory effect on Ca^{2+}-ATPase activity [19].

It is well known that the second messenger diacylglycerol (DAG), regulates PMCA by an indirect way. DAG is produced from phosphatidyl inositol 4,5 bis-phosphate hydrolysis by phospholipase C (PLC). One product, inositol trisphosphate ($InsP_3$), diffuses into the cytosol and stimulates the release of Ca^{2+} from the endoplasmic reticulum, associated with the plasma membrane and activates PKC [21], which in turn phosphorylates the PMCA [9]. It is well known that the action of this kinase and CaM on the PMCA are not additive but instead mutually exclusive [22,23]. In this work we studied the effect of DAG on the PMCA regulation, taking in consideration that this messenger lipid also possesses a free "hy-

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[1] *Abbreviations used*: BFP, blue fluorescent protein; CaM, calmodulin; DAG, diacylglycerol; DMSO, dimethylsulfoxide; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; IOV, Inside-out plasma membrane vesicles; $InsP_3$, inositol trisphosphate; PIP2, phosphatidyl inositol 4,5 bis-phosphate; PKC, protein-kinase C; PLC, phospholipase C; PMA, phorbol myristate acetate; PMCA, plasma membrane Ca^{2+}-ATPase; TNP-ATP, 2′(3′)-O-2,4,6-triphenyladenosine 5′-triphosphate.

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droxyl" group and a general structure that resembles ceramide. We show here that DAG is able to increase the activity of the PMCA in a manner which is additive to the stimulation induced by PKC and interacting directly with the enzyme.

Materials and methods

Chemicals

All the reagents were of the highest purity available. Diacylglycerol (1,2-dioctanoyl-sn-glycerol) and phorbol 12-myristate 13-acetate (PMA) were purchased from SIGMA and from Avanti Polar Inc. Other reagents were from Sigma. Rat brain protein-kinase C (isoforms α, β_1, β_2 and γ) and bis-indoliyl-maleimide I-HCl were purchased from CALBIOCHEM. Stocks of concentrated lipids were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO in the reaction mixture was always below 1%. Calmodulin was purified from bovine brain according to the method of Guerini et al. [24] with the modifications introduced in [25].

Purification of the erythrocytes Ca^{2+}-ATPase

Plasma membrane Ca^{2+}-ATPase was purified from human erythrocyte membranes [26] by using a calmodulin affinity column as has described before [27]. A coupled-enzyme assay system was used to determinate the Ca^{2+}-ATPase activity during purification of the enzyme, as described in [28]. The medium contained 1 U/mL pyruvate kinase, 1 U/mL lactic dehydrogenase, 100 mM KCl, 30 mM Hepes/KOH (pH 7.4), 2.5 mM $MgCl_2$, 0.2 mM NADH, 0.5 mM phosphoenolpyruvate, 1 mM ATP and 50 μM $CaCl_2$. The reaction was monitored following the difference in absorbance between 366 and 550 nm, using a dual-wavelength spectrophotometer (AMINCO DW-2a) at 37 °C in a final volume of 1 mL. The purified enzyme was stored, after N_2 bubbling, at −70 °C at a concentration of 100–200 μg/mL in a buffer containing 130 mM KCl, 20 mM Hepes/KOH (pH 7.2), 2 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol, 0.05% Triton X-100, 0.5 mg/mL phosphatidylcholine, 50 μM $CaCl_2$ and 5% glycerol (v/v).

Determination of the ATPase activity

Aliquots of purified Ca^{2+}-ATPase (about 1–2 μg of protein/mL) were incubated in a medium containing 130 mM KCl, 20 mM Hepes/KOH (pH 7.4), 1 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP and the appropriate quantity of $CaCl_2$, to obtain the desired free calcium concentrations. The final concentration of ionic calcium was calculated using an iterative computer program modified by Fabiato and Fabiato [29].

The reaction was incubated for 45 min at 37 °C, and stopped by the addition of cold trichloroacetic acid at 8% v/v (final concentration). The phosphate produced by ATP hydrolysis was determined according to the method of Fiske and SubbaRow [30], but using $FeSO_4$ as the reducing agent. Appropriate blanks were included to correct any interference with the colorimetric method.

Ca^{2+} transport by inside-out plasma membrane vesicles from human erythrocytes

Inside-out plasma membrane vesicles (IOV's) from erythrocytes were prepared as described by Sarkadi et al. [31]. Ca^{2+} transport was determined by the use of arsenazo III as calcium indicator using the wavelength pair 675–685 nm, following the methodology previously described [19]. Briefly, aliquots of IOV's was diluted in a final volume of 1 mL of a buffer containing 160 mM KCl, 50 μM arsenazo III, 0.5 mM $MgCl_2$, 25 μM $CaCl_2$ and 10 mM Tris–HCl (pH 7.4) at 37 °C. The Ca^{2+}-transport was initiated by the addition of 0.5 mM ATP. The system was calibrated by successive additions of known concentrations of $CaCl_2$.

Protein determination

The protein concentration of the plasma membrane fraction was determined by the Biuret assay [32] in the presence of deoxycholate, using bovine serum albumin as standard. The method of Lowry et al. [33] was used for the purified protein. To avoid interference from detergents and phospholipids, the protein was precipitated with trichloroacetic acid in the presence of deoxycholate [34].

Fluorescence methods

Steady-state fluorescence spectra were recorded on a HITACHI F-2000 spectrofluorimeter in a 0.5 cm path length cuvette, with a 10 nm excitation and emission band-pass, in a stirred cell holder thermostated at 22 °C. Titration experiments were performed by adding 5 μL aliquots of a working solution to 500 μL of the protein solution. Excitation and emission wavelengths are indicated for each experiment. The measured fluorescence intensity was corrected for light scattering by background subtraction. The inner filter was corrected at both, excitation and emission wavelengths, as describe elsewhere [35]. In the case of the quenching of intrinsic fluorescence, the effect of the dilution of the protein on the fluorescence was considered.

Data analysis

All calculation, manipulation and correction of experimental data were performed in Windows Excel 2000 (Microsoft Corp.). The values of K_m and V_{max} were determined using Eadie–Hofstee plots and the computer program Enzfitter 1.03 (Elsevier Biosoft). The symbols in the plots represent the average from n (indicated in the figure) independent determination ± SD. Statistical significance was determined by Student's t test. Significance was considered for $P < 0.05$. Plotting were performed using Origin 6.0 (Microcal Software Inc.).

Results

To investigate the effect of diacylglycerol on the Ca^{2+}-ATPase activity, aliquots of the purified enzyme from human erythrocytes were incubated with different concentrations of DAG. As shown in Fig. 1, DAG stimulates Ca^{2+}-ATPase activity in a dose-dependent manner. The maximal stimulation was observed at a concentration of 30 μM, in the same range as reported for the stimulation of the PKC [23] (Fig. 1). We also studied the effect of DAG in the presence of an optimal concentration of CaM and of ethanol, taking into consideration that this alcohol produces a marked increase on the PMCA activity [13,36]. The results showed an additive effect of

Table 1
Effect of diacylglycerol, calmodulin and ethanol on the $K_m(Ca^{2+})$ and V_{max} of the plasma membrane Ca^{2+}-ATPase.

Condition	V_{max} (μmol Pi mg^{-1} min^{-1})	$K_m(Ca^{2+})$ (μM)
Control	1.00 ± 0.05	1.05 ± 0.08
DAG	2.42 ± 0.18	0.25 ± 0.08
CaM	2.36 ± 0.20	0.56 ± 0.06
EtOH	2.01 ± 0.09	0.43 ± 0.07
CaM + DAG	3.60 ± 0.31	0.13 ± 0.07
EtOH + DAG	4.05 ± 0.38	0.15 ± 0,03

Values of the parameters V_{max} and K_m^{Ca} for the ATPase activity of PMCA. Experimental conditions presented in Figs. 2 and 3.

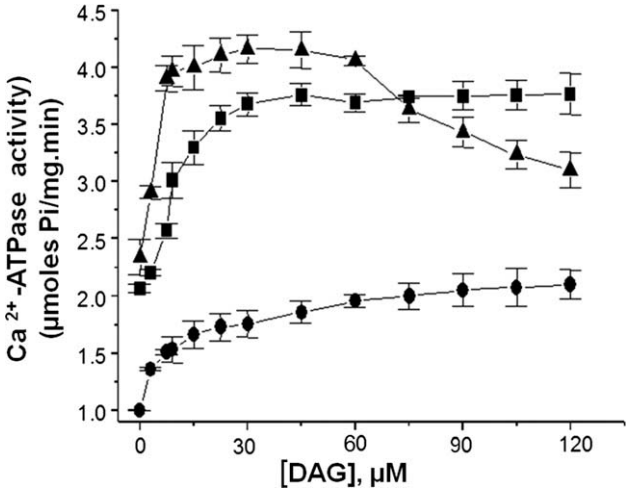

Fig. 1. Stimulation of plasma membrane Ca^{2+}-ATPase activity by DAG. The final Ca^{2+} concentration of was 10 µM. The determination of Ca^{2+}-ATPase activity was carried out by following the phosphate produced by ATP hydrolysis. The reaction was started by the addition of 1 µg/ml purified Ca^{2+}-ATPase in a final volume of 0.5 ml at 37 °C under continuous stirring. (●) Control, (■) 5 µg/mL CaM, and (▲) 5% ethanol. The reaction was incubated for 45 min and arrested by the addition of cold trichloroacetic acid (8%, final concentration). Other details under *Materials and methods*. Values represent the means ± SD. from at least five independent experiments.

DAG with CaM as well as with ethanol (Fig. 1). Thus, both effectors induced a large stimulation in addition to that provoked by the signaling lipid. The effect of DAG seems to be independent of the effect of CaM, since the 2-fold stimulation produced by the proteic modulator, is observed throughout the whole range of DAG tested (Table 1). On the other hand, the interaction of DAG with ethanol on the enzyme appears to be more complex. The 2.15-fold stimulation by ethanol alone, increase rapidly up to 3.1-fold and then decreased progressively in two phases to 1.5-fold (Fig. 1).

The effect of diacylglycerol on both the Ca^{2+} affinity and the V_{max} of the enzyme was determined in the presence of CaM and ethanol, incubating the enzyme with different free Ca^{2+} concentrations with or without 30 µM DAG. Fig. 2 shows the results of experiments performed with CaM, DAG and with both modulators present simultaneously. CaM increased both the V_{max} (from 1.0 ± 0.05 to 2.36 ± 0.2 µmol Pi mg^{-1} min^{-1}) and the affinity of the enzyme for Ca^{2+} (K_m from 1.05 ± 0.08 to 0.56 ± 0.06 µM) (Table 1). On the other hand, DAG produced an effect on the V_{max} which is similar to that exerted by CaM, but decreased the K_m for Ca^{2+} to 0.25 ± 0.08 µM. The simultaneous additions of CaM and DAG produced an increment in the V_{max} to values as high as 3.6 ± 0.31 µmol P_i mg^{-1} min^{-1}, and an associated decrease in K_m for Ca^{2+} down to 0.13 ± 0.07 µM. In regard to the effect of ethanol, Fig. 3 shows an increase in the V_{max} of the ATPase activity to 2.01 ± 0.09 µmol P_i mg^{-1} min^{-1}, with a concomitant decrease in the K_m for Ca^{2+} to 0.43 ± 0.07 µM. Addition of DAG and ethanol simultaneously, increased the V_{max} of the ATPase activity to 4.05 µmol P_i mg^{-1} min^{-1}, a value which is 4 times higher than the maximum activity of the control. On the other hand, the value of K_m for Ca^{2+} under this condition was 0.15 ± 0.03 µM, about one third of the value obtained by ethanol and one sixth of the value determined for the control samples.

It is also demonstrated that when DAG was used in combination with PKC, the two effector showed an additive effect on the enzyme. As can be seen in Fig. 4, the specific ATPase activity of the enzyme was 4-fold higher in the simultaneous presence of DAG and PKC, in comparison to the 2-fold stimulation induced by DAG alone or PKC with the phorbol ester PMA. We also tested the possible direct effect of PMA on Ca^{2+}-ATPase activity, observing that this phorbol ester did not produce any change (Fig. 4). To further dissect the component of stimulation by the direct action of DAG and via PKC in the activation of Ca^{2+}-ATPase, the experiments were conducted in the presence of bis-indolyl-maleimide, a known potent PKC inhibitor [37]. As observed, this compound, when added simultaneously with DAG and PKC, inhibited the PKC component of the stimulation, restoring the values to that obtained when DAG was added alone. In the same sequence of experiments, we tested the effect of the combination of DAG, PKC and PMA, showing that the phorbol ester does not produce any significant difference with respect to the same experiment performed with the combinations DAG and PKC, or DAG and CaM.

All the above results strongly suggest that DAG could be a possible modulator of the Ca^{2+}-ATPase. Thus, we next evaluated the possible physiological effect of this second messenger on the enzyme activity *in situ*. First, we studied the effect of DAG on the Ca^{2+}-ATPase activity on human red cell membrane fragments. The results were similar to those obtained with the purified and

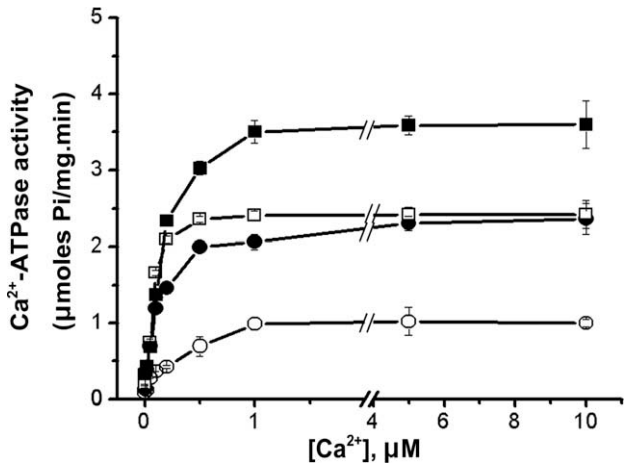

Fig. 2. Effect of DAG and calmodulin on Ca^{2+}-dependence of the ATPase activity of PMCA. An additive effect of DAG and calmodulin on the ATPase activity can be observed when both effector were added simultaneously. Experimental conditions were: (○) Control, (●) 5 µg/ml CaM, (□) 30 µM DAG, and (■) 30 µM DAG and 5 µg/ml CaM, in a reaction medium indicated in *Materials and methods*. Values represent the means ± SD. from at least five independent experiments.

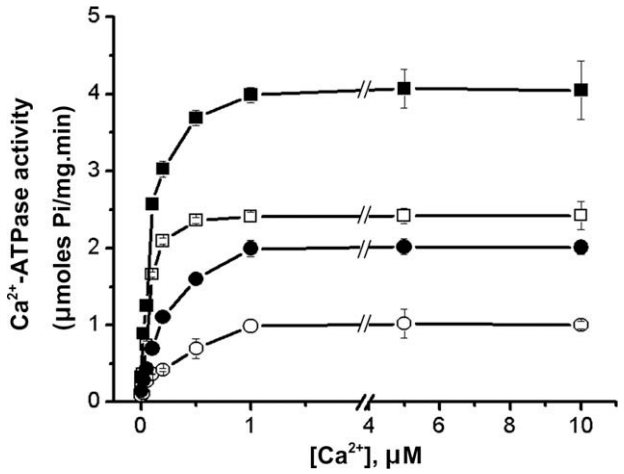

Fig. 3. Effect of DAG and ethanol on Ca^{2+}-dependence of the ATPase activity of PMCA. An additive effect of DAG and ethanol on the ATPase activity can be observed when both effector were added simultaneously. Experimental conditions were: (○) Control, (●) 5% ethanol (□) 30 µM DAG, (■) 30 µM DAG and 5% ethanol, in a reaction medium indicated *in Materials and methods*. Values represent the means ± SD. from at least five independent experiments.

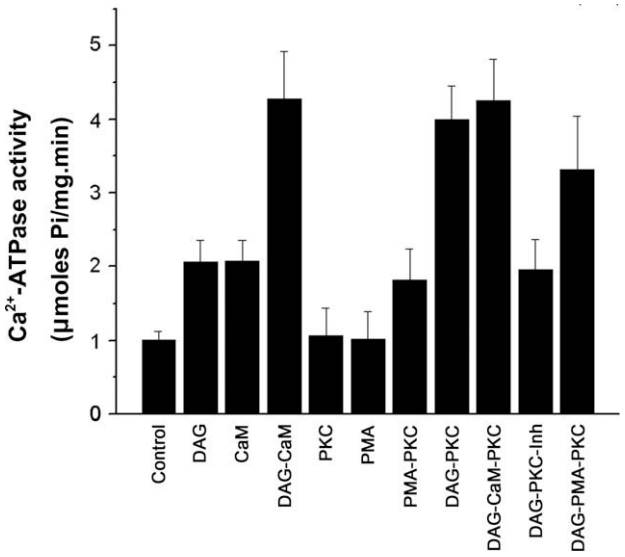

Fig. 4. Direct effect of DAG on Calcium pump. Several effectors were added alone or in different combinations to evaluate their results on the purified Ca^{2+}-ATPase activity. The reaction medium contained 130 mM KCl, 30 mM Hepes/KOH (pH 7.4), 0.5 mM $MgCl_2$, 10 µM $CaCl_2$. Additions were DAG (30 µM), CaM (5 µg/mL), PKC (120 U/mL), PMA (10 nM) and bis-indolyl-maleimide (20 nM) The reaction was carried out at 37 °C.

solubilized enzyme previously obtained (data not shown), and point out to a possible direct role of DAG in the native plasma membrane Ca^{2+}-ATPase. To further support this notion we studied the effect of DAG on the Ca^{2+} transport in human erythrocytes inside-out vesicles (IOVs). Fig. 5 shows that when ATP is added to this preparation, an immediate Ca^{2+} transport is initiated (observed by the decrease of extravesicular Ca^{2+}, as the cation is accumulated inside the vesicles). It can be observed that addition of DAG stimulated the velocity of the Ca^{2+} transport. Moreover, the additive effect of DAG and CaM can be readily observed on this figure, since upon addition of CaM a further increase in the Ca^{2+} transport velocity was obtained. The effect of the addition of the Ca^{2+} ionophore A23187 indicated that Ca^{2+} was indeed accumulated inside the vesicles.

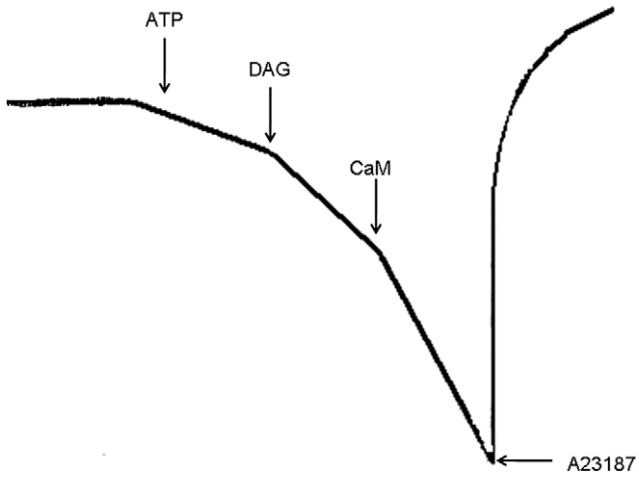

Fig. 5. Stimulation of Ca^{2+} transport by DAG in IOVs from erythrocytes. The additive effect of both compounds on Ca^{2+}-transport can be appreciated by the increase in the slope of the curve, when CaM was added after DAG. The reaction medium (1 mL) contained 130 mM KCl, 30 mM Hepes/KOH (pH 7.4), 0.5 mM $MgCl_2$, 50 µM arsenazo III, 10 µM $CaCl_2$ and 0.5 mg/mL IOVs. Additions were MgATP (0.5 mM), DAG (30 µM), CaM (5 µg/mL) and A23187 (1 µM). The reaction was carried out at 37 °C.

In order to further support a possible direct interaction of DAG with the PMCA, we carried out studies on the intrinsic fluorescence of the purified Ca^{2+}-ATPase. This result indicated that DAG binds to the calcium pump with high affinity, and induces conformational changes on the enzyme. For these experiments, we first estimated the quenching of the PMCA fluorescence by DAG. PMCA exhibits an emission band at 340 nm under excitation at 295 nm (Fig. 6, inset). The enzyme contains 7 Trps, 26 Tyrs and 49 Phe residues, but at this excitation wavelength, the unique absorbing aromatic residues are the Trps. Thus, the observed spectral bandwidth that was similar to that reported previously [11,12], effectively corresponding to the Trp emission.

DAG quenched the intrinsic fluorescence of the solubilized PMCA affecting the spectral properties of the emission, as could be evaluated by the spectral center of mass *cm*, defined by the equation

$$cm = \frac{\int v.F(v)dv}{\int F(v)dv} \quad (1)$$

being v the wavelength number (in cm^{-1}) and F the fluorescence intensity. In this regard, saturating concentration of DAG decreased the spectral center of mass from 28360 to 28220 cm^{-1}; i.e., a red-shifted distortion of 140 cm^{-1}, equivalent to a shift of 1.75 nm toward the right. Putting together all these data, the quenching of the intrinsic fluorescence and the spectral changes of the purified PMCA upon binding of DAG, as evidenced by the red-shifting, a direct interaction between DAG and PMCA is conceivable. This interaction would affect the conformational (local and/or global) structure of the protein, and consequently, expose the Trp emitters toward the aqueous media and/or inner polar micro-environments. Accordingly, we have reported variations of *cm* for the intrinsic PMCA fluorescence induced by effectors such as CaM, Ca^{2+} and vanadate [11].

The quenching of the intrinsic fluorescence of the PMCA was used to estimate the enzyme affinity for DAG. Under titration with DAG, the corrected fractional fluorescence (F/F_0) at 340 nm, showed a concentration-dependent saturable curve characteristic

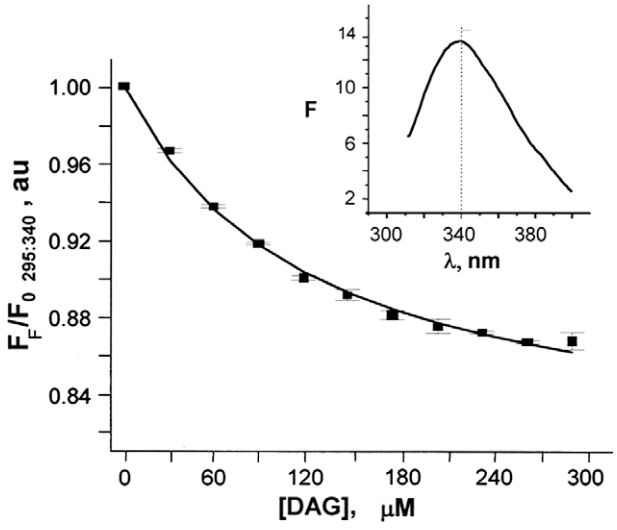

Fig. 6. Quenching of the intrinsic fluorescence of PMCA upon binding of DAG. The tryptophans PMCA fluorescence represent a dose-dependent quenching induced by DAG. PMCA (30 µg/mL) was titrated with DAG. Symbols represent the fractional fluorescence relative to the initial condition, F_0, in the absence of DAG, and are the average of three determinations ± SD, after correction by dilution and inner-filter effect. The curve represents the best computer-generated fit to the equation described in the text. Emission at 340 nm. *Inset*: Emission spectra of PMCA in buffer Tris–HCl (pH 7.4). In both excitation at 295 nm. T = 22 °C.

of a binding isotherm, as shown in Fig. 6. An analysis of the binding system requires a model; thus, considering that PMCA has a unique site for DAG binding,

$$E + DAG \underset{K_d^{DAG}}{\longleftrightarrow} E{\cdot}DAG$$

The expression that would relate the spectroscopy variable and the thermodynamic parameter of the binding would be represented by the equation

$$\frac{F}{F_0} = \frac{1 + \alpha^{DAG}.K_d^{DAG}.[DAG]}{1 + K_d^{DAG}.[DAG]} \quad (2)$$

being F, the corrected fluorescence; F_0, the corrected fluorescence in absence of DAG; K_d^{DAG}, the dissociation constant; α^{DAG}, the quenching factor; and $[DAG] \cong [DAG]_t$, the ligand total concentration, since $[DAG]_t$ is much larger than the total enzyme concentration, $[E]_t$. A nonlinear fitting of the experimental values for the equation [21], constrained to $\alpha^{DAG} < 1$ (since it is a quenching phenomenon), minimized the quadratic difference to $\chi^2 = 1 \times 10^{-5}$, and reported the following parameters as best estimated: $K_d^{DAG} = 118.1$ μM (40.67 μg/mL) and $\alpha^{DAG} = 0.81$.

Additionally, we observed the differential effect of DAG and CaM on the interaction of TNP-ATP with PMCA. The ATP analog, TNP-ATP, has been one of the fluorescence derivatives most used as reporter molecule sensitive to changes in the micro-environment of the nucleotide-binding domain of ATPases [38]. In fact, we found that TNP-ATP interacts with PMCA increasing its quantum yield by 12 times at 530 nm, and shifting the emission maximum in −15 nm with a $K_d^{TNP-ATP}$ of about 2 μM (manuscript in preparation). Taking advantage of this interaction, we evaluated the sensitized emission of TNP-ATP under excitation of the tryptophan residues. As shown in Fig. 7, the excitation of the bound TNP-ATP at 295 nm, quenched the emission band of the Trps fluorescence at 340 nm, and exhibited an emission (sensitized emission) at 535 nm, characteristic of the TNP-ATP emission. Fig. 8 shows the titration curves of the sensitized emission of bound TNP-ATP, measured as the enhancement ratio, F/F_0, at 535 nm, under excitation at 295 nm, in three conditions tested. It can be observed that the sensitized emission of TNP-ATP is significantly different for the DAG condition, in comparison with the control and CaM conditions.

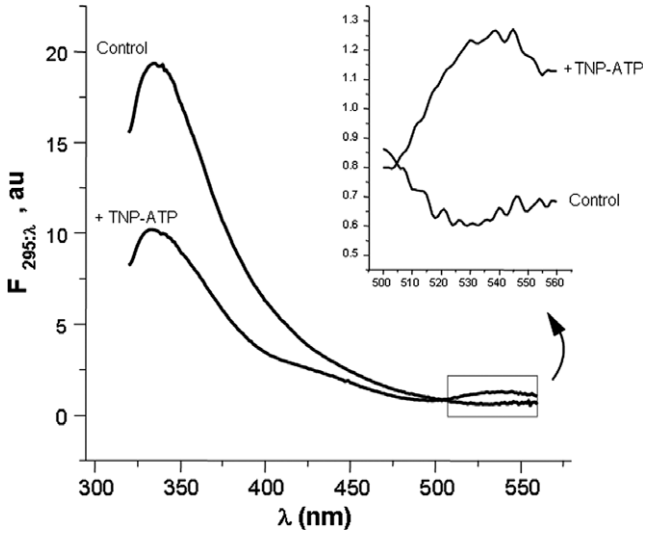

Fig. 7. Quenching of the intrinsic PMCA fluorescence on the interaction with TNP-ATP. The binding of the ATP analog TNP-ATP to the PMCA attenuates the Trp fluorescence emission. The fluorescence emission spectrum of PMCA (36 μg/mL) was registered in the absence (Control) and presence of 20 μM TNP-ATP (+TNP-ATP), previous subtraction of their respective blanks. *Inset*: Zoom in of the range corresponding to the emission band of the TNP-ATP. Excitation at 295 nm. $T = 22$ °C.

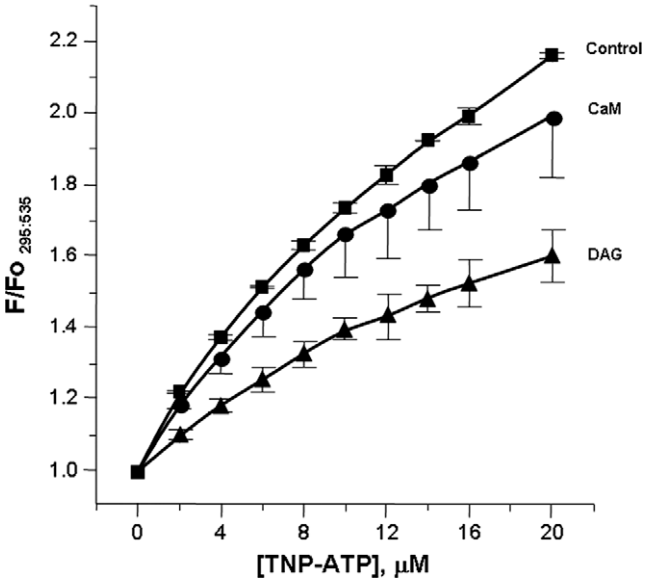

Fig. 8. Sensitized emission of TNP-ATP molecule bound to the PMCA under excitation of the Trps. TNP-ATP emits fluorescence resonance energy transfer (FRET) from the Trp donors. Fluorescence titration of PMCA (36 μg/mL) with TNP-ATP in the conditions: in buffer (■, Control), with 5 μg/mL of CaM (●, CaM) and with 174 μM of DAG (▲, DAG). The symbols represent the average corrected fluorescence (F), in term of the fluorescence in the absence of TNP-ATP (F_0), for three repetitions ($n = 3$) ± SD. Excitation at 295 nm and emission at 535 nm. $T = 22$ °C.

Discussion

The work presented here points to the stimulation of the plasma membrane Ca^{2+}-ATPase by DAG, which produces an increment in the V_{max} of the enzyme and in the affinity of the protein for Ca^{2+}. The stimulation induced by DAG is additive with the effect of both CaM and ethanol. This last result was unexpected since we were looking for a natural substitute for ethanol, as a stimulator of this Ca^{2+}-ATPase. However, all the results indicate that this is not the case, since both CaM and ethanol stimulate the enzyme by interaction with different domains [13,14] and such domains appear to be, at least partially distinct to the DAG binding region. Thus, the stimulation of Ca^{2+}-ATPase by CaM is accepted to be due to the interaction of CaM with an autoinhibitory domain (9 kD) present in the carboxyl end of the ATPase, [27,39] and it has also been demonstrated that ethanol interacts with the enzyme in a different site. Thus, different isoforms of the enzyme have distinct sensibility to ethanol [14]. By the use of truncated forms of this ATPase, it has been demonstrated that a segment of 95 aa in the carboxyl terminal end of the protein endows the ATPase with ethanol sensitivity, since its removal produces the loss of the ethanol effect [14]. This domain appears to be superimposed partially with the domain of interaction with CaM, but different to it. On the other hand, the sensitivity to acidic phospholipids, has been located in two domains of the enzyme, one close to the carboxyl terminus of the protein, partially shared with the site for CaM binding, and the other, a lysine rich cytoplasmic domain located between transmembrane domains 2 and 3 [40,41].

The mechanism of action of DAG on the enzyme appears different to that produced by PKC, since its effect is also additive to the stimulation produced by the latter, ruling out that it corresponds to the phosphorylation of the enzyme mediated by this kinase. The addition of phorbol esters to the incubation media in the absence of PKC did not have any effect on the enzyme activity, ruling out any possible small contamination of the preparation with PKC.

Interestingly, the putative C1 domain, which consists of a conserved sequence of 50 amino acids bearing the $HX_{11-12}CX_2CX_{12-14}$

$CX_2CX_4HX_2CX_{6-7}C$ motif, found in all proteins described so far, that bind phorbol esters and/or DAG [42], is absent in the PMCA primary structure. However, this site of interaction has been reported to be relevant for the transportation of PKC (and other enzymes) from the cytosol to the plasma membrane [43–45]. Since the PMCA is already located at the plasma membrane, the presence of this region in the enzyme is not needed. There is at least another evidence where DAG interacts directly with a protein and the phorbol esters have no effect. Thus, Beck et al. [46] have recently shown a stimulation by DAG on a receptor-operated Ca^{2+} channel (TRPC7) in human keratinocytes. The authors demonstrated that the stimulation of the Ca^{2+} current induced by DAG is by a direct interaction, since addition of phorbol myristate acetate or specific PKC inhibitors showed little effect on this current. Accordingly with our argument, this Ca^{2+} channel is already located at the plasma membrane, and thus, similarly to the PMCA, it is not required to be recruited by interaction of DAG or PMA, by interacting to the C1 motif mentioned above.

We demonstrated in this work that DAG quenched the Trp fluorescence of PMCA. The mechanism of quenching might be due to increasing the exposure of emitters to the aqueous environment and/or a conformational change which moves emitters close to some amino acid side chain functional groups, both mechanisms acting on the Trp emitters differentially once DAG is bound to the enzyme. Whatever the mechanism, the binding of DAG must induce a conformational change in the PMCA associated with the increase in the ATPase activity. In this sense, it has been a usual practice monitoring changes in the quantum yield of fluorophores to characterize the binding of ligand to macromolecules, so, we used the intrinsic fluorescence of the PMCA as a reporter variable to estimate the DAG affinity. The K_d^{DAG} obtained by this means is in the same order of magnitude than the apparent K_m^{DAG} (at 1 mM MgATP and 10 μM Ca^{2+}) for the ATPase activity. The above results strongly support a direct interaction between the PMCA and DAG. The functional consequence of the stimulation of the Ca^{2+}-ATPase activity and the related Ca^{2+} transport by DAG in an additive manner with CaM, strongly suggest that this second messenger could be involved in Ca^{2+} homeostasis.

On the other hand, the fluorescent ATP analog, TNP-ATP, binds to PMCA with high affinity, as monitored by its quantum yield enhancement, but it is not a substrate for the PMCA (data not shown). The sensitized emission at 535 nm by TNP-ATP under excitation at 295 nm, seems to have its origin in the phenomenon of energy transfer between the excited donors (Trp residues) and the absorbing acceptor (TNP-ATP bound). The quenching at 340 nm (at the Trp emission) and the spectral overlapping between the Trp emission spectrum and the TNP-ATP absorbing spectrum, fulfill the energy transfer requirement. However, this phenomenon needs also of geometrical fulfillments, so that spatial proximity between the molecules, among others, is imperative to consider in order to be feasible this kind of dipole–dipole interaction. Few works have been carried out with the pair (Trp, TNP-ATP) for FRET experiments. We can mention one by Liu et al. [47] on the P-glycoprotein, and the observed FRET in vesicles of sarcoplasmic reticulum [48]. On the other hand, it was not observed FRET between Trps and TNP-ATP on the annexin IV but on annexin VI [49].

In our experimental system, native PMCA with or without TNP-ATP no-covalently bound, it is not possible to measure the efficiency of the energy transfer because of the presence of multiple donors (in principle, all the Trps are potential donors). However, the global signal can be still a qualitative index of the effectiveness of the energy transfer among these probes.

The minor sensitizing emission of the TNP-ATP in the presence of DAG might be interpreted as an induced conformational change that increases the effective distance and/or on the average relative orientation between the electronic centers of the donors and the acceptor. Changes in the efficiency of the energy transfers between Trps and TNP-ATP, associated to the interaction of Pgp with several drugs, were reported by Liu et al. [47]. Hence, conformational changes associated to the ATPase activation by DAG and CaM, seems to be reasonable. Accordingly, Corradi et al. [50] reported variation of the efficiency of energy transfer between a double-labeled PMCA (BFP-PMCA-GFP) on CaM activation, indicating changes in the relative positions of the probes because of the activation of the PMCA. Furthermore, the differential effect between both effectors in the sensitizing emission of the TNP-ATP here observed suggests that the mechanisms of action of both effectors are different. In this aspect, It has been reported that gangliosides modulate the PMCA activity and induce conformational changes, evidenced by differential accessibility to collisional quenchers to labeled PMCA, by a mechanism different from that of CaM [51].

This work reports biochemical and biophysical supports that allow to conclude that the second messenger diacylglycerol, which until now had been implicated in an indirect regulation of the plasma membrane Ca^{2+} pump via PKC, indeed stimulates the enzyme by direct binding, increasing the V_{max} of the ATPase activity, the affinity of the enzyme for Ca^{2+} and the rate of Ca^{2+} transport, probably by a distinct mechanism to that the produced by CaM and ethanol.

One apparent functional contradiction between our results and some evidences obtained before is the fact that PIP2, the precursors of DAG, is a potent stimulator of the PMCA. In fact, PIP2 has been shown to increase the affinity of the enzyme for Ca^{2+} to a larger extent than CaM and any other acidic phospholipids so far studied [7]. However, different from DAG, the V_{max} of the enzyme reached by this effector is the same to that attained by CaM or PKC, and is not additive to these effectors. Thus, the physiological meaning of this stimulation is uncertain. Even more, the concentration of PIP2 at the plasma membrane is low and quite stable. Therefore, is not easy to interpret in terms of the functional Ca^{2+} homeostasis. In contrast, the over-stimulation of that PMCA induced by DAG in the presence of CaM and/or PKC is easier to infer in terms of the characteristic rapid peak in the intracellular Ca^{2+} concentration typically observed after any stimulation related to the production of IP_3. Thus, upon the increase in the $[Ca^{2+}]_i$ obtained after any signal conducting to an elevation of IP_3 and the concomitant peak in the cytoplasmic Ca^{2+} concentration, the cell is committed to rapidly reduce this high level of Ca^{2+} to a much lower steady-state level, which is a balance between the Ca^{2+} homeostatic mechanisms (PMCA, SERCA and mitochondria, functioning to reduce the $[Ca^{2+}]_i$ and the opening of the capacitative Ca^{2+} entry at the plasma membrane, which induce the cell to elevate the $[Ca^{2+}]_i$. The overall picture that emerge is a typical plateau, well below the initial peak, but significantly higher than the previous basal Ca^{2+} concentration (for example, see [15]). It is likely that the Ca^{2+} signal that the cell actually recognize is not only the initial peak, which stays only for few seconds (less than a minute), but instead the steady-state level after the peak recovery, which remains for longer time (about one hour or so). In this sense, DAG would be responsible for the fast reduction in the intracellular Ca^{2+} concentration to this steady-state level. Supporting this model, the stimulation of the PMCA by CaM and PKC, which would act simultaneously, are not additive among them, but indeed are additive to the stimulation by DAG. So that an additional over-stimulation would warrant the rapid Ca^{2+} transient typically observed after PLC stimulation, via receptor and G protein or by tyrosine phosphorylation of a PLC-associated receptor.

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