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Regulation of plasma membrane Ca^{2+}-ATPase activity by acetylated tubulin: Influence of the lipid environment

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ABSTRACT

We demonstrated previously that acetylated tubulin inhibits plasma membrane Ca^{2+}-ATPase (PMCA) activity in plasma membrane vesicles (PMVs) of rat brain through a reversible interaction. Dissociation of the PMCA/tubulin complex leads to restoration of ATPase activity. We now report that, when the enzyme is reconstituted in phosphatidylcholine vesicles containing acidic or neutral lipids, tubulin not only loses its inhibitory effect but is also capable of activating PMCA. This alteration of the PMCA-inhibitory effect of tubulin was dependent on concentrations of both lipids and tubulin. Tubulin (300 μg/ml) in combination with acidic lipids at concentrations >10%, increased PMCA activity up to 27-fold. The neutral lipid diacylglycerol (DAG), in combination with 50 μg/ml tubulin, increased PMCA activity >12-fold, whereas tubulin alone at high concentration (≥300 μg/ml) produced only 80% increase. When DAG was generated *in situ* by phospholipase C incubation of PMVs pre-treated with exogenous tubulin, the inhibitory effect of tubulin on PMCA activity (ATP hydrolysis, and Ca^{2+} transport within vesicles) was reversed. These findings indicate that PMCA is activated independently of surrounding lipid composition at low tubulin concentrations (<50 μg/ml), whereas PMCA is activated mainly by reconstitution in acidic lipids at high tubulin concentrations. Regulation of PMCA activity by tubulin is thus dependent on both membrane lipid composition and tubulin concentration.

1. Introduction

PMCA (plasma membrane Ca^{2+} ATPase) is a member of the family of P-type ATPases. It is responsible for active transport of Ca^{2+} ions, using energy from ATP hydrolysis, in many cell types. There are four PMCA isoforms (PMCA 1–4), each encoded by a different gene, and each isoform has multiple subtypes based on alternative splicing of its mRNA [1]. Regulation of PMCA activity has been extensively studied. Calmodulin is the main activator [2]; the enzyme can also be activated by phosphorylation of kinase A or C, partial proteolysis, or acidic lipids. PMCA activity appears to be affected by composition of phospholipids in the surrounding plasma membrane [3,4]. In human erythrocyte membranes, PMCA can be activated by a variety of acidic phospholipids, whereas neutral phospholipids have no effect [5,6]. Diacylglycerol (DAG) is a potent activator of erythrocyte PMCA, through direct interaction [7]. Activity of PMCA from the brain was increased by the level of phosphatidylinositol 4,5-bisphosphate [5].

We demonstrated previously that acetylated tubulin inhibits the ATPase activity of PMCA, as well as the activity of various isolated P-ATPases. Na^+,K^+-ATPase is inhibited by acetylated tubulin in brain plasma membrane *in vitro* [8], and in neuronal [9] and non-neural cells [10] *in vivo*. Tubulin must be acetylated in order to display such enzyme-inhibitory effect [11]. Tubulin forms a complex with Na^+,K^+-ATPase through interaction with the fifth cytoplasmic domain [12]. In erythrocytes from hypertensive patients, formation of a complex with acetylated tubulin decreased the activity of Na^+, K^+-ATPase [13]. In *Saccharomyces cerevisiae*, acetylated tubulin inhibited plasma membrane H^+-ATPase *in vivo* and *in vitro*. The enzyme and tubulin were shown to be part of a protein complex which is dissociated during glucose catabolism in cells, leading to activation of the enzyme [14]. PMCA in brain plasma membrane is inhibited by acetylated tubulin, and both proteins are part of the same complex [15]. Calmodulin and ethanol dissociate the acetylated tubulin/PMCA complex and thereby activating PMCA [16–18]. In order to interact with and inhibit PMCA, tubulin must be acetylated at Lys40 of the α-chain [15].

The present study addressed the combined effect of lipids and tubulin on PMCA activity. In the presence of neutral or acidic lipids, at

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Abbreviations: PMCA, plasma membrane Ca^{2+} ATPase; PC, L-α phosphatidylcholine type XVI-E from fresh egg yolk; BE, lipidic extract from bovine brain containing acidic lipids; PA, L-α-phosphatidic acid from egg yolk; DAG, diacylglycerol; $C_{12}E_{10}$, polyoxyethylene-10-laurylether; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PLC, phospholipase C; PMSF, phenylmethyl-sulfonyl-fluoride; p-NPPC, para-nitro-phenyl-phosphatidyl choline; PMVs, plasma membrane vesicles.

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certain concentrations, tubulin strongly activated PMCA, rather than inhibiting it.

2. Materials and methods

2.1. Materials

ATP, anti-mouse IgG conjugated with peroxidase, anti-α-tubulin mouse mAb (ascites fluid) DM1-A, anti-PMCA mAb 5F10, anti-acetylated tubulin mouse mAb 6-11B-1, L-α phosphatidylcholine type XVI-E from egg yolk, brain extract (BE) type Folch fraction 1 from bovine brain containing ~10% phosphatidylinositol, 50% phosphatidylserine and other lipids, L-α phosphatidic acid from egg yolk, calmodulin-agarose, 1,2-Dicapryloyl-sn-glycerol (DAG) and polyoxyethylene-10-laurylether ($C_{12}E_{10}$) were from Sigma Chemical Co. (St. Louis, MO, USA). Fura 2-AM was from Molecular Probes (Eugene, OR, USA).

2.2. PMCA purification

Plasma membrane vesicles (PMVs) were isolated from rat brain by the method of Michaelis et al. [19], with slight modification [15]. PMCA was purified from rat brain plasma membrane by the method of Salvador and Mata [20], with modification. Plasma membrane (45 mg protein) was suspended in 10 ml of 20 mM HEPES/KOH, pH 7.40, 20% glycerol, 130 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, and 0.5 mM $CaCl_2$ ("purification buffer"). Membrane was solubilized for 10 min at 4 °C by slow addition of $C_{12}E_{10}$ (2 mg detergent per mg total membrane protein) with 0.1% PC. The detergent-solubilized membrane was centrifuged at 100,000×*g* for 30 min at 4 °C. The supernatant fraction was used to purify PMCA by calmodulin-affinity chromatography, as described by Niggli et al. [21]. Contaminant proteins were eliminated, and PMCA was eluted with a buffer similar to "purification buffer" except that 0.5 mM $CaCl_2$ was replaced by 1 mM EGTA, and $C_{12}E_{10}$ was included (0.05% final concentration). Eluted fractions containing high concentrations of PMCA (as detected by Western blot), were pooled, aliquoted, and kept frozen in liquid N_2. According to immunoblotting with anti-calmodulin (clone 2D1 + 6D4 + 1F11, Sigma) and ECL detection, the PMCA preparation was free of calmodulin.

All protocols and procedures for animal experiments were reviewed and approved by the Ethics Committee of the granting institution (CONICET; Res. # 1806/04).

2.3. Tubulin preparation

Brains from 30 to 60-day-old rats were homogenized at 4 °C in one volume of MEM buffer (0.1 M Mes/NaOH, pH 6.7, containing 1 mM EGTA and 1 mM $MgCl_2$). The homogenate was centrifuged at 100,000×*g* for 45 min, and the pellet was discarded. Tubulin was purified by one assembly/disassembly cycle, followed by phosphocellulose chromatography, as described previously [22]. Concentration was adjusted to 1 mg/ml with MEM buffer, and tubulin was used immediately. According to immunoblotting with anti-calmodulin (clone 2D1 + 6D4 + 1F11, Sigma) and ECL detection, the tubulin preparation was free of calmodulin.

2.4. Reconstitution of PMCA with lipids, and PMCA activity assay

The method of Palacios et al. [23] was used, with slight modification. Purified PMCA (150–300 μg) containing 0.05% $C_{12}E_{10}$, in a volume of 100 μl, was added to tubes containing dried lipid (PC, or PC mixed with other lipids as stated) to give a lipid/protein ratio of 5.3:1. Contents of tubes were thoroughly mixed by agitation, pre-incubated for 10 min on ice, and diluted with PMCA assay medium (1 ml final volume). Reaction at 340 nm, using a coupled enzyme assay, was measured spectrophotometrically to assess PMCA activity [5,24]. The reaction mixture contained PMCA (5–10 μg protein) reconstituted in lipids, in 0.34 ml assay buffer (50 mM HEPES/KOH, pH 7.4, 100 mM KCl, 5 mM NaN_3, 2 mM $MgCl_2$, 0.22 mM NADH, 0.42 mM phosphoenolpyruvate, 3 I.U. pyruvate kinase, 8 I.U. lactate dehydrogenase, and $CaCl_2$ sufficient to give free Ca^{2+} concentration 2.4 μM). Defined concentrations of free Ca^{2+} were established using $CaCl_2$/EGTA solutions, and calculated using WEBMAXC Standard software. Samples were kept 5 min at 37 °C, and the reaction was started by addition of 1 mM ATP. PMCA activity was calculated as the difference in ATP hydrolysis between samples incubated in the presence *vs.* absence of Ca^{2+}. For assay of enzyme activity in the presence of tubulin, the tubulin was pre-incubated 20 min with PMCA reconstituted with lipids, and then added to the reaction mixture. The determination of PMCA activity in PMVs (0.25 mg protein) was similar to that described for enzyme reconstituted in lipids. Additionally, we performed two controls to demonstrate that PMVs did not contain significant enzymatic activities SERCA and SPCA. These controls were the measurement of ATPase activity in the presence and absence of 1 μM thapsigargin (inhibitor of SERCA) to determine the activity of SERCA, and the measurement of ATPase activity in the presence and absence of 1 μM thapsigargin and 2 mM vanadate to inhibit SERCA and PMCA (remaining activity is due to the SPCA).

2.5. Determination of Ca^{2+} transport in PMVs

Ca^{2+} transport was determined by the Fura-2AM method of Jeremic et al. [25], with some modification. PMVs obtained as described above were resuspended in 10 mM HEPES, pH 7.4, incubated with 10 μM Fura-2AM for 30 min at 30 °C with gentle agitation, washed twice by centrifugation, and resuspended in 10 mM HEPES to eliminate excess Fura-2AM. 0.1 mg sample of PMVs was resuspended in "transport buffer" (50 mM Tris–HCl, pH 7.3, 100 mM KCl, 75 μM EGTA, 5 mM NaN_3, 400 nM thapsigargin, 2.5 mM $MgCl_2$, and $CaCl_2$ sufficient to give free Ca^{2+} concentration 4 μM), with final volume of 1 ml. Samples were kept 5 min at 37 °C, and the reaction was started by addition of 1.5 mM ATP. Excitation wavelengths were 340 and 380 nm, and emission was measured at 510 nm. Specific Ca^{2+} transport by PMCA activity was measured in the presence and absence of vanadate.

2.6. PLC purification, and enzyme activity assay

Hemolytic phospholipase C, obtained from supernatant of *Pseudomonas aeruginosa* (NCTC, fides III) culture medium, was purified by reversed-phase chromatography on diatomaceous earth (Celite-545, Mallinckrodt Baker, NJ, USA), and PLC activity was determined using synthetic substrate p-NPPC as described by Lucchesi et al. [26]. One PLC unit was defined as the amount of enzyme liberating 1 nmol p-nitrophenol from p-NPPC per minute at 37 °C.

2.7. Isolation and determination of hydrophobic tubulin

Hydrophobic tubulin was isolated from Triton X-114 phase as described previously [27], with slight modification. Reaction mixtures containing PMCA (10 μg protein) were reconstituted with lipids and tubulin at the indicated concentrations, and pre-incubated 20 min at 37 °C. Triton X-114 was added (1% final concentration), and the mixture was heated 5 min at 37 °C and centrifuged at 600×*g* for 5 min for phase separation. Detergent-rich lower phase, containing hydrophobic tubulin, was washed with NaCl/Tris buffer (50 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl). Aliquots were subjected to electrophoresis and immunoblotting as below for determination of acetylated and total tubulin.

2.8. Electrophoresis and immunoblotting

Proteins were separated by SDS-PAGE on 8–10% polyacrylamide slab gels [28], transferred to nitrocellulose, and reacted with anti-α-tubulin mouse mAb DM1A (dilution 1:1000), anti-acetylated tubulin mouse mAb 6-11B-1 (dilution 1:1000) [29], and anti-PMCA mouse mAb 5F10 (dilution 1:300) [30]. The nitrocellulose sheet was reacted with anti-mouse IgG conjugated with peroxidase.

3. Results

3.1. Effect of lipids on PMCA activity of rat brain plasma membrane

We showed previously that PMCA interacts with tubulin in membranes of brain cells, resulting in reduced enzyme activity [15]. Studies on effect of lipids on PMCA activity by other groups have mainly used PMCA purified from erythrocytes, in which isoform 4 is predominant. In the present study, we used PMCA purified from rat brain, in which all isoforms (1 to 4) are present [31]. The purified PMCA was reconstituted in PC with addition of various concentrations of DAG, BE, or PA, and enzyme activity was determined based on hydrolysis of ATP. PMCA activity increased in proportion to the amount of enzyme reconstituted in PC, and in the presence of each of the three lipids at 20% concentration (Fig. 1A). PA was the most efficient of the three lipids, reaching an activation factor of 7 (Fig. 1A, Table 1). Similar promoting effects of PA and other acidic phospholipids were previously observed for PMCA in brain cells and erythrocytes [5,6]. We found that DAG, a neutral phospholipid, increases activity of PMCA from brain >4-fold (Fig. 1A, Table 1), consistent with previous studies. PMCA activity was dependent on the concentration of acidic or neutral lipid involved in reconstitution of the enzyme (Fig. 1B). The PMCA preparation used in these experiments was depleted of tubulin, as shown by the control (Fig. 1C). These findings, taken together, indicate that PMCA purified from rat brain membranes and reconstituted into PC is activated by acidic or neutral lipids in the absence of tubulin. In subsequent experiments, we examined the combined effect of lipids and purified tubulin on PMCA activity.

3.2. Effect of tubulin on PMCA activity in the presence of lipids

Various amounts of tubulin were pre-incubated for 20 min with PMCA previously reconstituted with various types of lipids and then diluted in the assay medium. PMCA reconstituted in 100% PC showed low activity (2 nmol Pi/min/mg protein), which doubled at low tubulin concentrations (<25 μg/ml) and returned to initial value at higher concentrations (≥50 μg/ml) (Fig. 2A, Table 1). Similarly, reconstitution of the enzyme in PC mixed with various amounts of DAG resulted in increased PMCA activity at low tubulin concentrations, and reduction of activity at tubulin concentrations >25 μg/ml (Fig. 2A). The effect of tubulin varied depending on the proportions of BE and PA mixed with PC during reconstitution. PMCA activity gradually increased at low tubulin concentrations (<25 μg/ml) and low percentage of BE or PA (up to 3% during reconstitution), and then decreased at higher tubulin concentrations (Fig. 2B, C). When higher BE or PA proportions (≥10%) were used during reconstitution, PMCA activity increased continuously along with tubulin concentration, even up to 300 μg/ml (Fig. 2B, C). At low (<50 μg/ml) tubulin concentration, in PMCA reconstituted in 80% PC and 20% other lipids, enzyme activity was increased maximally by PA (21-fold), and to lesser degrees by DAG (12-fold) and BE (10-fold) (Table 1). At high concentration of tubulin (300 μg/ml), PMCA activity was increased 27-fold by 20% PA, and 21-fold by BE (Table 1). For PMCA reconstituted in DAG, increased tubulin concentration caused reduced enzyme activity (Fig. 2A), which eventually reached a level corresponding to 0% DAG (*i.e.*, 100% PC) (Table 1). Absence of PMCA in purified tubulin preparations used in these experiments was represented by the

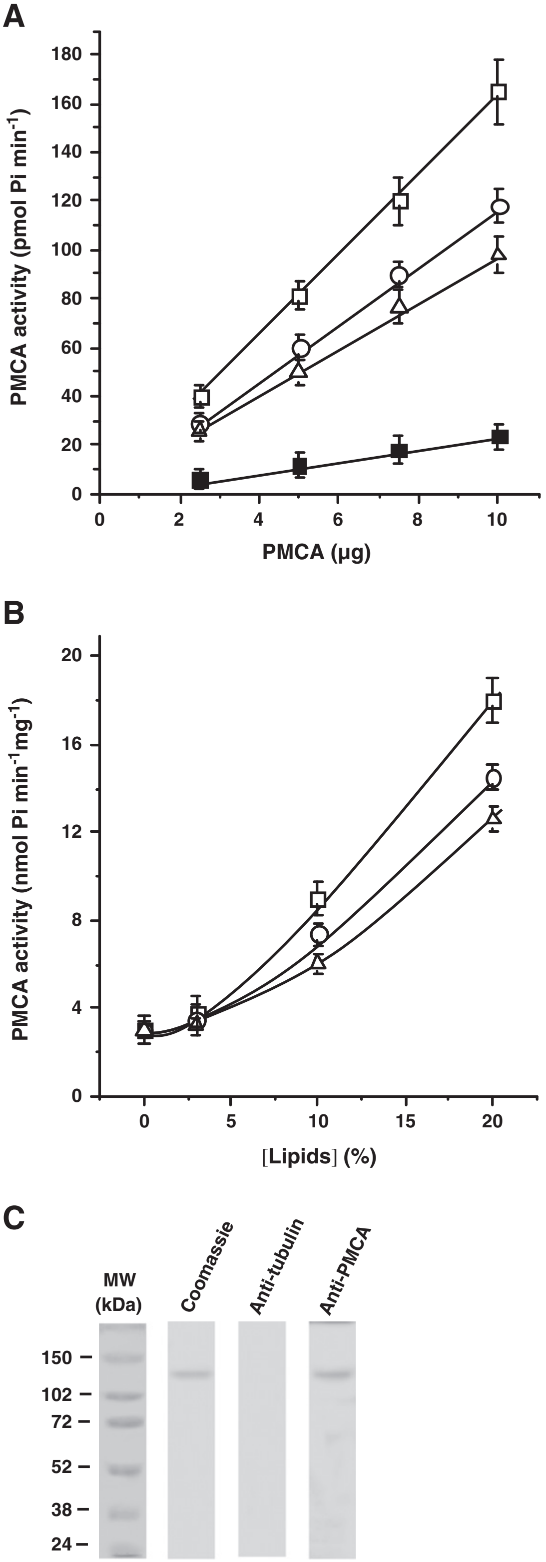


Fig. 1. Effect of lipids on activity of rat brain PMCA reconstituted in PC. PMCA was purified from rat brain, reconstituted in various lipids, and activity was determined by ATP hydrolysis as described in the Materials and methods section. (A) Activity of PMCA (at the indicated amounts) reconstituted in 100% PC (■), or in 80% PC plus 20% DAG (△), BE (○), or PA (□). (B) Activity of PMCA reconstituted in DAG (△), BE (○), or PA (□) at the indicated concentrations, to complete 100% of the lipids with PC. (C) PMCA purified from rat brain (5 μg protein) was analyzed by SDS-PAGE stained with Coomassie Blue and Western blotting with staining of lanes by anti-α-tubulin mAb DM1A and, anti-PMCA mAb 5F10. Values shown are mean ± SD from three independent experiments.

Table 1
Effect of amounts of tubulin and lipids on PMCA activity.

	PMCA activity							
	PC[a]		PC + DAG[a]		PC + BE[a]		PC + PA[a]	
	Ae[b]	% of PC[c]	Ae[b]	% of PC[c]	Ae[b]	% of PC[c]	Ae[b]	% of PC[c]
− Tub	2.3 ± 0.5	100	9.7 ± 0.7	430 ± 65	12 ± 0.7	525 ± 49	16.5 ± 1.3	732 ± 105
+ Tub (50 μg/ml)	5.1 ± 0.2	227 ± 41	28 ± 3.0	1237 ± 141	22 ± 2.0	974 ± 127	50 ± 3.0	2226 ± 361
+ Tub (300 μg/ml)	2.5 ± 0.4	109 ± 6.5	4.2 ± 0.3	186 ± 28	33 ± 3.0	1461 ± 192	62 ± 5.0	2751 ± 389

[a] PMCA (7 μg protein) was reconstituted in 100% PC, or in 80% PC plus 20% of the indicated phospholipids.

[b] Ae: PMCA activity in nmol Pi min^{-1} mg protein^{-1}.

[c] PMCA activity expressed as percentage of sample reconstituted in 100% PC.

control (Fig. 2D). Coomassie Blue staining showed a nearly-homogenous 55-kDa protein band, coinciding with the tubulin band revealed by anti-tubulin. Note that the tubulin preparation lacked PMCA.

Acetylated tubulin inhibits PMCA activity by interacting with the enzyme. We previously demonstrated the formation of acetylated tubulin/PMCA complex by immunoprecipitation experiments, and by passage of acetylated tubulin from aqueous to detergent phase upon

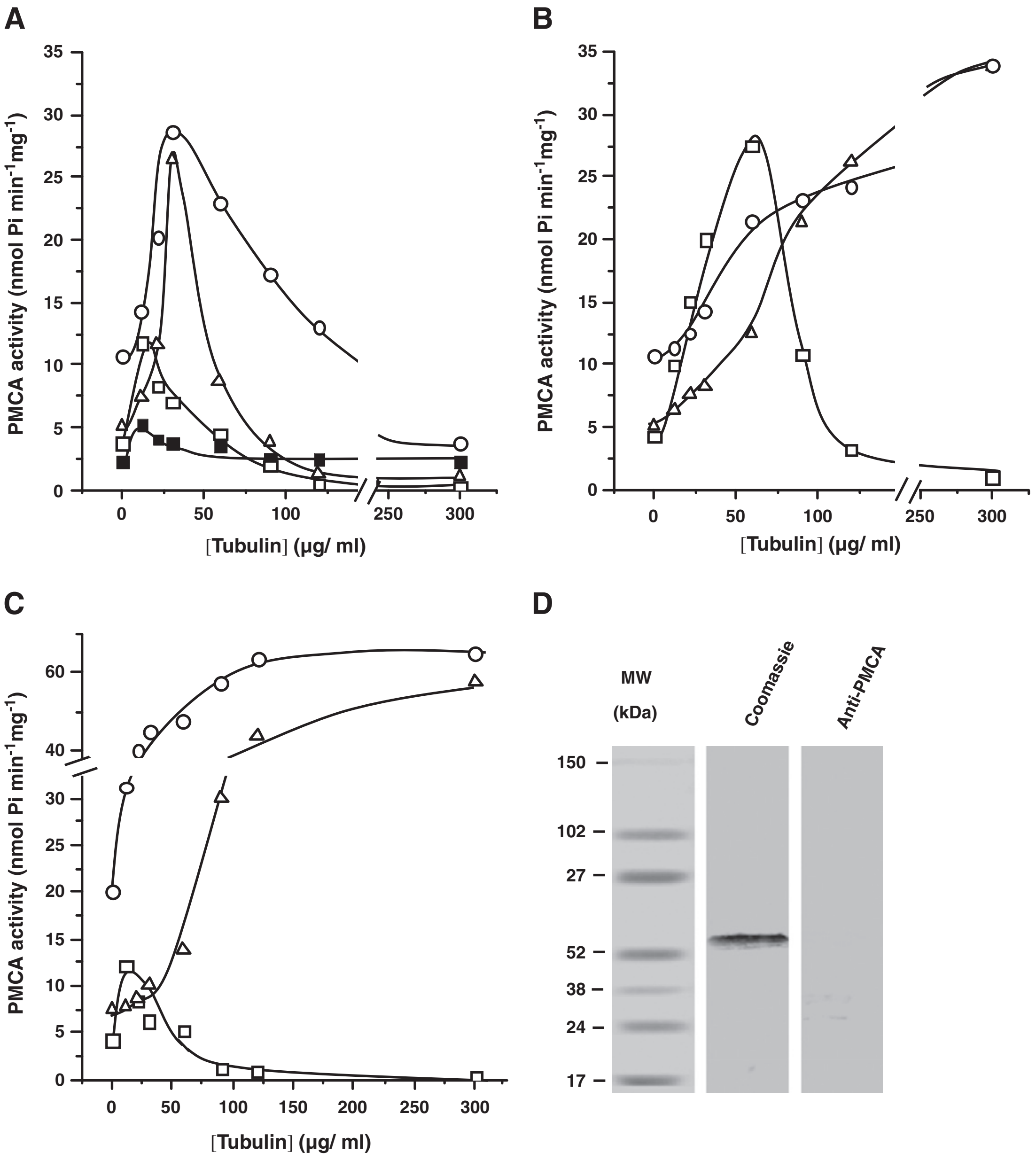


Fig. 2. Effect of tubulin on activity of rat brain PMCA reconstituted in phospholipids. PMCA (5 μg) was purified from rat brain, and reconstituted in 100% PC (■, A), or in PC with 3% (□), 10% (△), or 20% (○) DAG (A), BE (B), or PA (C). PMCA activity was determined by ATP hydrolysis at the indicated tubulin concentration as described in materials and methods. Values shown are mean ± SD from three independent experiments. (D) Tubulin (5 μg protein) from rat brain was analyzed by SDS-PAGE stained with Coomassie Blue and Western blotting with staining by anti-PMCA mAb 5F10.

binding to PMCA [15]. To test whether the effects of tubulin on PMCA reconstituted in lipids in the present study were due to changes in tubulin/PMCA interaction, we measured amounts of acetylated tubulin/PMCA complex in experiments corresponding to those shown in Fig. 2. For this, we determined amounts of "hydrophobic acetylated tubulin" (HAT) (acetylated tubulin found in detergent phase), since the complex behaves as a hydrophobic entity during partition with Triton X-114 [27], while non-interacting acetylated tubulin remains in aqueous phase. Increased content of acetylated tubulin in the hydrophobic fraction indicates higher level of acetylated tubulin/PMCA complex [15]. Levels of total and acetylated hydrophobic tubulin in mixtures of PMCA (reconstituted with various phospholipids) and various amounts of tubulin are shown in Fig. 3. PMCA reconstituted in 100% PC did not bind acetylated tubulin at either low or high tubulin concentrations. Consistent with this finding, PMCA activity was minimally affected by tubulin at low or high concentrations under these conditions (Fig. 2A). In contrast, when PMCA was reconstituted in PC plus 20% DAG, it bound to tubulin at high concentrations but not at low concentrations, consistent with effects of high and low tubulin concentrations in Fig. 2. Results for PMCA reconstituted in PC plus 20% BE were similar to those with DAG. Results for PMCA reconstituted with PC plus 20% PA were unclear. A reduced amount of acetylated tubulin seemed to interact with PMCA (Fig. 3). Under these conditions, the increased PMCA activity shown in Fig. 2 cannot be explained by interaction of the enzyme with tubulin; if such interaction existed, it should be inhibitory rather than promoting.

3.3. Effect of *in situ* generation of DAG in plasma membrane vesicles (PMVs) on PMCA activity and Ca^{2+} transport

Increased concentrations of DAG, BE, or PA resulted in increased PMCA activity, as shown in Figs. 1 and 2 and Table 1. We therefore investigated effects of altered lipid concentrations on activity of PMCA in a more natural environment, *i.e.*, intact membrane system. Rat brain PMVs were incubated in the presence *vs*. absence of purified tubulin, and then in the presence *vs*. absence of phospholipase C (PLC), to increase the *in situ* level of DAG. PLC hydrolyzes phospholipids such as PC, and thereby generates DAG and phosphorylcholine [32]. PMCA activity was measured immediately after addition of PLC, or after incubation with PLC for 30 min at 37 °C. Aliquots of incubation mixtures were centrifuged to isolate PMVs, and total tubulin was determined by Western blot. In the absence of tubulin, increased DAG did not affect PMCA activity. In the absence of PLC preincubation, exogenous addition of tubulin inhibited PMCA activity by >80% (Fig. 4A), and increased tubulin concentration in PMVs by 4-fold (Fig. 4B). When DAG content in PMVs was increased by PLC incubation, PMCA activity was clearly stimulated by the presence of tubulin (Fig. 4A), even when tubulin remained associated with vesicles (Fig. 4B).

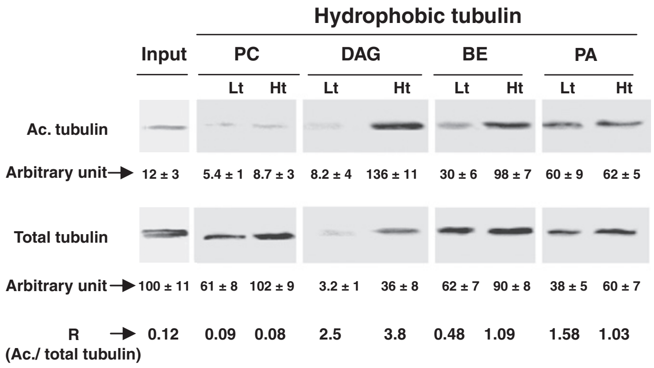


Fig. 3. Quantification of acetylated tubulin/PMCA complex as hydrophobic acetylated tubulin (HAT) in PMCA reconstituted in lipids after incubation with tubulin. PMCA was purified from rat brain, reconstituted in 100% PC, or in 80% PC plus 20% of the indicated lipids, and incubated for 20 min at 37 °C with low (25 μg/ml, Lt) or high (250 μg/ml, Ht) tubulin concentration. The incubation systems were partitioned as described in the Materials and methods section. Detergent fractions were immunoblotted and revealed with mAb DM1A for total tubulin, and mAb 6-11B-1 for acetylated (Ac.) tubulin. Tubulin (5 μg protein) used in the experiment was immunoblotted and revealed with the same antibodies (Input). Tubulin bands were scanned, and values are shown as arbitrary units. Values are mean ± SD from three independent experiments. R = ratio between acetylated and total tubulin in the detergent fraction.

To determine whether PMCA-dependent Ca^{2+} transport was affected by DAG generation in PMVs incubated with PLC, PMVs were treated with or without exogenous tubulin, and Ca^{2+}-transport was measured as described in the Materials and methods section, in the presence *vs*. absence of PLC. PMVs were treated with Fura-2AM, a Ca^{2+} chelator, and Ca^{2+} incorporation following addition of ATP in the presence *vs*. absence of tubulin and PLC was determined by fluorescence intensity (Fig. 5A). Increased rate of Ca^{2+} transport is indicated by a change of slope in the curve of intensity *vs*. incubation time. Addition of tubulin caused 80% decrease in Ca^{2+} transport in PMVs, and ~4-fold increase in amount of tubulin bound to vesicles. PLC incubation of tubulin-treated PMVs caused a 97% increase in Ca^{2+} transport (Fig. 5), without a significant change in amount of tubulin bound to vesicles. Similar experiments using vanadate, a potent inhibitor of P-ATPases, did not result in significant changes in Ca^{2+} transport of (data not shown). These findings indicate that Ca^{2+} transport within PMVs, which affects both tubulin and PLC levels, is PMCA-dependent.

The possibility that the effect of PLC treatment on PMCA activity in PMVs (Figs. 4 and 5) was due to the released acidic moieties instead of DAG was discarded since 100 μM of phosphorylcholine or phosphoryl ethanol amine had no effect PMCA reconstituted in 100% PC (result not shown).

4. Discussion

Intracellular Ca^{2+} is a second messenger involved in important physiological cell processes including proliferation, differentiation, and apoptosis [33–35]. The Ca^{2+} pump of plasma membranes is the main protein that regulates intracellular Ca^{2+} concentration, through intracellular signaling. PMCA activity is regulated by various factors, including lipids, proteolysis, calmodulin, and ethanol [3,4,36,37]. We showed previously that PMCA of rat brain membranes and CAD cells is inhibited by acetylated tubulin *in vitro*. When acetylated tubulin and PMCA become associated to form a protein complex, enzyme activity is inhibited, and activity is restored when such complex is dissociated by calmodulin or ethanol [15].

We show clearly here that the lipid environment influences the interaction between acetylated tubulin and PMCA and consequently its enzyme activity, however, we are at present unable to explain the intimate mechanism of this interaction and how this regulates the enzymatic activity. Several facts point out the complexity of this regulatory mechanism. In general, we found activation of PMCA by tubulin under certain conditions (nature and concentration of the lipids surrounding PMCA and tubulin concentration) even when, according to previous published results [15], inhibition rather than activation was expected to occur. If surrounding lipids are mainly acidic, the presence of tubulin favor enzymatic activity, while if they are neutral lipids, the effect on enzymatic activity is activatory provided that tubulin is present at low concentration, and it is inhibitory if tubulin is at higher concentration. Key observations supporting this concept are: (a) Tubulin at concentrations <50 μg/ml activated PMCA of PMVs reconstituted in PC, or in PC with DAG, BE, or PA (Fig. 2, Table 1); (b) At high tubulin concentrations, PMCA purified from PMVs was activated when reconstituted in PC with acidic lipids (BE, PA), but was inhibited when reconstituted in PC, or in PC with neutral lipid DAG (Fig. 2, Table 1); (c) PMCA was inhibited by increased concentration of tubulin [15] when embedded in PMVs; (d) *In vitro*

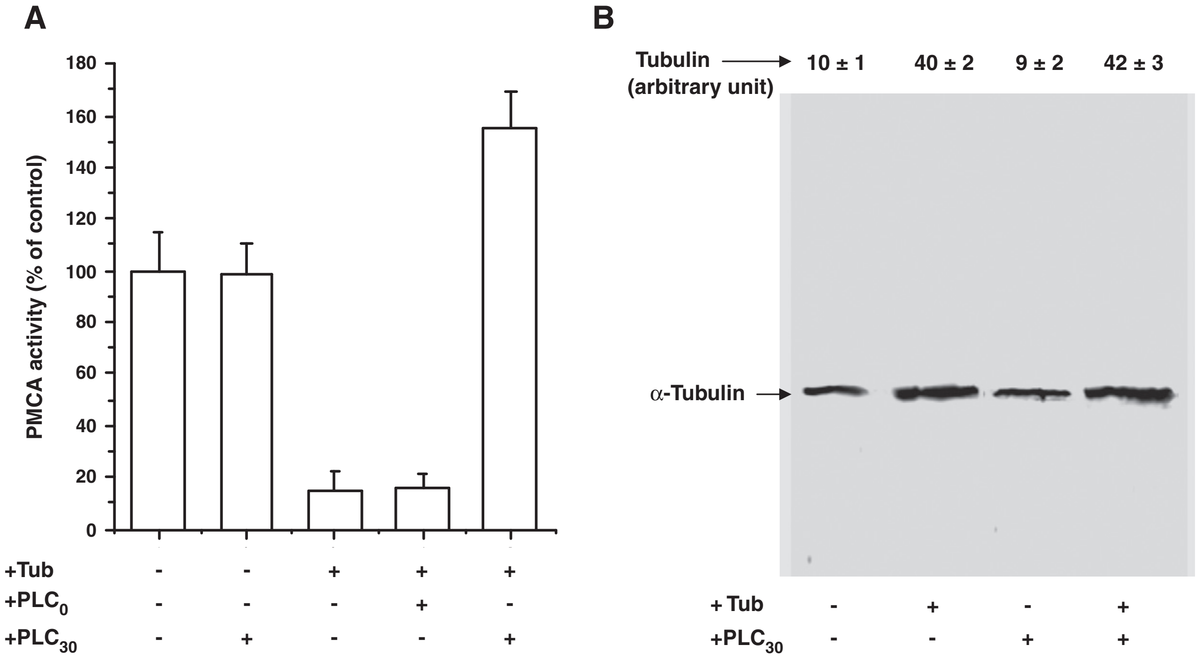


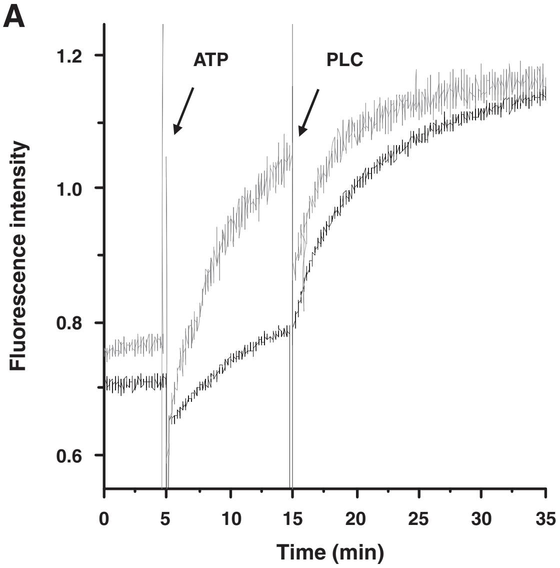
Fig. 4. Effect on PMCA activity by DAG generation by PLC incubation in plasma membrane. PMVs (0.25 mg protein) were incubated for 30 min at 37 °C in a final volume of 0.34 ml assay buffer, in the presence (+) or absence (−) of exogenous tubulin (300 μg/ml), and added with or without PLC (4 IU). (A) PMCA activity in 100 μl aliquot was determined immediately after addition of PLC (PLC$_0$), or after incubation for 30 min at 37 °C (PLC$_{30}$). PMCA activity in the absence of exogenous tubulin and PLC was 0.25 ± 0.03 nmol Pi min^{-1} mg of prot^{-1} determined by ATP hydrolysis (see Materials and methods section). (B) Another 100 μl aliquot was centrifuged at 100,000 ×g for 20 min at 37 °C to eliminate excess tubulin, and pellets were resuspended in original volume with assay buffer, immunoblotted, and revealed with anti-α-tubulin mAb. Tubulin bands were scanned, and values shown as arbitrary unit. Values are mean ± SD from three independent experiments.

generation of DAG in PMVs by action of PLC eliminated the inhibitory effect of tubulin on ATP hydrolysis capacity of PMCA, and on PMCA-dependent Ca^{2+} transport; (e) Tubulin interacts with other P-ATPases in plasma membrane, inhibiting their enzyme activity [38]. When PMVs were incubated in the presence of 300 μg/ml tubulin, the ATP-hydrolyzing activity of PMCA was reduced by about 80% (Fig. 4). When Ca^{2+}-transport activity into PMVs was measured under similar conditions (Fig. 5), same degree of inhibition (80%) was obtained. This suggests that Ca^{2+}-transport inhibition is caused by inhibition of ATP-hydrolyzing activity of PMCA. When PMVs were pre-treated with PLC to increase DAG concentration, subsequent treatment with high tubulin concentration did not inhibit ATP-hydrolyzing activity of PMCA (Fig. 4A) or Ca^{2+}-transport activity (Fig. 5).

Calmodulin, ethanol, and acidic lipids are activators of PMCA [16,18]. In the present study, PA increased PMCA activity by 7-fold (Table 1). Tubulin works together with lipid to regulate PMCA activity. Tubulin in combination with acidic lipids is the most potent activator of PMCA even when compared with calmodulin which activates PMCA by a factor of 2 [4], whereas high-concentration tubulin in combination with neutral lipid is the most potent inhibitor of the enzyme. This conclusion is supported by studies of PMCA reconstituted in lipids, and in PMVs. When the enzyme was reconstituted in PC, PMCA activity was increased 7-fold by PA. Tubulin without PA had no effect on enzyme activity. A combination of PA plus tubulin increased enzyme activity almost 27-fold, illustrating their synergistic effect. DAG produced 4-fold increase of PMCA activity, whereas the combination of DAG plus tubulin, eliminated >50% of this increase. ATP hydrolysis and PMCA-dependent Ca^{2+} transport in PMVs were reduced ~80% by tubulin, and were restored by generation of DAG by PLC incubation (Figs. 4, 5).

Tubulin forms a complex with PMCA, since the two proteins were immunoprecipitated together by an anti-tubulin antibody [15]. In the present study, both purified proteins were used for experiments on the effect of tubulin on PMCA reconstituted in lipids (Figs. 1, 2, 3). However, it is not yet clear whether there is a direct interaction between these proteins. One possibility is that they interact directly, with some cytoplasmic domain of PMCA as the site of interaction with tubulin, as we previously found for the interaction between tubulin and Na^+,K^+-ATPase [12]. Another possibility is that interaction between tubulin and PMCA is not direct but mediated by lipids; if this were the case, it would be reasonable to think that PMCA activity could be influenced by lipid composition. We found that tubulin is associated with PMCA in the presence of the several lipids tested in the present work including PA (Fig. 3). However, to obtain an inhibitory effect, PMCA should associate with tubulin of the acetylated isotype. Observe in Fig. 3 that the formation of the acetylated tubulin/PMCA complex is more specific with PMCA reconstituted in DAG since in the detergent (hydrophobic) phase there is total tubulin that is mainly acetylated (ratio 3.8). This indicates that when PMCA was reconstituted in DAG and subsequently added with tubulin, the acetylated isotype was preferentially associated suggesting that a complex with similar characteristics found in PMVs was formed [15]. Instead, PMCA reconstituted in 20% PA, even when it did not inhibit the insertion of tubulin in the detergent phase, seems to form a lower amount of acetylated/PMCA complex (ratio 1.03) as compared with DAG (ratio 3.8). This difference could be due to a negative electric environment by PA diminishing the specific association of the acetylated tubulin with PMCA.

The joint effect of lipids and tubulin may have a key role in cell physiology. In this sense, it is know that lipids are not homogeneously distributed in cell membranes. The nature of the lipids constituent of membranes depends on cell type, particular regions of the cell, physiological state of the cell, *etc.* Furthermore, PMCA is also asymmetrically distributed. So, it is not unreasonable to speculate that the resulting PMCA activity in different regions of the cell depends on the nature of the lipids and the concentration of tubulin (or microtubules) in each region. Furthermore, cell signaling mechanisms could



B

	Ca^{2+}-transport (% of control)		PMVs tubulin (arbitrary unit)	
	-PLC	+PLC	-PLC	+PLC
-Tubulin	100	99 ± 12	10 ± 2	9 ± 3
+Tubulin	21 ± 5	97 ± 11	42 ± 5	43 ± 4

Fig. 5. Effect of tubulin and PLC on PMCA-dependent Ca^{2+} transport in PMVs. PMVs (1 mg protein) were incubated for 30 min at 37 °C in a final volume of 1 ml transport buffer in the presence (black lines in A; +Tubulin in B) or absence (gray lines in A; −Tubulin in B) of exogenous tubulin (300 μg/ml). (A) Calcium level in PMVs, as a function of incubation time, was estimated as relative fluorescence intensity, using Fura-2AM as indicator, as described in the Materials and methods section. ATP (final concentration 1.5 mM) and PLC (16 IU) were added at the times indicated by arrows. (B) Ca^{2+} transport was estimated based on the curve, in the first 5 min of incubation after addition of ATP (−PLC), or after addition of PLC (+PLC) in A. Values shown are percentage of the control without tubulin or PLC. 100 μl aliquots of −PLC and +PLC samples were centrifuged at 100,000 ×g for 20 min at 37 °C, to eliminate excess tubulin, and pellets were resuspended in original volume with assay buffer, immunoblotted, and revealed with anti-α-tubulin mAb. Tubulin bands were scanned, and values shown as arbitrary units. Values are mean ± SD from three independent experiments.

eventually use either a change in nature or concentration of lipids and/or tubulin concentration, provoking in this way inhibiting or activating responses on PMCA. This leads us to consider that not only the nature or concentration of lipids could be important to modulate PMCA activity but also changes in the dynamics of membrane structure [39,40]. One example could be the activation of PLC which catalyzes the production of DAG with the consequent activation or inhibition of PMCA depending on tubulin concentration (Fig. 2A). Instead, if PLD is activated, PA would be produced with the consequent activation of PMCA regardless of tubulin concentration (Fig. 2C).

The study of this PMCA regulatory mechanism is in preliminary stages. The important several functions in which PMCA is involved in different cell types indicate that significant efforts from the biochemical, biophysical, cellular and physiological fields should be done. In order to validate this PMCA regulatory mechanism, we are currently trying to verify whether some signaling pathway induces changes in lipidic composition or tubulin concentration that resulted in alteration of local PMCA activity.

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