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PMCA activity and membrane tubulin affect deformability of erythrocytes from normal and hypertensive human subjects☆

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ABSTRACT

Our previous studies demonstrated formation of a complex between acetylated tubulin and brain plasma membrane Ca^{2+}-ATPase (PMCA), and the effect of the lipid environment on structure of this complex and on PMCA activity. Deformability of erythrocytes from hypertensive human subjects was reduced by an increase in membrane tubulin content. In the present study, we examined the regulation of PMCA activity by tubulin in normotensive and hypertensive erythrocytes, and the effect of exogenously added diacylglycerol (DAG) and phosphatidic acid (PA) on erythrocyte deformability. Some of the key findings were that: (i) PMCA was associated with tubulin in normotensive and hypertensive erythrocytes, (ii) PMCA enzyme activity was directly correlated with erythrocyte deformability, and (iii) when tubulin was present in the erythrocyte membrane, treatment with DAG or PA led to increased deformability and associated PMCA activity. Taken together, our findings indicate that PMCA activity is involved in deformability of both normotensive and hypertensive erythrocytes. This rheological property of erythrocytes is affected by acetylated tubulin and its lipid environment because both regulate PMCA activity.

1. Introduction

Hypertension is a pathological state associated with multiple factors, including changes in the properties of erythrocytes and in their hemorheological parameters. These changes include alterations in intracellular levels of calcium and sodium, erythrocyte deformability index, and membrane fluidity [1–5]. Intracellular calcium regulates numerous erythrocyte membrane functions, including control of shape, lipid composition, and cation permeability [6,7]. Plasma membrane Ca^{2+}-ATPase (PMCA) is a P-type ATPase that plays a crucial role in regulation of cell calcium homeostasis [8]. We demonstrated in 2008 that tubulin (primarily the acetylated isotype, termed AcTub) is capable of interacting with PMCA and thereby inhibiting its enzyme activity [9]. However, the regulatory effect of tubulin on PMCA is not always inhibitory, and is modulated by the lipids that surround PMCA. In a follow-up 2012 study, we showed that PMCA activity is enhanced by diacylglycerol (DAG), phosphatidic acid (PA), or an acidic lipid mixture, and that this enhancing effect is further promoted by the presence of tubulin [10]. When tubulin purified from rat brain (containing the natural mixture of isotypes) was mixed with purified PMCA in the presence of acidic or neutral lipids, a tubulin/PMCA complex was formed that showed a preference for AcTub. The lipid fractions evidently stimulated recruitment of AcTub during complex formation, because the above preference was not observed in the absence of the lipids [10].

Tubulin in human erythrocytes is distributed into three operationally distinct fractions that can be isolated by centrifugation procedures: cytosolic fraction, membrane fraction, and a fraction that sediment at 100,000 ×*g* [11]. We found that hypertensive subjects, in comparison with normal subjects, had a higher level of tubulin in the membrane fraction. Total tubulin content was essentially the same, but the protein was redistributed from the sedimentable fraction to the membrane fraction. The increased content of tubulin in membrane was correlated with inhibition of Na^+,K^+-ATPase (NKA), another P-type ATPase. In erythrocytes from spontaneously hypertensive rats, the progressive increase of tubulin content in erythrocyte membranes was correlated with disease development and reduced erythrocyte deformability [12].

The present study was focused on the roles of tubulin, lipids, and PMCA activity in human erythrocyte deformability. Erythrocyte deformability was reduced or increased, respectively, by inhibition or promotion of PMCA activity. AcTub and lipid membrane properties both affected PMCA activity and thereby regulated erythrocyte deformability.

Abbreviations: AcTub, acetylated tubulin; DAG, diacylglycerol; PA, phosphatidic acid; PMCA, plasma membrane Ca^{2+}-ATPase.

☆ In memory of Marina Rafaela Amaiden ("Rafa"), who will remain forever in our hearts.

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Table 1
Demographic and hemodynamic data from the studied populations of normotensive (N) and hypertensive (H) patients.

Parameters	N (n = 30)	H (n = 45)
Age (years)	64 ± 11	62 ± 10
Sex (male/female)	18/12	20/25
Weights (Kg)	77 ± 11	80 ± 12
Systolic BP (mm Hg)	110 ± 18	165 ± 22
Diastolic BP (mm Hg)	86 ± 10	103 ± 9
Diabetes	Negative	Negative
Hypercholesterolemia	0/30	5/45
Antihypertensive treatment	–	20/45
Urea (mg/dl)	35 ± 7	34 ± 8
Creatinine (md/dl)	0.72 ± 0.12	0.78 ± 0.11
Na^+ (mM)	141 ± 5	139 ± 10
K^+ (mM)	6 ± 2	5 ± 1

2. Material and methods

2.1. Materials

ATP, mouse anti-α-tubulin mAb DM1A, anti-PMCA mAb 5 F10, anti-AcTub mAb 6-11B-1, peroxidase-conjugated mouse IgG, fluorescein-conjugated mouse IgG, 1,2-dicapryloyl-sn-glycerol (DAG), L-α-PA from egg yolk, nitrocellulose membrane, nocodazole, paclitaxel (taxol), calcium ionophore A23187 (calcimycin), ouabain, and o-vanadate were from Sigma Chemical Co. (St. Louis, MO, USA). Anti-phosphatidylinositol 3-kinase (PI3K) p110 mAb Ci was from Santa Cruz Biotechnology. Lumigen PS-3 detection kit and high-performance chemiluminescence film were from GE Healthcare Life Sciences (Piscataway, NJ, USA).

2.2. Preparation of human erythrocytes

Male and female patients in the San Antonio de Padua Hospital of Rio Cuarto (Cordoba, Argentina) were recruited for the study using a protocol of informed consent approved by the Human Ethics Committee of the National University of Rio Cuarto. Demographic data and hemodynamic of patients are summarized in Table 1. The fresh blood samples were obtained from healthy volunteers and hypertensive patients' vacutainer tubes (Becton-Dickinson, Plymouth, UK) using 1 mg/ml of EDTA as anticoagulant and stored for 96 h at 4 °C. Erythrocytes isolated by conventional centrifugal separation and immediately are used for experiments. Hypertensive patients were selected on the basis of personal history of hypertension (according to WHO criteria) consistently >160/110 mm Hg.

The ~50% of hypertensive patients were treated with antihypertensive drugs. In Figs. 1 and 3 and Table 2 a patient from hypertension group was treated with anti-hypertensive and in Figs. 2 and 4 two patients in the group of hypertensive patients were treated with anti-hypertensive.

2.3. Isolation of erythrocyte membranes

Erythrocytes isolated from 2 ml blood were resuspended in 4 ml lysis buffer (7.5 mM sodium phosphate, pH 7.5, containing 1 mM EDTA, and 20 mg/ml PMSF) and incubated for 15 min in ice. The lysate was centrifuged at 20,000 ×*g* for 20 min. The pellet was washed three times with 6 ml lysis buffer without PMSF, resuspended in 0.3 ml lysis buffer ("membrane fraction"), and stored at −20 °C until use.

2.4. Preparation of Sepharose-linked antibody

Anti-α-tubulin mAb DM1A and anti-PI3K p110 mAb Ci were covalently linked to CNBr-activated Sepharose 4B as described previously [13], with slight modification. Sepharose beads were washed with 100 volumes of 1 mM HCl at 21 °C. The resulting packed beads (1 ml) were mixed with ascites fluid (2.5 mg protein) containing mAb DM1A or mAb Ci in 1 ml coupling buffer (0.5 M NaCl containing 0.2 M $NaHCO_3$, pH 8.2). The mixture was shaken on a platform rocker for 4 h at 21 °C and loaded into a small chromatographic column. Unbound antibody was removed by washing with 5 ml coupling buffer. Antibody-bound beads were transferred to a beaker and suspended in 1 ml coupling buffer containing 0.2 M glycine to block unreacted Sepharose sites. The mixture was shaken for 2 h at 21 °C, and unbound glycine was removed by washing the beads with 10 ml coupling buffer. The resulting Sepharose-linked antibody was washed with 1.5 ml of 0.01 M Tris/HCl, pH 8.0, containing 0.14 M NaCl and 0.025% NaN_3, and stored at 4 °C until use (maximum 2 days).

2.5. Immunoprecipitation procedure

One volume membrane preparation (5 mg protein/ml) was mixed with one volume NaCl/Tris (150 mM NaCl, 50 mM Tris/HCl, pH 7.4) containing 1% Triton X-100 (NaCl/Tris-Triton) and centrifuged to eliminate residual insoluble material. Aliquots (0.15 ml) were mixed with 0.15 ml packed antibody-Sepharose beads and incubated 4 h at 20 °C with gentle shaking. Samples were centrifuged, the supernatant was removed, and precipitated material was washed five times with NaCl/Tris-Triton. Fractions (50 μl) of packed beads and supernatant were resuspended in 50 μl Laemmli sample buffer, heated to 50 °C for 15 min, and centrifuged. Aliquots (20 μl) of soluble fractions were subjected to SDS-PAGE. A control was run in parallel using anti-PI3K p110 mAb Ci, instead of anti-α-tubulin mAb DM1A, linked to Sepharose.

2.6. Preparation and addition of exogenous phospholipids

DAG and PA were kept as 1000-fold concentrated stock solutions in chloroform, dried prior to use under N_2 stream, and emulsified by sonication for 10 min in 150 mM NaCl, 10 mM Tris–HCl, pH 8.0. DAG and PA in vesicles were added to intact erythrocytes to a final concentration of 50 μM [14,15].

2.7. PMCA activity assay

Enzyme activity of PMCA from erythrocyte membranes (0.5–1.0 mg protein) was determined using a coupled enzyme assay as described by Salvador and Mata [16]. In brief, membranes were incubated for 5 min at 37 °C in a reaction mixture containing 50 mM Tris–HCl, pH 7.4, 20 mM KCl, 100 mM NaCl, 2.5 mM $MgCl_2$, 1 mM ouabain, 0.16 mM NADH, 0.45 mM phosphoenolpyruvate, 10 IU pyruvate kinase, 28 IU 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 Webmax C Standard software. The enzymatic reaction was initiated 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+} in a final volume of 0.34 ml. NADH oxidation was measured for 15 min at room temperature at wavelength 340 nm in a recording spectrophotometer.

2.8. Determination of erythrocyte deformability

Erythrocyte deformability was determined using the filtration method described by Cabrales [17]. In brief, polycarbonate filters (Nuclepore/Whatman; Pleasanton, CA, USA) with a pore size of 5 μm, diameter of 13 mm, and pore density of $4 \times 10^3/mm^2$ were perfused with diluted cell suspensions using an infusion pump. Cell suspensions or medium (buffer) were delivered through the filter at rates of 0.5, 0.6, 0.7, 0.8, and 0.9 ml/min. The pressure drop across the filter was measured with a differential pressure transducer. Erythrocyte deformability was assessed by determining the parameters β and ψ. β is the ratio of (i) the resistance to flow through a filter pore calculated according to the method of Skalak et al. [18,19], using the ratio of the initial pressure

drop across the filter in cell suspension, to (ii) the pressure drop with buffer alone.

$$\beta = (Pi/Po-1)v/h + 1$$

where Pi = pressure drop across the filter; Po = cells present to the pressure drop; υ = ratio of mean cell volume to pore volume, and h = volume packed erythrocyte fraction of the perfusate. Filtration measurements were made using different filters, and averaged. The value of β depends on the flow used to deform the cells. ψ is the slope of the curve of β *vs.* flow. An increase of ψ reflects a decrease of erythrocyte deformability and is independent of the flow used to deform the cells.

2.9. Immunofluorescence microscopy

Erythrocytes were fixed on coverslips with anhydrous methanol at −20 °C. Samples were rehydrated, incubated with 2% BSA (bovine serum albumin) in NaCl/Pi (PBS) for 60 min, and stained by indirect immunofluorescence using mouse anti-α-tubulin mAb DM1A (dilution 1:100) or anti-AcTub mAb 6-11B-1 (dilution 1:200) in NaCl/Pi containing 1% BSA. Fluorescein-conjugated anti-mouse IgG (dilution 1:200) was used as secondary antibody. Coverslips were mounted on Fluor-Save and observed for epifluorescence with a fluorescence microscope (model H600L; Nikon; Tokyo, Japan). As we demonstrated previously [11], uniform fluorescence distribution in erythrocytes indicates high tubulin content in membrane, whereas a ring pattern of fluorescence indicates localization of tubulin in the sedimentable fraction.

2.10. Immunoblotting

Proteins were separated by SDS-PAGE (8% gel for PMCA; 14% gel for separate tubulin) by the method of Laemmli [20], and transferred to a nitrocellulose sheet. Blots were reacted with anti-α-tubulin mAb DM1A (dilution 1:1000), anti-AcTub mAb 6-11B-1 (dilution 1:1000) [21], or anti-PMCA mAb 5 F10 (dilution 1:1000) [22]. The sheet was reacted with the corresponding peroxidase-conjugated anti-IgG antibody (dilution 1:500), and stained using the 4-chloro-1-naphthol method or the ECL system. Band intensities were quantified using the Scion Image software program.

2.11. Protein determination

Protein concentration was determined by the method of Bradford [23] using BSA as standard.

3. Results

3.1. Tubulin/PMCA complex in erythrocytes from normotensive and hypertensive subjects

Our previous studies showed that plasma membrane tubulin content in erythrocytes from normotensive and hypertensive subjects affects ion transport [11], and that tubulin in brain membranes forms a complex with PMCA, resulting in reduced enzyme activity [9]. Furthermore, Postnov YV et al. found that calcium transport is altered in erythrocyte membranes with essential hypertension [24]. We therefore investigated the possibility that tubulin/PMCA complex is involved in PMCA activity regulation in erythrocytes and thereby promotes development of hypertension. We immunoprecipitated the complex from detergent-solubilized erythrocyte membranes using Sepharose beads coupled to anti-α-tubulin mAb DM1A. PMCA in the immunoprecipitate and that remaining in the supernatant were detected by Western blotting (Fig. 1). In normotensive erythrocytes, 65% of total PMCA present in the membrane fraction was precipitated by DM1a (Precipitate "N"), indicating that most of the enzyme is associated with tubulin. Remaining non-associated PMCA was recovered in the supernatant fraction (Supernatant "N"). In hypertensive erythrocytes, ~90% of total PMCA in the membrane fraction was associated with tubulin (Precipitate "H"). These findings indicate that the content of tubulin/PMCA complex in erythrocyte membranes is higher for hypertensive than for normotensive subjects. Therefore, a diminished PMCA activity should be expected in erythrocytes from hypertensive subjects. In effect, PMCA activity was 40% lower in hypertensive than in normotensive erythrocytes (right panel). Taken together, these findings suggest that (i) tubulin and PMCA are associated in a complex in erythrocyte membranes, (ii) PMCA activity is regulated by the degree of such association, and (iii) increased complex formation is the cause of reduced PMCA activity in hypertensive erythrocytes.

3.2. Effects of DAG and PA on PMCA activity and erythrocyte deformability

Erythrocyte deformability, *i.e.*, their capacity for changing shape while traveling through blood vessels, is reduced in hypertensive

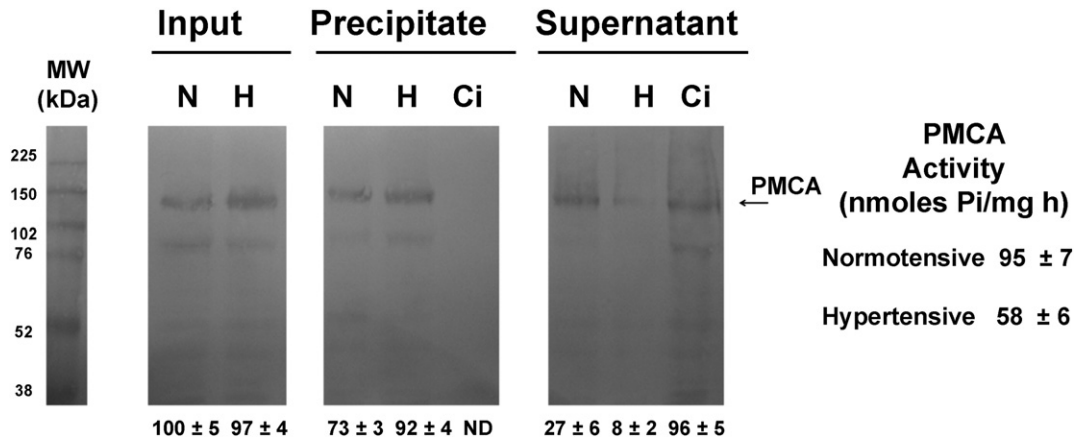
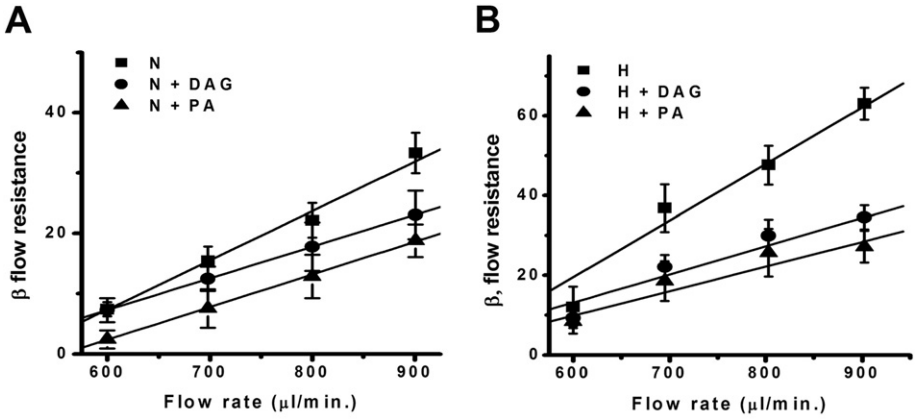


Fig. 1. Tubulin/PMCA complex and enzyme activity in membranes of hypertensive (H) and normotensive (N) erythrocytes. Input: membranes were solubilized with 0.5% Triton X-100 and analyzed by immunoblotting with anti-PMCA mAb 5 F10. Precipitate: membranes solubilized in detergent were immunoprecipitated with Sepharose linked to anti-α-tubulin mAb DM1A or to anti-PI3K mAb Ci as control. Immunoprecipitate was analyzed by immunoblotting with mAb 5 F10. Supernatant: upper fraction of immunoprecipitate. An equal quantity of membrane protein was loaded in each lane. Optical densities corresponding to PMCA bands were quantified using the Scion Image software program. Values are shown as arbitrary units (A.U.). mAb 5 F10 recognized additional bands at regions corresponding to MW 95 and 52 KDa; these may represent proteolysis of the pump [38,39]. PMCA activity was measured by ATP hydrolysis in erythrocyte membrane fractions as described in M&M. Values shown are mean ± SD from three independent experiments.



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	Normotensive		Hypertensive	
	Deformability (ψ)	PMCA Activity (nmoles Pi/mg/h)	Deformability (ψ)	PMCA Activity (nmoles Pi/mg/h)
Untreated	80 ± 7	95 ± 16	141 ± 18	58 ± 6
+ DAG	53 ± 1	116 ± 8	68 ± 9	75 ± 7
+ PA	54 ± 2	118 ± 7	61 ± 9	123 ± 8

Fig. 2. Effects of DAG and PA on erythrocyte deformability and PMCA activity. Normotensive (A) and hypertensive (B) erythrocytes (quantity 1 ml for each) were incubated at 37 °C for 30 min in the absence (■) or presence of 50 μM DAG (●) or PA (▲). Flow resistance (β) through filter pores was determined as described in M&M. (C) Deformability (ψ) values were calculated from the slopes of the curves in A and B. PMCA activity was determined by ATP hydrolysis as described in M&M. Values shown are mean ± SD from four independent experiments.

subjects [25,26]. Erythrocyte deformability is affected by intracellular levels of sodium and calcium ions [12,27]. Our previous studies have shown that PMCA and NKA activities are regulated by tubulin [9,10,28–30], and that the regulatory effect on PMCA activity depends on surrounding lipids [10]. In the present study, we examined the regulatory effects of DAG and PA on PMCA activity and consequently on erythrocyte deformability. For determination of erythrocyte deformability, we measured β (resistance to flow) in normotensive and hypertensive subjects (Fig. 2A, B) and calculated ψ (Fig. 2C). Both β and ψ were lower for normotensive than for hypertensive erythrocytes, indicating greater deformability. Consistent with our previous findings [12], deformability was ~50% lower for hypertensive than for normotensive erythrocytes (Fig. 2C, ψ values). Following addition of PA or DAG, deformability of normotensive erythrocytes increased ~40%, and PMCA activity increased ~20% (Fig. 2C). DAG or PA treatment of hypertensive erythrocytes caused a ~55% increase of deformability and a significant increase in PMCA activity (Fig. 2C).

The observed correlation between increased PMCA activity caused by DAG and PA and increased erythrocyte deformability suggested that PMCA activity is involved in the deformability. To test this hypothesis, we assessed deformability following treatment of erythrocytes with P-ATPase inhibitors. In the presence of 2 mM O-vanadate (a general P-ATPase inhibitor), the ψ value of normotensive erythrocytes increased ~55% (Table 2), suggesting that deformability may be dependent on P-ATPase (NKA or PMCA) activity. We next examined erythrocyte deformability in the presence of ouabain (1 mM), a selective inhibitor of NKA activity. Ouabain had no effect on deformability of either normotensive or hypertensive erythrocytes (Table 2), indicating that NKA does not play a major role in regulation of deformability. To investigate the role of PMCA, we determined deformability following incubation of erythrocytes with the calcium ionophore A23187 (calcimycin) and free calcium, a condition simulating the high calcium concentration that would occur within erythrocytes in the absence of PMCA activity. A23187 treatment caused a ~60% decrease in deformability of normotensive erythrocytes, but had no effect on deformability of hypertensive erythrocytes (in which PMCA activity is ~40% lower than in normotensive erythrocytes) (Table 2). These findings indicate that PMCA activity plays an important role in erythrocyte deformability.

3.3. The effects of DAG on PMCA activity and erythrocyte deformability depend on membrane tubulin content

The promoting effect of DAG on PMCA activity and erythrocyte deformability was more pronounced in hypertensive than in normotensive erythrocytes (Fig. 2C). The fact that membrane tubulin content is higher in hypertensive erythrocytes [6] suggests that the strong effect of DAG on deformability is due to a higher content of inhibited PMCA (resulting from a higher level of tubulin/PMCA complex; Fig. 1) and thus a greater amount of PMCA available to be re-activated. We hypothesized that tubulin/PMCA complex helps regulate erythrocyte deformability through inhibition or de-inhibition of PMCA activity.

Table 2
Effects of PMCA inhibitors on erythrocyte deformability.

	Deformability (Ψ)	
	Normotensive	Hypertensive
Untreated	80 ± 7	141 ± 18
Vanadate	124 ± 13	140 ± 28
Ouabain	75 ± 11	131 ± 17
A23187	81 ± 13	129 ± 18
A2387 + Ca^{2+}	142 ± 17	140 ± 18

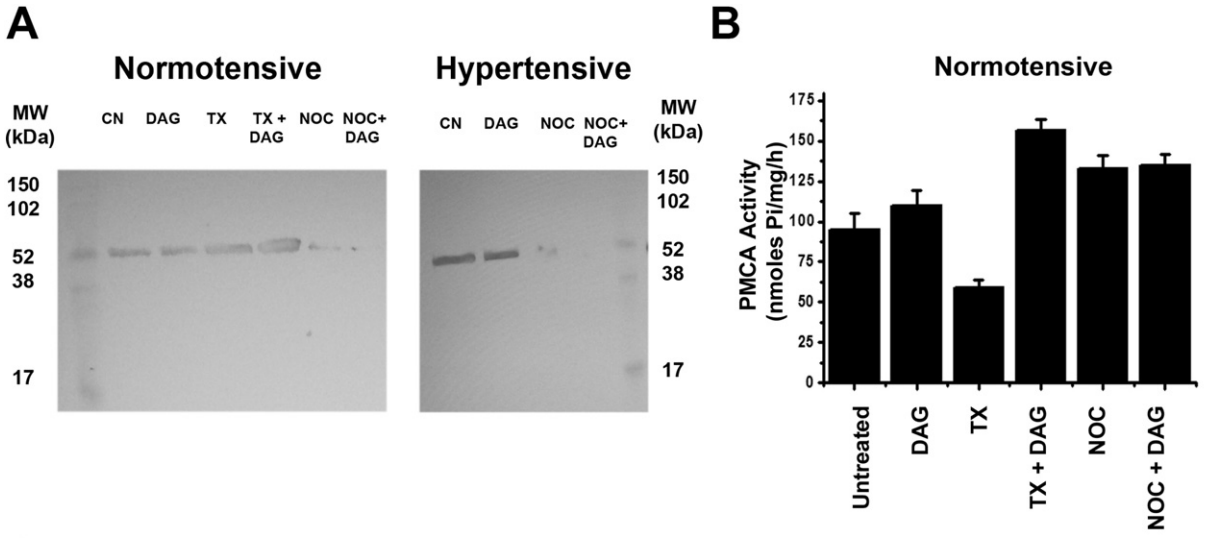
Hypertensive and normotensive erythrocytes were incubated at 37 °C for 30 min in the presence of 1 mM vanadate or 1 mM ouabain. In the experiment with Ca^{2+} ionophore A23187, cells were incubated with 50 μM of A23187 and then at 37 °C for 30 min in the absence or presence of sufficient $CaCl_2$ to reach a free Ca^{2+} concentration of 100 μM. Cells were washed after treatment and deformability (ψ) was determined as described in M&M. Values shown are mean ± SD from three independent experiments.

To test this hypothesis, we increased or reduced membrane tubulin content in normotensive erythrocytes by treatment with taxol or nocodazole, respectively [12], and then evaluated the effect of DAG on erythrocyte deformability. Taxol treatment resulted in a ~40% increase in tubulin content (Fig. 3A,C), in agreement with our previous findings [12], and a reduction in PMCA activity and cell deformability (Fig. 3B,C). Subsequent addition of DAG produced a ~100% increase in PMCA activity (Fig. 3B), and ~50% increase in deformability (Fig. 3C). In experiments with hypertensive erythrocytes, DAG treatment (without taxol pre-treatment) increased deformability and PMCA activity (Figs. 2C, 3C). When cells were pre-treated with nocodazole to reduce membrane tubulin content (Fig. 3A, C), DAG had no effect on deformability or PMCA activity of normotensive erythrocytes (Fig. 3B). When the various experiments described above were performed with PA instead of DAG, the results obtained were similar to those with DAG (data not shown). Taken together, our findings indicate that the effect of DAG on erythrocyte deformability and PMCA activity depends on membrane tubulin content; the higher the tubulin content, the greater the promoting effect of DAG on deformability.

3.4. Effects of DAG and PA on distributions of total tubulin and AcTub in erythrocytes

In our previous studies on rat brain membranes, regulation of PMCA activity through tubulin/PMCA complex showed a preference for the AcTub isotype [9]. In *in vitro* experiments using artificial vesicles, acidic or neutral lipids increased AcTub content in the hydrophobic phase when PMCA was present [10]. The effect of DAG on erythrocyte deformability observed in the present study may therefore be due to a lipid-induced change in AcTub distribution, since AcTub is involved in regulation of PMCA activity. This possibility was tested by treating normotensive and hypertensive erythrocytes with DAG or PA and analyzing distribution of total tubulin and AcTub by indirect immunofluorescence with mAbs DM1A and 6-11B-1, respectively. Tubulin in erythrocytes appears as a sedimentable structure in membrane and soluble fractions [11,12]. In immunofluorescence techniques, the sedimentable structure is seen as a fluorescent ring in the erythrocyte periphery, whereas membrane tubulin is seen uniformly in the entire erythrocyte [11,12]. In normotensive erythrocytes, DAG or PA treatment had no effect on total tubulin distribution (Fig. 4). Following DM1A staining, fluorescence was concentrated at the periphery, indicating that total tubulin was mostly in sedimentable form (Fig. 4A). In contrast, DAG or PA treatment did affect AcTub distribution; AcTub (as revealed by 6-11B-1 staining) was concentrated at the periphery before DAG or PA treatment but migrated to the membrane following treatment (Fig. 4A).

In hypertensive erythrocytes, total tubulin was found mainly in the membrane. Following DAG or PA treatment, total tubulin was translocated to sedimentable structures, whereas AcTub remained at the membrane (Fig. 4A). To confirm this finding, membranes from normotensive and hypertensive erythrocytes were isolated, and membrane AcTub was quantified by immunoblotting. DAG or PA treatment caused



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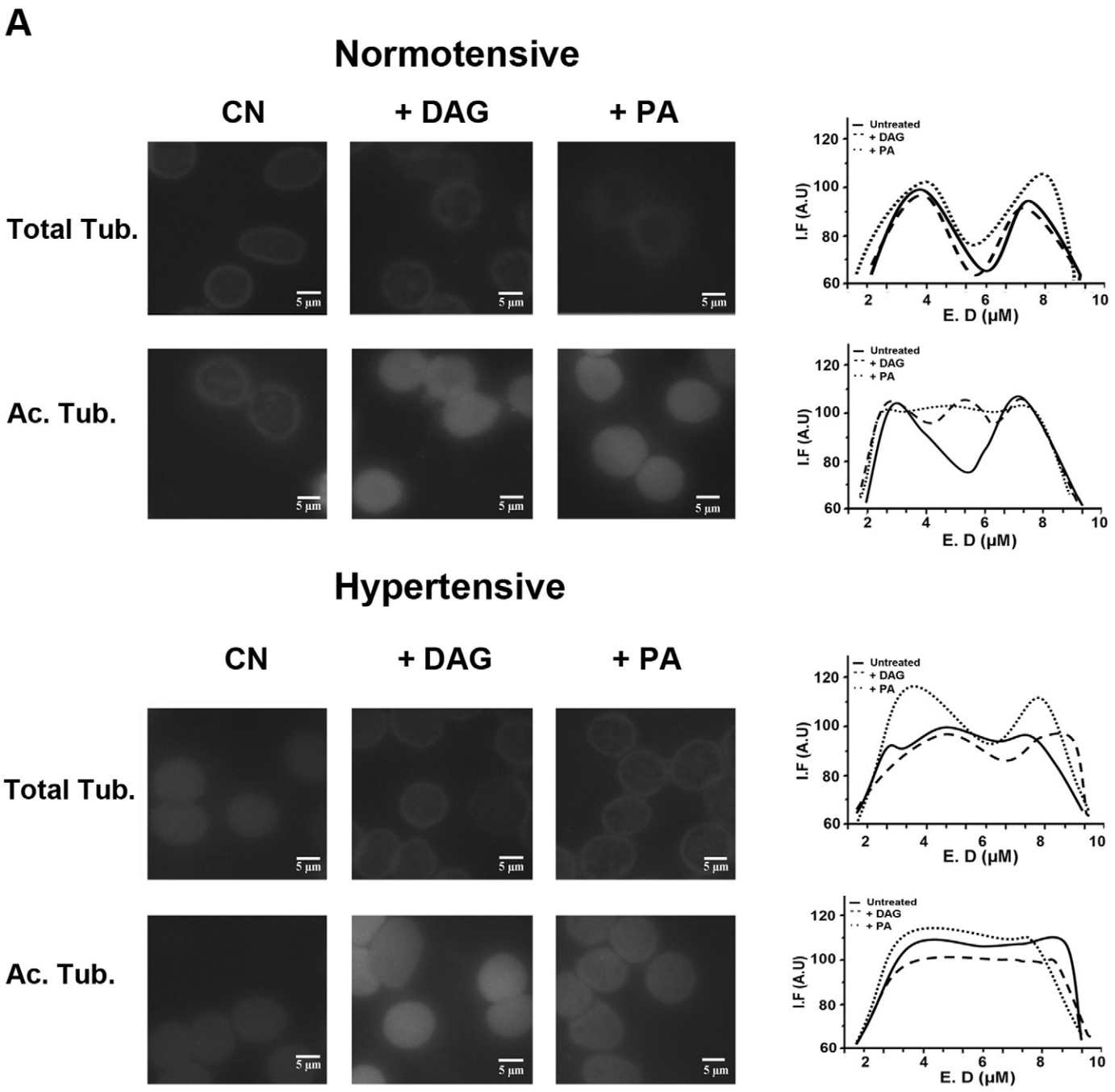
	Normotensive						Hypertensive			
	CN	DAG	TX	TX + DAG	NOC	NOC+ DAG	CN	DAG	NOC	NOC+ DAG
Tubulin Arbitrary Units (A.U)	100 ± 5	118 ± 4	140 ± 7	136 ± 12	10 ± 4	13 ± 5	100 ± 4	103 ± 2	10 ± 4	11± 5
Deformability (Ψ)	80 ± 7	53 ± 1	116 ± 3	62 ± 10	57± 5	59 ± 7	141 ± 18	68 ± 9	66 ± 6	68 ± 5

Fig. 3. Effects of taxol, nocodazole, and DAG on erythrocyte deformability, PMCA activity, and membrane tubulin content. Normotensive and hypertensive erythrocytes were incubated for 1 h at 37 °C in the absence (CN) or presence of 50 μM nocodazole (NOC). Normotensive (but not hypertensive) erythrocytes were incubated with 5 μM taxol (TX). Following these treatments, the cells were incubated for 30 min with 50 μM DAG. (A) Membranes were obtained from an aliquot of erythrocyte suspension and subjected to immunoblotting and staining with anti-tubulin mAb DM1A. Volumes loaded on each well were calculated to represent equivalent amounts of erythrocytes. Optical densities were determined by the Scion Image program, and tubulin contents for each lane were expressed as percentages of corresponding values for untreated (CN) normotensive and hypertensive erythrocytes. (B) Membranes isolated from another aliquot of normotensive erythrocyte suspension were subjected to determination of PMCA activity by ATP hydrolysis as described in M&M. (C) Top line: values for tubulin bands as in Panel A (three independent experiments) were quantified and expressed as arbitrary units (A.U.). Bottom line: Other aliquots of erythrocyte suspensions were subjected to determination of flow resistance (β; not shown) and deformability (Ψ). Values shown are mean ± SD from three independent experiments.


A
Normotensive
CN
+ DAG
+ PA
Total Tub.
Ac. Tub.
5 µm
Untreated
+ DAG
+ PA
I.F (A.U)
E. D (µM)
Hypertensive
B
Normotensive
Hypertensive
MW (kDa)
Cn + DAG + PA
HTA + DAG + PA
102
52
38
17
Acetylated Tubulin
Ac. Tub. (A.U)
10±2 65 ± 3 71± 4
49 ± 5 48 ± 6 56±5

an increase of membrane AcTub in normotensive but not in hypertensive erythrocytes (Fig. 4B). These findings indicate that DAG and PA treatment caused a redistribution of both total tubulin and AcTub in erythrocytes. These lipids increased AcTub content in erythrocyte membranes, similarly to our previous findings for acidic and neutral lipids in artificial vesicles [10].

4. Discussions

In hypertension, altered transport of sodium and calcium ions through the erythrocyte membrane leads to changes in characteristics and physical properties of erythrocytes [26]. Findings from our previous studies [9–12] and the present study indicate that both membrane tubulin and membrane lipid composition are involved in regulation of erythrocyte deformability through their effects on PMCA activity. Erythrocyte deformability was directly correlated with PMCA activity. Deformability was minimal when PMCA was inhibited, either by increased membrane tubulin in hypertensive erythrocytes (Fig. 2B [compare ψ and PMCA activity values], Fig. 3A), or by enzyme inhibitors such as vanadate (Table 2). Deformability was maximal when PMCA activity was increased by reduction of membrane tubulin content in normotensive erythrocytes (Figs. 2, 3), or in the presence of DAG and tubulin (Fig. 3). Erythrocyte deformability thus appears to be controlled by membrane tubulin content and lipid composition, both of which regulate PMCA activity. We found that erythrocytes of spontaneously hypertensive rats displayed lower deformability and greater resistance to flow, resulting in increased blood pressure. Treatment of these rats with nocodazole (which reduces membrane tubulin content) resulted in blood pressure values similar to those of control (Wistar) rats [12]. Altered deformability of hypertensive erythrocytes through modification of PMCA activity by lipid addition or tubulin redistribution, as in the present study, presumably also has a direct effect on blood pressure.

PMCA activity is regulated by phosphorylation (by protein kinases A and C), interaction with acidic phospholipids, the dimerization (oligomerization) process, autoinhibition, interaction with a variety of partner proteins (including tubulin, actin, MAGUK, CLP36, NOS-1, and NHERF2) and, most importantly, interaction with calmodulin [9,31–34]. We showed recently that tubulin inhibits PMCA embedded in homogenate of rat brain cell plasma membranes [9]. This inhibitory effect was dependent on the lipid composition of the membrane in which the enzyme was embedded. In the presence of acidic or neutral lipids, tubulin activated, rather than inhibited, the enzyme [10]. The present study gave similar findings in human erythrocytes. High membrane tubulin content (as in hypertensive erythrocytes [11]) caused inhibition of PMCA activity (Fig. 1). DAG or PA treatment of such high-tubulin cells increased the PMCA activity (Fig. 2). In previous studies, the promoting effect of DAG and PA on PMCA activity was observed even in the absence of tubulin [10,35]. In contrast, the promoting effect of DAG and PA on enzyme activity in the present study was more pronounced in the presence of tubulin (Fig. 3). This finding suggests that regulation of PMCA activity in erythrocytes depends primarily on formation of tubulin/PMCA complex. Increased level of the complex resulted in lower enzyme activity. The enzyme activity could be increased again either by removing membrane tubulin or adding acidic or neutral lipids.

Our previous study demonstrated that AcTub acquires hydrophobic properties when it interacts with PMCA. When partitioned with Triton X-114 in artificial vesicles containing PMCA, hydrophilic AcTub was partitioned into the hydrophobic phase because of its association with PMCA [10]. In artificial vesicles containing PMCA and acidic or neutral lipids, the lipids caused an increased content of AcTub in the hydrophobic phase [10]. Similar results were obtained with human erythrocytes in the present study. DAG and PA both increased membrane AcTub content in normotensive and hypertensive erythrocytes (Fig. 4A). Acidic and neutral lipids evidently promoted association of AcTub with the erythrocyte membrane and thereby promoted PMCA activity. Conversely, recruitment of lipid-free AcTub to the membrane inhibited enzyme activity. The molecular mechanisms underlying these effects remain unclear. We have observed that tubulin binds to cytoplasmic domain (CD) 2 and 3 of PMCA (Carbajal A, Arce C, unpubl. data). Furthermore, the acidic phospholipids bind to two sites in the enzyme, one in the calmodulin-binding site (in the C-terminus), the other in CD2 [36]. One possibility is that acidic lipids prevent interaction of tubulin with the CD3 site, in which case tubulin is still capable of anchoring to PMCA (because of its binding to CD2) but its inhibitory effect is lost. PMCA would still be activated, primarily because CD3 is the domain for ATP hydrolysis [36]. In the absence of acidic lipids, tubulin could bind to the CD2 and CD3 sites and thereby inhibit the enzyme activity. Experiments to test this hypothesis are underway.

Numerous biochemical and hematological parameters are altered in hypertensive patients. Here we only take into account the criteria of high blood pressure and membrane tubulin and we found that tubulin/PMCA/DAG regulates erythrocytes deformability regardless of the abovementioned parameters. It is possible that differences in the above parameters can affect patients PMCA activity (and thus the deformability of erythrocytes) but the data presented here (Figs. 2 and 3) suggest that membrane tubulin and DAG affect the erythrocyte deformability. Subsequent studies in groups with differences in biochemical and hematological parameters will elucidate other pathways of affecting the deformability of erythrocytes, affecting either PMCA activity or not.

The change in erythrocyte deformability has been attributed to changes in the fluidity of the cell membrane, flexibility of the membrane skeleton, the viscosity of the cytoplasm, and the cell membrane surface area to cell volume ratio [37]. Furthermore, aggregation structures called “rouleaux” in human erythrocytes containing high concentration of intracellular calcium were observed [27]. Kaczmarska et al. [37] determined that erythrocytes from patients with essential hypertension increase the fraction of oval cells and these cells also added more easily. We have not found these structures in samples of erythrocytes of hypertensive patients (Fig. 4), because the methodology we use does not allow us to visualize the findings of these authors. Anyway, the difference in hematocrit between patients was less than 3% (data not shown) which does not seem significantly different in deformability. This allows us to speculate that neither aggregation nor increased erythrocytes would cause the decrease of its deformability. We hypothesized that the increased membrane tubulin with inhibiting PMCA, leading to an increase in intracellular calcium, might affect the rheological properties of the membrane (for example, membrane fluidity and elasticity) which leads to a change in erythrocyte deformability. Experiments are in progress to elucidate this aspect.

The combined effects of lipids and tubulin play essential roles in cell physiology. Membrane lipid composition and PMCA distribution vary depending on the cell type, the region of the cell, and the physiological state of the cell. Cellular signaling mechanisms may be modified by changes in lipid type and concentration and/or tubulin concentration, resulting in promotion or inhibition of PMCA activity. Modulation of PMCA activity depends not only on lipid type and concentration but also on changes in membrane structure and consequent alteration

Fig. 4. Effects of DAG and PA on tubulin distribution in erythrocytes. (A) Left: distributions of α-tubulin (Total Tub.) and AcTub in normotensive and hypertensive erythrocytes were determined by indirect immunofluorescence using anti-α-tubulin mAb DM1A and anti-AcTub mAb 6-11-B1 as described in M&M. Right: distribution of fluorescence within erythrocytes (three individual cells for each case). Fluorescence intensity is expressed as arbitrary units (A.U). E.D: erythrocyte diameter. (B) Membrane fractions were obtained from 2 ml blood as described in M&M. AcTub amount was determined for an aliquot from each treatment by immunoblotting with mAb 6-11-B1. Volumes loaded on each well were calculated to represent equivalent amounts of erythrocytes. The result from a typical experiment is shown. Tubulin bands were quantified using the Scion Image program and values are expressed as arbitrary units (mean ± SD from three independent experiments).

of rheological erythrocyte deformability as observed in the present study. For example, such rheological changes could be triggered by activation of phospholipase C or D which catalyze DAG production, and subsequent promotion or inhibition of PMCA activity as a function of tubulin concentration [10]. Studies are underway in our laboratory to identify signaling pathways that induce changes in lipid composition or tubulin concentration, resulting in alteration of localized PMCA activity and erythrocyte deformability.

Conflicts of interest

The study was supported by grants from Agencia Nacional de Promocion Cientıfica y Tecnologica de la Secretarıa de Ciencia y Tecnologıa del Ministerio de Cultura y Educacion en el marco del Programa de Modernizacion Tecnologica (PICT 00–00000–01338/08 to Dr Cesar Casale and PICT 1439/; 0826/12) to Dr Carlos A. Arce, Consejo Nacional de Investigaciones Cientıficas y Tecnicas (Conicet), Secretarıa de Ciencia y Tecnica de la Universidad Nacional de Rıo Cuarto y de la Universidad Nacional de Cordoba y Ministerio de Ciencia y Tecnica de la Provincia de Cordoba.

Disclosures

None declared.

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