A New Steroidal Saponin from *Agave brittoniana* and Its Biphasic Effect on the Na^+-ATPase Activity

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A new steroidal saponin, 3-{(*O*-6-deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-[*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy}-6-hydroxy-(3β,5α,6α,25*R*)-spirostan-12-one, was isolated from *Agave brittoniana* Trel. The structure was determined by extensive NMR spectroscopy studies and chemical conversions. Its effects on the Na^+-ATPase and (Na^++K^+)-ATPase activities of the proximal tubule from pig kidney were evaluated. It was observed that this steroidal saponin exerts a biphasic effect on the Na^+-ATPase activity. It is concluded that the effect of the aqueous extract as a diuretic is due, at least in part, to the action of saponin on the ouabain-insensitive Na^+-ATPase.

Key words: *Agave brittoniana,* Steroidal Saponin, Na^+-ATPase

Introduction

Alternative medicines such as herbal products are increasingly being used for preventive and therapeutic purposes. *Agave brittoniana* Trel. (Agavaceae), commonly called "agave dragão" in Brazil, is a plant native from tropical regions and used with ornamental purpose (Cruz, 1965; Manfred, 1947). In the traditional medicine, the aerial parts of this plant are taken as diuretic. Nonetheless, no chemical studies were carried out on the constituents of this species. Several *Agave* species contain varying amounts of steroidal saponins, from which some natural precursors, used in synthesis of steroidal hormones, particularly corticosteroids, are obtained (Agrawal *et al*., 1985; Ding *et al*., 1989). Saponins are widespread natural substances possessing amphipathic features, containing a hydrophobic steroidal nucleus and a hydrophilic carbohydrate moiety (Lacaille-Dubois and Wagner, 1996), and are known to have several biological and pharmacological activities, depending on their chemical structures, such as antifungal, antibacterial, anti-inflammatory, hypocholesterolemic and anticarcinogenic properties, haemolytic effects, alteration of membrane permeability and, in particular, modulation of renal sodium excretion (Francis *et al*., 2002; Haruna *et al*., 1995; Mimaki *et al*., 1999).

Most of the sodium filtered in the glomerulus is reabsorbed in the proximal tubule, thus small changes in this process will have major consequences for the overall body sodium metabolism. The (Na^++K^+)-ATPase plays a crucial role in the sodium reabsorption producing an electrochemical gradient for the transepithelial transport of this cation (Féraille and Doucet, 2001). However, it is not an unique primary active transporter in the kidney. Whittembury and Proverbio (1970) described a second sodium pump, potassium independent, ouabain-insensitive and furosemide-sensitive, in rat kidney (Proverbio *et al*., 1989). This enzyme was further identified in other animals and tissues (Moretti *et al*., 1991), and may play an important role in fine tone sodium excretion (Caruso-Neves *et al*., 1999; Lara *et al*., 2002; Rangel *et al*., 1999). The amount of sodium excreted may be altered by several factors in agreement with the physiological condition of the individual. The increase of renal sodium excretion is, in general, accompanied with augmentation in water elimination in the urine. Thus, substances that modulate

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the renal sodium transport will have a direct effect on the water content in the urine. Notwithstanding the use of "agave dragão" in popular medicine, no studies correlating the pharmacological properties of this species with the chemical constituents of the aqueous extract were reported. In the present study, we isolated a new steroidal saponin, 3-{(*O*-6-deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-[*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy}-6-hydroxy-(3β,5α,6α,25*R*)-spirostan-12-one (**1**) (Fig. 1), present in aerial parts of *A. brittoniana*. Its effects on the Na^+-ATPase and (Na^++K^+)-ATPase activities of the proximal tubule from pig kidney were evaluated.

Materials and Methods

General procedures

Melting points were determined by an Electrothermal 9200 micro-melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin Elmer 243B polarimeter. IR spectra were measured on a Perkin Elmer 599B spectrometer. The MALDI-TOFMS was conducted using a Perseptive Voyager RP mass spectrometer. GC-MS analyses were performed with a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer using an ionization voltage of 70 eV and an ionization current of 60 μA for EI. GC was carried out with FID using a DB-1 glass capillary column (0.25 × 25 m, 0.25 micron; J. & W. Scientific Incorporated, Folsom, CA, USA). NMR spectra were measured in pyridine-d_5 (100 mg of **1** in 0.5 ml) at 25 °C with a Varian Gemini 200 NMR spectrometer, with tetramethylsilane ($\delta = 0.00$) used as internal standard. ^{1}H NMR spectra were recorded at 200 MHz and ^{13}C NMR spectra at 50 MHz. Silica gel columns (230–400 mesh ASTM; Merck) and Sephadex LH-20 (Pharmacia) were used for CC. TLC was performed on silica gel plates (Kieselgel 60F_{254}; Merck) using the following solvent systems: (A) $CHCl_3$/MeOH/H_2O (65:35:10 v/v/v, lower phase) for steroidal saponin **1**, (B) $CHCl_3$/MeOH (95:5) for sapogenin and (C) *n*-BuOH/C_5H_5N/H_2O (60:40:30) for monosaccharides. Spray reagents were orcinol/H_2SO_4 for steroidal saponin **1** and monosaccharides, and $CeSO_4$ for sapogenin.

Plant material

Fresh leaves of *Agave brittoniana* were obtained from the ornamental plant garden of the Federal University of Rio de Janeiro, Rio de Janeiro, in January 2000 and a voucher specimen is maintained in the Laboratory of Chemistry of Medicinal Plants at this University.

Extraction and isolation

The fresh leaves of the plant (3 kg) were extracted with MeOH (6 l) followed by concentration. The residue (37 g) was dissolved in water (600 ml) and extraction with an equal volume of *n*-BuOH gave a crude material (12.5 g). It was roughly chromatographed on Sephadex LH-20 with MeOH to give the crude steroidal saponin (2.5 g). Further purification by chromatography on a silica gel column eluted with $CHCl_3$/MeOH/H_2O (70:30:10 v/v/v) afforded one TLC-homogeneous compound **1** (635 mg, R_f 0.43) which gave a dark green color with orcinol/H_2SO_4.

Compound **1**

Colorless needles; m.p. 280–285 °C (dec.). – $[\alpha]_D^{25}$ 40° (*c* 1.0, MeOH). – IR: ν_{max} (KBr) = 3422, 2929, 2874, 1707, 1647, 1456, 1429, 1377, 1317, 1261, 1243, 1229, 1159, 1073, 1038, 982, 919, 898, 867 cm^{-1} [(25*R*)-spirostanol, intensity 919 < 898]. – MALDI-TOFMS: *m/z* = 1425.6027 $[M+Na]^+$ (high resolution). – ^{1}H and ^{13}C NMR: see Tables I and II.

Acid hydrolysis of **1**

Compound **1** (200 mg) was hydrolyzed with 2 M HCl/1,4-dioxane (1:1; 10 ml) in a sealed tube for 3 h at 100 °C. After cooling, the reaction mixture was neutralized with 3% KOH/MeOH and evaporated to dryness. The salts that deposited on addition of MeOH were filtered off and the filtrate was passed through a Sephadex LH-20 column with MeOH to give the hydrolysate (192 mg) which was chromatographed by silica gel CC with $CHCl_3$/MeOH/H_2O (7:3:0.2 v/v/v) to yield the sapogenin (43 mg) and a sugar mixture. The sapogenin was identified as 3,6-dihydroxy-(3β,5α,6α,25*R*)-spirostan-12-one by comparison of $[\alpha]_D$, IR, ^{1}H and ^{13}C NMR and EI-MS data with the literature (Yokosuka *et al.*, 2000). The sugar mixture was dissolved in pyridine and analyzed by silica gel TLC in the above described solvent system. After spraying, rhamnose gave a green spot at R_f 0.75,

glucose gave a blue spot at R_f 0.70 and galactose gave a purple spot at R_f 0.66.

Molar carbohydrate composition and D,L configurations

The molar carbohydrate composition of compound **1** was determined by GC-MS analysis of its monosaccharides as their trimethylsilylated methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation (Kamerling *et al.*, 1975). The configurations of the glycosides were established by capillary GC of their trimethylsilylated (–)-2-butylglycosides (Gerwig *et al.*, 1978).

Methylation analysis

Compound **1** was methylated with dimethyl sulfoxide-lithium methylsulfinyl carbanion-methyl iodide (Parente *et al.*, 1985). The methyl ethers were obtained after hydrolysis (4 N trifluoroacetic acid, 2 h, 100 °C) and analyzed as partially alditol acetates by GC-MS (Sawardeker *et al.*, 1965).

Preparation of isolated basolateral membranes (BLM) from proximal tubule

The proximal tubule basolateral membranes (BLM) were prepared from adult pig kidneys as previously described (Grassl and Aronson, 1986). The kidneys were removed immediately after the animal's death and maintained in a cold solution containing (mM): sucrose (250), 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)/[tris(hydroxymethyl)-aminomethane] hydrochloride (TRIS-HCl), pH 7.6 (10), ethylenediamine tetraacetic acid (EDTA) (2), and phenylmethylsulfonyl fluoride (PMSF) (1). The microsomal fraction was obtained by differential centrifugation. The fraction containing the BLM was isolated by the Percoll gradient method. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 2–3 mg of protein ml^{-1} and stored at –4 °C.

Measurement of ATPase activity

The ATPase activity was measured according to the method described by Grubmeyer and Penefsky (1981). Except as noted under "Results and Discussion", the composition of the standard assay medium (0.1 ml) was: 10 mM $MgCl_2$, 5 mM [γ^{32}P]ATP, 20 mM HEPES/TRIS-HCl (pH = 7.0), and 120 mM NaCl for the measurement of the (Na^++K^+)-ATPase activity. The Na^+-ATPase activity was calculated from the difference between the [^{32}P]Pi released in the absence and in the presence of 2 mM furosemide. Furosemide is a loop diuretic sulfonamide-derivate, that has been shown to inhibit Na^+-ATPase, without any modification on the activity of the (Na^++K^+)-ATPase activity. In addition, the assay is made in the presence of 2 mM ouabain. The (Na^++K^+)-ATPase activity was calculated from the difference between the [^{32}P]Pi released in the absence and in the presence of 1 mM ouabain (Caruso-Neves *et al.*, 2002; Proverbio *et al.*, 1989). Protein concentrations were determined by the Folin phenol method using bovine serum albumin as a standard (Lowry *et al.*, 1951). The significance of the differences was verified by the Bonferroni t-test.

Results and Discussion

The fresh leaves of *A. brittoniana* were extracted with methanol. After concentration under reduced pressure, the methanol extract was partitioned between water and *n*-butanol. Chromatographic separations of the organic phase on Sephadex LH-20 and silica gel gave compound **1** which was detected with a orcinol/H_2SO_4 reagent. Compound **1** was obtained as colorless needles and gave a positive Liebermann-Burchard test for a steroidal saponin. It revealed a quasi-molecular ion peak at *m/z* 1425.6027 [M+Na^+] in the MALDI-TOFMS. In the ^{13}C NMR spectrum (Table II), 63 carbon signals observed are 5 methyl, 14 methylene (6 of which were oxygenated), 40 methine (33 of which were oxygenated) and 3 quaternary carbons (one of which was oxygenated). On the basis of the above mentioned MS and NMR spectral data, compound **1** was supposed to be a saponin with the molecular formula $C_{63}H_{102}O_{34}$, bearing a chain of six sugar moieties.

In addition to this, the spirostanol glycosidic nature of compound **1** was indicated by the strong absorption bands at 3422 and 1073 cm^{-1} and a 25*R*-spirostan steroidal structure (867, 898 and 919 cm^{-1}, intensity 919 < 898) in the IR spectrum (Wall *et al.*, 1952), confirmed by ^{1}H and ^{13}C NMR spectra (Tables I and II) (Itabashi *et al.*, 2000; Kuroda *et al.*, 2001; Ohtsuki *et al.*, 2004; Yokosuka *et al.*, 2000). The ^{1}H NMR spectral data (Table I) contained signals for two hydrogen atoms at δ 3.85 (m) and 3.53 (br d, J = 8.8 Hz), two secondary

Table I. Selected ^1H NMR assignments [δ in ppm (J in Hz)] of compound **1** in C_5D_5N.

Aglycone characteristic proton signals			Sugar anomeric proton signals		
Position	Compound **1**	^1H-^1H-COSY	Position	Compound **1**	^1H-^1H-COSY
H-3	3.85 (m)	H-2, H-4	Gal–H-1	4.84 (d, 7.7)	Gal–H-2
H-6	3.53 (d, 8.8)	H-5, H-7	Glc–H-1	5.08 (d, 7.8)	Glc–H-2
Me-18	1.04 (s)		Glc–H-1′	5.50 (d, 7.5)	Glc–H-2′
Me-19	0.74 (s)		Glc–H-1″	5.05 (d, 7.5)	Glc–H-2″
Me-21	1.33 (d, 6.9)	H-20	Glc–H-1‴	5.14 (d, 8.0)	Glc–H-2‴
Me-27	0.71 (d, 5.8)	H-25	Rha–H-1	5.75 (s)	Rha–H-2

Table II. ^{13}C NMR data of the aglycone and carbohydrate moieties of compound **1** in C_5D_5N[a].

C	**1**	C	**1**
1	35.84	Glc 1	104.40
2	29.52	Glc 2	80.31
3	77.70	Glc 3	87.45
4	34.21	Glc 4	70.13
5	53.84	Glc 5	76.88
6	71.29	Glc 6	62.43
7	41.30	Glc 1′	103.59
8	32.92	Glc 2′	74.23
9	54.94	Glc 3′	87.45
10	35.84	Glc 4′	68.94
11	37.58	Glc 5′	77.47
12	212.53	Glc 6′	61.65
13	54.94	Glc 1″	104.92
14	55.52	Glc 2″	75.04
15	31.01	Glc3″	77.47
16	79.27	Glc 4″	71.09
17	55.14	Glc 5″	77.89
18	16.16	Glc 6″	62.03
19	13.10	Glc 1‴	104.12
20	42.20	Glc 2‴	75.04
21	13.92	Glc 3‴	76.01
22	108.91	Glc 4‴	77.47
23	31.37	Glc 5‴	76.67
24	28.80	Glc6‴	60.74
25	30.10	Rha 1	103.42
26	66.54	Rha 2	71.95
27	16.90	Rha 3	72.14
Gal 1	102.09	Rha 4	73.38
Gal 2	72.60	Rha 5	69.90
Gal 3	75.04	Rha 6	18.00
Gal 4	79.60		
Gal 5	74.82		
Gal 6	60.28		

[a] The assignments were made on the basis of DEPT, HETCOR and COLOC experiments.

methyl hydrogen atoms at δ 1.33 (d, J = 6.9 Hz) and 0.71 (d, J = 5.8 Hz) and two angular methyl hydrogen atoms at δ 1.04 and 0.74 (each s). The above ^1H NMR spectral data and a comparison of the ^{13}C NMR signals of the aglycone moiety of **1** (Table II) with those described in the literature (Yokosuka *et al*., 2000) suggested the structure of the aglycone to be 3,6-dihydroxy-(3β,5α,6α,25*R*)-spirostan-12-one. In addition to this, the ^1H NMR spectrum of **1** showed six anomeric hydrogen atoms at δ 4.84 (d, J = 7.7 Hz), 5.05 (d, J = 7.5 Hz), 5.08 (d, J = 7.8 Hz), 5.14 (d, J = 8.0 Hz), 5.50 (d, J = 7.5 Hz) and 5.75 (s) corresponding to Gal–H-1, Glc–H-1″, Glc–H-1, Glc–H-1‴, Glc–H-1′ and Rha–H-1, respectively.

In the ^{13}C NMR spectrum of compound **1**, a 1,4-linked inner β-D-galactopyranosyl unit, a 1,2,3-linked inner β-D-glucopyranosyl unit, a 1,3-linked inner β-D-glucopyranosyl unit, a 1,4-linked inner β-D-glucopyranosyl unit, a terminal β-D-glucopyranosyl unit and a terminal α-L-rhamnopyranosyl unit were observed. As shown in Tables I and II, ^1H and ^{13}C NMR chemical shift assignments were made by standard 1D and 2D NMR techniques. Its COLOC spectrum displayed long range couplings between galactose–H-1 at δ 4.84 and aglycone–C-3 at δ 77.70, between glucose–H-1″ at δ 5.05 and glucose–C-3′ at δ 87.45, between glucose–H-1 at δ 5.08 and galactose–C-4 at δ 79.60, between glucose–H-1‴ at δ 5.14 and glucose–C-3 at δ 87.45, between glucose–H-1′ at δ 5.50 and glucose–C-2 at δ 80.31 and between rhamnose–H-1 at δ 5.75 and glucose–C-4‴ at δ 77.47. In addition to this, the methylation analysis of compound **1** furnished 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl glucitol. These results indicated that compound **1** is undoubtedly as shown in Fig. 1.

On acid hydrolysis, compound **1** gave a sapogenin, galactose, glucose and rhamnose. The sapogenin was identified as 3,6-dihydroxy-(3β,5α,6α,25*R*)-spirostan-12-one by direct comparison of $[\alpha]_D$, IR, ^1H and ^{13}C NMR and EI-MS data with the litera-

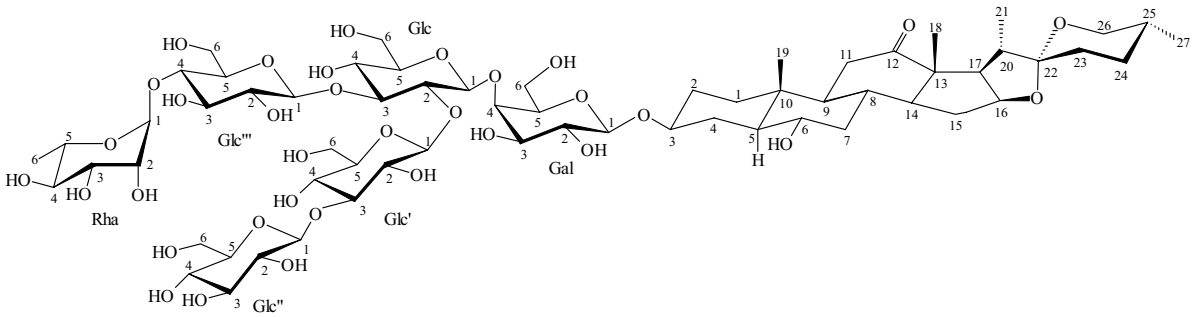

Fig. 1. Chemical structure of compound **1**.

ture (Yokosuka *et al.*, 2000). The molar carbohydrate composition of compound **1** indicated the presence of six neutral monosaccharides: galactose/glucose/rhamnose (1:4:1). Their absolute configurations were determined by GC of their trimethylsilylated (–)-2-butylglycosides (Kamerling *et al.*, 1975; Gerwig *et al.*, 1978). D-Galactose, D-glucose and L-rhamnose were identified. Consequently, on the basis of IR, ^1H and ^{13}C NMR spectroscopy, MALDI-TOFMS and chemical reactions, the structure of compound **1** was established as 3-{(*O*-6-deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→3)-*O*-[*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy}-6-hydroxy-(3β,5α,6α,25*R*)-spirostan-12-one.

In a previous paper (De Souza *et al.*, 2004), we described the evaluation of two steroidal saponins isolated from the rhizomes of *Costus spicatus* on the Na^+-ATPase and (Na^++K^+)-ATPase activities of the proximal tubule from pig kidneys. It was observed that both saponins, the furostanol saponin and the spirostanol saponin, inhibited specifically the Na^+-ATPase activity. This result prompted us to evaluate the effect of saponin **1** (Fig. 1 and 2) isolated from *Agave brittoniana* on the Na^+-ATPase and (Na^++K^+)-ATPase activities of MBL. The effect of saponin **1** from *A. brittoniana* was tested in the presence of saturating or subsaturating concentrations of Na^+ and K^+. On both conditions, the (Na^++K^+)-ATPase activity does not change by the increase in concentrations of saponin **1**. This result is in accordance with that reported by De Souza *et al.* (2004).

On the other hand, saponin **1** from *A. brittoniana* has a biphasic effect on the ouabain-insensitive Na^+-ATPase activity of BLM (Fig. 3). The enzyme activity was stimulated by saponin **1** at low concentrations, with a maximal effect at 10^{-11} M

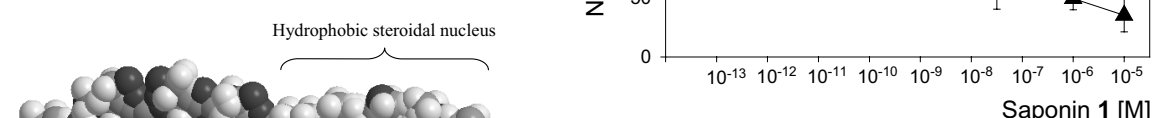

Fig. 2. Threedimensional representation of compound **1** (black label, oxygen atoms; dark gray label, carbon atoms; light gray label, hydrogen atoms).

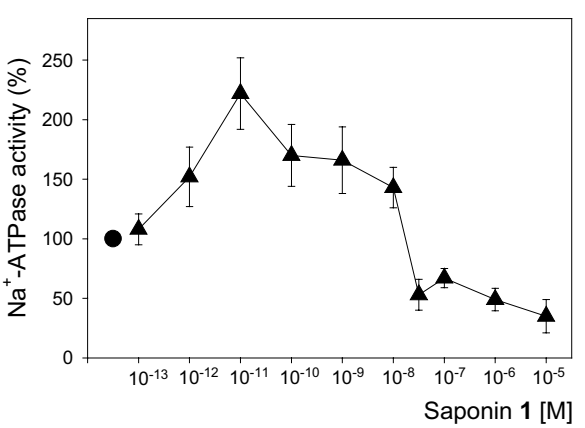

Fig. 3. Saponin **1** dependence on the Na^+-ATPase activity. The Na^+-ATPase activity was measured as the difference between ATPase activity in the presence and in the absence of furosemide, 2 mM, both in the presence of 2 mM ouabain. The absolute value of the Na^+-ATPase activity is 32.0 ± 2.4 nmol Pi mg^{-1} min^{-1}. For each saponin concentration used it was performed a parallel assay of measuring the Na^+-ATPase activity in the absence of saponin. The control is represented by (●). All experiments were done in duplicate.

and inhibited at high concentrations with a maximal effect observed at 10^{-7} M. This dual response may reflect different interactions between the saponin and BLM. It has been shown that the saponin could be correlated to a change in the membrane fluidity or interactions between phospholipids and protein (Haruna *et al*., 1995) which can modify the enzyme activity. However, we cannot exclude a possible involvement of a membrane receptor in mediation of this effect, since the stimulatory phase occurs at very low concentrations of saponin **1**.

In order to analyze the effect of saponin **1** from *A. brittoniana* on the kinetic parameters of the Na^+-ATPase, we measured the enzyme activity in different Na^+ concentrations (Fig. 4). The kinetic parameters were calculated using the Michaelis-Menten equation. The values of the kinetic parameters are means of those obtained by fitting the data for each experiment. The Na^+ concentration that promotes half maximal stimulation ($K_{0.5}$) was 5.8 ± 0.5 mM and the maximal rate (v_{max}) was 28.6 ± 1.4 nmol Pi mg^{-1} min^{-1}. At the stimulatory phase, in the presence of saponin **1**, $K_{0.5}$ for Na^+ increases to 9.2 ± 1.6 mM and v_{max} to 62.8 ± 4.12 nmol Pi mg^{-1} min^{-1}. In contrast, at the inhibitory phase, saponin **1** decreases $K_{0.5}$ to 2.76 ± 0.6 mM and v_{max} to 17.6 ± 1.32 nmol Pi mg^{-1} min^{-1}. The different effects observed in stimulatory and inhibitory phases could reflect the modifications in chemical structures that permit different forms of interaction between phospholipids and protein or a change in the membrane fluidity.

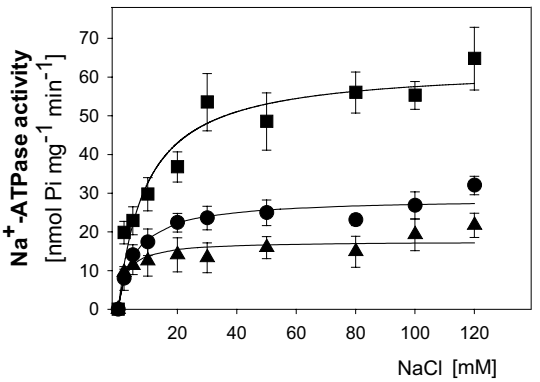

Fig. 4. Na^+ concentration dependence of the Na^+-ATPase activity in proximal tubule BLM in the presence of 2 mM ouabain. (●) Control; (■) 10^{-11} M saponin **1**; (▲) 10^{-7} M saponin **1**. The kinetic parameters were calculated by the following equation: $v = v_{max} \times [S]/K_{0.5} + [S]$. The data (mean ± SE) correspond to the difference between parallel assays performed in the absence or in the presence of each Na^+ concentration. All experiments were done in duplicate ($n = 7$).

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