

Modulation of Sodium Pumps by Steroidal Saponins

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Costus spicatus, used in Brazilian traditional medicine to expel kidney stones, contains steroidal saponins with different chemical characteristics. In spite of its popular utilization as potent diuretic, no scientific reports correlate this activity with the chemical constituents of the extract. Therefore, two steroidal saponins ($3\beta,22\alpha,25R$)-26-(β -D-glucopyranosyloxy)-2-methoxyfurost-5-en-3-yl *O*-D-apio- β -D-furanosyl-(1 \rightarrow 2)-*O*-[6-deoxy- α -L-mannopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside (**1**) and ($3\beta,22\alpha,25R$)-spirostan-3-yl *O*-D-apio- β -D-furanosyl-(1 \rightarrow 2)-*O*-[6-deoxy- α -L-mannopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside (**1a**), were isolated from the rhizomes of this plant and their effects on the Na⁺-ATPase and (Na⁺+K⁺)-ATPase activities of the proximal tubule from pig kidney were evaluated. It was observed that **1** and **1a** inhibit specifically the Na⁺-ATPase activity.

Key words: *Costus spicatus*, Steroidal Saponins, Na⁺-ATPase, (Na⁺+K⁺)-ATPase

Introduction

Alternative medicines such as herbal products are increasingly being used for preventive and therapeutic purposes. *Costus spicatus* Swartz (Costaceae), commonly called “cana-do-brejo” in Brazil, is a wide-spread plant found in wet coastal forests. In the traditional medicine, the rhizome of this plant is boiled and taken as diuretic. Its extracts are used in the treatment of complaints of the bladder and urethra and to expel kidney stones (Cruz, 1965; Manfred, 1947). Nonetheless, only recently chemical studies were carried out on the constituents of this species, in which steroidal saponins were isolated and characterized (da Silva *et al.*, 1999a, 1999b). The saponins are wide-spread natural substances possessing amphipathic features, containing a hydrophobic steroidal nucleus and a hydrophilic carbohydrate moiety (Lacaille-Dubois and Wagner, 1996). Saponins are known to have several physiological activities, depending on their chemical structures, such as haemolytic properties, alteration of membrane permeability and, in particular, the modulation of renal sodium excretion (Haruna *et al.*, 1995).

The renal sodium excretion depends on the balance between sodium filtration in glomerulus and its reabsorption along different nephron segments. Since the most filtered sodium is reabsorbed in the proximal tubule small changes in this process will include major consequences for the overall body sodium metabolism. Transcellular sodium reabsorption in the proximal tubule involves two primary activity transporters: the ouabain-sensitive (Na⁺+K⁺)-ATPase and the ouabain-insensitive, furosemide-sensitive Na⁺-ATPase (Caruso-Neves *et al.*, 2002; Proverbio *et al.*, 1989). These enzymes are located in basolateral membranes and they are involved in the genesis of the Na⁺ electrochemical gradient.

Regardless of the utilization of “cana-do-brejo” in popular medicine, no studies correlating the pharmacological properties of this species with the chemical constituents of the extract were reported. In the present study, we isolated two steroidal saponins present in the methanolic extract of rhizomes of *Costus spicatus* and their effects on the Na⁺-ATPase and (Na⁺+K⁺)-ATPase activities of proximal tubule basolateral membranes from pig kidney were evaluated.

Materials and Methods

Plant material

Rhizomes of *Costus spicatus* were collected at Ilha do Fundão, Rio de Janeiro in January 2000, and identified by Luci Senna Valle. A voucher specimen (no. R192950) is deposited at the herbarium of the National Museum, Rio de Janeiro, Brazil.

Extraction and isolation

The isolation and structural characterization of the steroidal saponins were made according to techniques described in the literature (da Silva *et al.*, 1999a). The powdered rhizomes of the plant (1 kg) were extracted with MeOH (5 l) followed by concentration and extraction with an equal volume of *n*-BuOH, which gave a crude material after concentration at reduced pressure (5 g). It was roughly chromatographed on Sephadex LH-20 with MeOH to give the crude steroidal glycoside (1.5 g). Further purification by chromatography on a silica gel column eluted with CHCl₃/MeOH/H₂O (70:30:10 v/v/v) afforded the TLC homogeneous compound **1** (350 mg) and **1a** (50 mg).

Preparation of isolated basolateral membranes 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 cold solution containing (mM): sucrose (250), HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid]/TRIS ([tris(hydroxymethyl)aminomethane] hydrochloride), pH 7.6 (10), EDTA (ethylenediaminetetraacetic acid) (2) and PMSF (phenylmethylsulfonyl fluoride) (1). The microsomal fraction was obtained by differential centrifugation. The fraction containing the basolateral membranes was isolated by the Percoll gradient method. The membrane preparation was resuspended in 250 mM sucrose at a final concentration of 20–30 mg of protein per ml and stored at –4 °C.

Measurement of ATPase activity

Except as noted under “Results and Discussion”, the composition of the standard assay medium (0.2 ml) was: 10 mM MgCl₂, 5 mM [γ -³²P]ATP, 20 mM HEPES/TRIS (pH 7.0), 5 mM sodium azide,

and 120 mM NaCl for the measurement of the Na⁺-ATPase activity or 120 mM NaCl and 30 mM KCl for the measurement of the (Na⁺+K⁺)-ATPase activity. The ATPase activity was measured according to the method described in the literature (Grubmeyer and Penefsky, 1981). The Na⁺-ATPase activity was calculated from the difference between the [³²P] Pi released in the absence and in the presence of 2 mM furosemide or in the presence and in the absence of Na⁺; all measurements were in the presence of 2 mM ouabain.

The (Na⁺+K⁺)-ATPase activity was calculated from the difference between the [³²P] 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

According to techniques described in the literature (da Silva *et al.*, 1999a), on the basis of spectroscopic methods and chemical reactions, the structures of the steroidal saponins were established as (3 β ,22 α ,25R)-26-(β -D-glucopyranosyloxy)-2-methoxyfurost-5-en-3-yl *O*-D-apio- β -D-furanosyl-(1 \rightarrow 2)-*O*-[6-deoxy- α -L-mannopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside (**1**) and (3 β ,22 α ,25R)-spirostan-3-yl *O*-D-apio- β -D-furanosyl-(1 \rightarrow 2)-*O*-[6-deoxy- α -L-mannopyranosyl-(1 \rightarrow 4)]- β -D-glucopyranoside (**1a**) (Fig. 1).

Since it has been shown that the effect of some compounds on the (Na⁺+K⁺)-ATPase activity depends on the concentration of Na⁺ and K⁺, the effect of both saponins in the presence of saturating or subsaturating concentrations of Na⁺ and K⁺ was tested. On both conditions, the (Na⁺+K⁺)-ATPase activity does not change by the increase in concentrations of saponins **1** and **1a** from 10⁻⁸ to 10⁻⁵ M. It has been shown that the effect of saponins on the isolated (Na⁺+K⁺)-ATPase of dog kidney depends on their structures (Kuroda *et al.*, 1995; Mimaki *et al.*, 1999).

Fig. 2 shows the effect of saponins **1** and **1a** on the ouabain-insensitive Na⁺-ATPase activity of BLM. The enzyme activity was inhibited by saponin **1** in a dose-dependent manner. The maximal effect was observed at 10⁻⁶ M showing the Na⁺-ATPase activity inhibited by 70%. The apparent

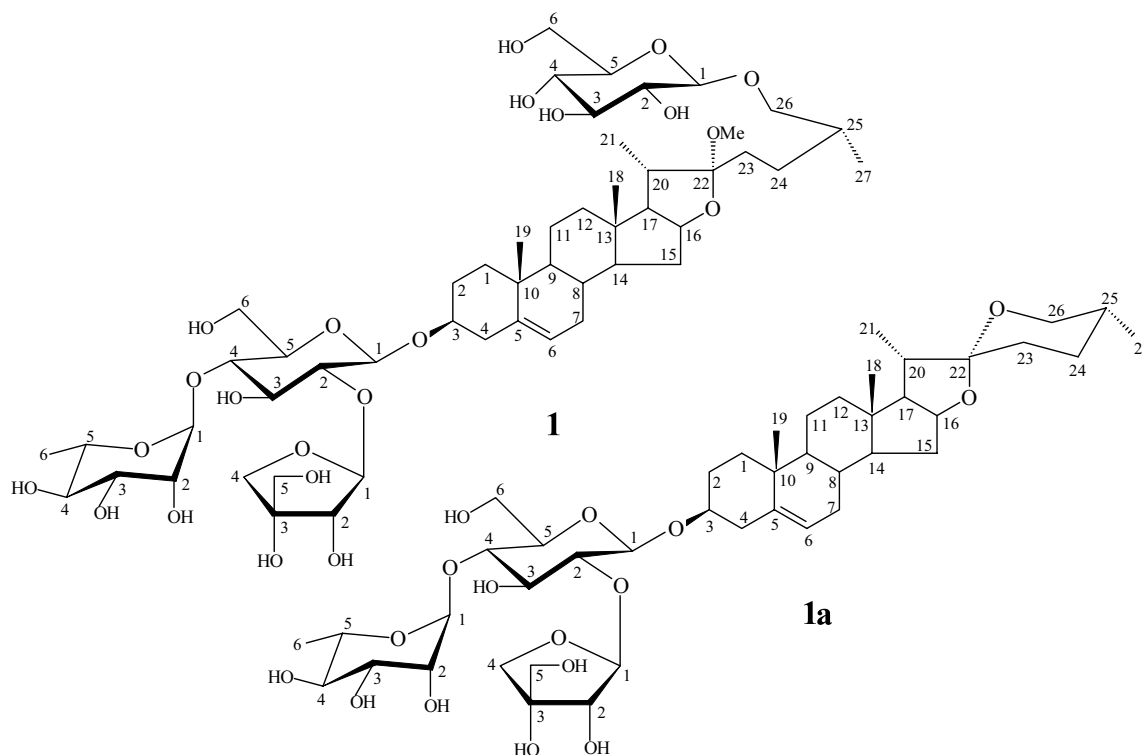


Fig. 1. Chemical structures of the steroidal saponins **1** and **1a**.

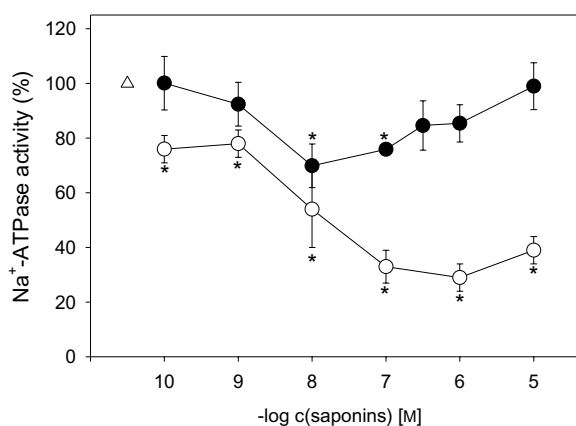


Fig. 2. Dependence of the saponins **1** (open circle) and **1a** (close circle) on the $\text{Na}^+\text{-ATPase}$ activity. The $\text{Na}^+\text{-ATPase}$ activity was measured as the difference between ATPase activity in the presence and in the absence of Na^+ , both in the presence of 2 mM ouabain. The absolute value of the $\text{Na}^+\text{-ATPase}$ activity is $32.5 \pm 1.6 \text{ nmol Pi mg}^{-1} \text{ min}^{-1}$. For each saponin concentration used it was performed a parallel assay of measuring the $\text{Na}^+\text{-ATPase}$ activity in the absence of saponin (control). All experiments were done in duplicate. *Statistically significant when compared to control ($n = 8$).

IC_{50} value, obtained by extrapolation of the half maximal inhibition, is about 10^{-8} M . On the other hand, saponin **1a**, which does not have a glucose unit at position 26, has a biphasic effect on the $\text{Na}^+\text{-ATPase}$ activity. Initially, the increase in the concentration from 10^{-10} to 10^{-8} M inhibited the enzyme activity by 25% showing the maximal effect obtained at 10^{-8} M . The apparent IC_{50} value, obtained by extrapolation of the half maximal inhibition, is about 10^{-9} M . Further increase in concentration of this compound promotes the return of the enzyme activity to the control value. It is important to note that saponin **1a** has a lower effective effect than saponin **1** in high concentrations. This difference could be explained by the presence of a glucose unit at position 26 in saponin **1**. This arrangement could impair the binding of the steroid unit on the enzyme structure. On the other hand, the inhibitory effect observed by both saponins could be correlated to changes in the membrane fluidity or interactions between phospholipids and protein. Haruna *et al.* (1995) showed that *Gleditsia* saponins interact with human erythrocyte membranes changing of membrane fluid-

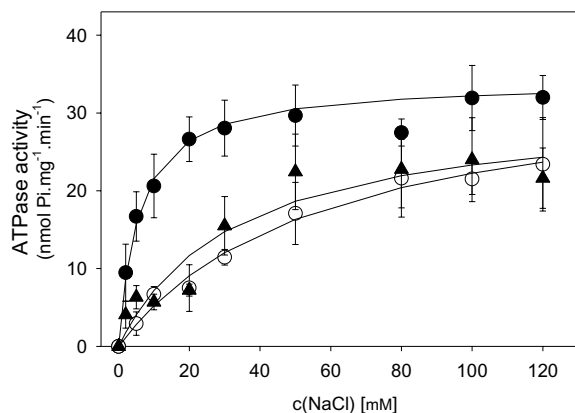


Fig. 3. Na^+ concentration dependence of the ATPase activity in proximal tubule basolateral membranes in the presence of 2 mM ouabain. Close circle, control; open circle, 10^{-8} M saponin **1**; close triangle, 10^{-8} M saponin **1a**. The kinetic parameters were calculated by following equation: $v = V_{\max} \times [S]/K_{0.5} + [S]$. The data (mean \pm 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 ($n = 4$).

ity leading to inhibition of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity.

In order to analyze the effect of these saponins on the kinetic parameters of the $\text{Na}^+\text{-ATPase}$, we measured the enzyme activity in different Na^+ concentrations (Fig. 3). 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 34.1 ± 1.6 mmol Pi \cdot mg⁻¹ \cdot min⁻¹. Both saponins decrease the $K_{0.5}$ to 56.7 ± 10.2 mM (10^{-8} M saponin **1**) and 33.2 ± 13.3 mM (10^{-8} M saponin **1a**), but do not change the v_{\max} . The observation that the effect of saponins is the same if the $\text{Na}^+\text{-ATPase}$ ac-

tivity was measured through furosemide-sensitive or Na^+ -stimulation ATPase activity indicates that the effect of saponins is specific to $\text{Na}^+\text{-ATPase}$.

In the past decade, a second $\text{Na}^+\text{-ATPase}$, which is insensitive to ouabain and sensitive to furosemide, was described in several animal tissues (Proverbio *et al.*, 1989; Moretti *et al.*, 1991). In spite of several papers published on this enzyme, its physiological role remains to be elucidated. Recently, our laboratory proposed that this enzyme involved in the regulation of Na^+ reabsorption in the proximal tubule is the primary active transport target for natriuretic and antinatriuretic peptides (Rangel *et al.*, 1999; Lara *et al.*, 2002). Furthermore, it has been observed that diuretic compounds such as furosemide and ethacrinic acid inhibit the $\text{Na}^+\text{-ATPase}$. We showed that furosemide inhibits the $\text{Na}^+\text{-ATPase}$ in the same preparation used in this paper with IC_{50} of 1.1 mM. In the present paper, we show that both saponins **1** and **1a**, isolated from the methanolic extract of rhizomes of *Costus spicatus*, are also able to inhibit the enzyme but with higher affinity than furosemide. Then, it is possible to postulate that the effect of *Costus spicatus*, used in Brazilian traditional medicine as a diuretic compound, is due to action of saponins, at least in part, on the ouabain-insensitive $\text{Na}^+\text{-ATPase}$ activity.

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