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Insights into the mechanism of Na^+,K^+-ATPase inhibition by 2-methoxy-3,8,9-trihydroxy coumestan

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ABSTRACT

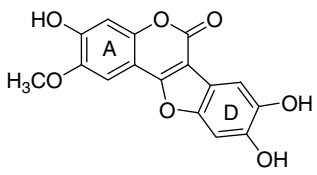
The molecular mechanisms involved in Na^+,K^+-ATPase inhibition by 2-methoxy-3,8,9-trihydroxy coumestan were investigated. We show that this compound decreases the free sulfydryl groups present in the enzyme and that its inhibitory effect is prevented by dithiothreitol and other two sulfydryl containing reagents. We propose a redox cycle culminating with the irreversible oxidation of sulfydryl groups essential for the catalytic activity of this enzyme and of two other related P-type ATPases.

1. Introduction

The Na^+,K^+-ATPase is a membrane protein that uses the energy derived from the hydrolysis of ATP to transport Na^+ and K^+ ions across the plasma membrane of higher eukaryotes. This active transport is essential for the maintenance of the resting potential of all cells, particularly important in neuronal and muscle functions. It also provides the driven force for the transport of amino acids, sugars, and neurotransmitters, the extrusion of Ca^{2+} and regulates many essential cellular functions such as cell volume, heat production, and intracellular pH regulation. Besides that, the Na^+,K^+-ATPase is the molecular receptor of cardiac glycosides which have been used for centuries in the treatment of congestive heart failure due to their positive inotropic effect. This cardiac effect involves the inhibition of Na^+,K^+-ATPase activity, resulting in increased intracellular sodium concentrations and decreased driven force for the Na^+/Ca^{2+} exchanger so that less Ca^{2+} is extruded from the cell. As a consequence, more Ca^{2+} is accumulated in the sarcoplasmic reticulum and is available for calcium-induced calcium-release, increasing the cardiac force of contraction.[1] However, despite their widespread use in the therapeutics, cardiac glycosides present a low therapeutic index and some important adverse effects that justify the need for drug monitoring and support the search for new inhibitors of Na^+,K^+-ATPase. This is particularly relevant due to the prevalence and incidence of heart failure, that are high in developed countries and increasing in developing countries.

Recent studies pointed to the search for non-steroidal or altered steroid-like inhibitors of Na^+,K^+-ATPase,[2–4] since failure to improve the therapeutic index of digoxin has been attributed to the preservation of the C/D-*cis* junction in the steroid backbone, unique to the cardiac glycosides.[5]

In 2001, we reported that wedelolactone, a naturally occurring coumestan isolated from *Eclipta prostrata*, as well as some derivatives synthesized in our laboratory, inhibited the Na^+,K^+-ATPase.[6] This was the first register of Na^+,K^+-ATPase inhibition by coumestans, a class of isoflavonoids. The mechanism of inhibition of this class of compounds was further studied using 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36), a synthetic isomer of wedelolac-



PCALC 36

Figure 1. Structure of 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36).

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tone (Fig. 1). We showed that this coumestan presented a mechanism of inhibition different from classical inhibitors of Na^+,K^+-ATPase such as ouabain and vanadate.[7]

As Na^+,K^+-ATPase has been reported to be inhibited by oxidation of its free sulfydryl groups,[8–11] we decided to study the influence of antioxidants on the inhibitory effect of PCALC36 in order to get insights into the molecular mechanism of action of our coumestan. We also investigated the selectivity of PCAL36 by studying its effect on $(Ca^{2+} + Mg^{2+})$-ATPase (SERCA) and H^+,K^+-ATPase, two other P-type ATPases.

2. Results

The inhibitory effect of ouabain, PCALC36, and dithiothreitol, a redox reagent known as Cleland's reagent (DTT), on Na^+,K^+-ATPase is shown in Figure 2. DTT by itself (1 and 5 mM) did not alter the Na^+,K^+-ATPase activity. However, it prevented the inhibition exerted by PCALC36 in a concentration-dependent manner, with 5 mM DTT blocking almost completely the inhibition. On the other hand, the inhibition promoted by 1 mM ouabain was not affected by DTT addition to the incubation medium.

The effect of DTT prompted us to test other antioxidants sharing the presence of sulfydryl groups in their structures (Table 1).

Cystein and β-mercaptoethanol also prevented the inhibition promoted by PCALC36. As for DTT, this protective effect was specific as these antioxidants did not affected the Na^+,K^+-ATPase activity or its inhibition by ouabain. In order to evaluate if sulfydryl reagents could not only prevent but also revert the inhibition elicited by PCALC36, the effect of β-mercaptoethanol was investigated after pre-incubation of the enzyme with this coumestan. As observed in Figure 3, β-mercaptoethanol did not revert the inhibition.

Once the oxidation of sulfydryl groups present in the catalytic site of the Na^+,K^+-ATPase leads to enzyme inhibition,[8–11] the protective effect of antioxidants containing a sulfydryl group indicates that PCALC36 could inhibit the enzyme through interaction with such groups. In order to check this hypothesis, the content of free sulfydryl groups was measured in the absence (control) and presence of PCALC36. As shown in Figure 4, pre-incubation with 30 μM PCALC36 lead to a significant decrease of free sulfydryl groups, as observed with mercury acetate, used as a positive control.

Since the presence of reduced sulfydryl groups in the catalytic site of other P-type ATPases also seems to be essential, we investigated the effect of PCALC36 on the activity of a rat $(Ca^{2+} + Mg^{2+})$-ATPase (SERCA type) and H^+,K^+-ATPase (Fig. 5). Both ATPases were inhibited by PCALC36, with an estimated IC_{50} of 10 and 30 μM for $(Ca^{2+} + Mg^{2+})$-ATPase and H^+,K^+-ATPase, respectively, that is, a value only slightly higher than the one previously reported for the Na^+,K^+-ATPase (3–11 μM).[7,12] These data reveal a somewhat unspecific inhibition of P-type ATPases, probably due to oxidation of sulfydryl groups in the relatively conserved catalytic site of these enzymes. Such mechanism has already been proposed for iron inhibition of rabbit heart SERCA.[13] Corroborating this idea, the inhibition of $(Ca^{2+} + Mg^{2+})$-ATPase by PCALC36 was also prevented in the presence of DTT (data not shown).

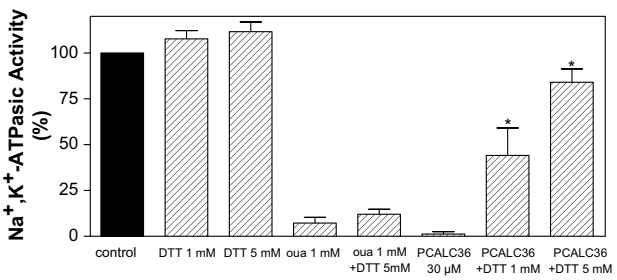


Figure 2. Influence of dithiothreitol (DTT) on Na^+,K^+-ATPase inhibition by PCALC36 and ouabain (oua). $^*p < 0.001$ compared to PCALC36, one-way ANOVA followed by Newman–Keuls multiple comparison analysis. Each bar represents mean ± SEM of three experiments performed in triplicate.

3. Discussion

It is well known that catechols can be oxidized by oxygen in a biological medium in the presence of transition state metals such as Fe^{3+}, Mn^{7+}, and Cu^{2+}, leading to the formation of superoxide and semiquinone radicals.[14] As an example, H_2O_2 production in cell culture has recently been demonstrated after the addition of cathecins and quercetin.[15] Since it is impossible to free laboratory solutions from contaminating metal ions,[14] the oxidation of PCALC36 leading to the corresponding semiquinone radical and superoxide anion could be the initial step of a redox cycle culminating with the oxidation of sulfydryl groups in the enzyme and its inactivation (Scheme 1). In the propagation step, the resulting superoxide anion can oxidize PCALC36 to the corresponding semiquinone, being reduced to H_2O_2. According to our interpretation, in the inhibition step the sulfydryl groups of Na^+, K^+-ATPase are oxidized by the semiquinone radical. Although H_2O_2 is poorly reactive, it is capable of inactivating directly some enzymes through oxidation of –SH groups essential for catalysis as in the case of Na^+, K^+-ATPase.[14] Note that the importance of a catechol in ring D of our coumestan (central to the reaction scheme proposed here) has been previously demonstrated[12] when we tested the inhibitory potency of ten coumestans bearing different patterns of substitution in the A- and D-rings.

We also considered the possibility of a nucleophilic addition of the sulfydryl groups to PCALC36, leading to a covalent adduct with the enzyme, as an alternative mechanism of action. In order to check this possibility, we studied the effect of 3,8-dibenzyloxi-9-methoxy coumestan, a protected coumestan with no free phenol group.

This compound did not inhibit the Na^+,K^+-ATPase and did not react with thiophenol in a buffered medium (Scheme 2), disclosing the hypothesis of enzyme inhibition through the formation of covalent adducts.

4. Conclusion

In the present study, we investigated the molecular mechanism of Na^+,K^+-ATPase inhibition by 2-methoxy-3,8,9-trihydroxy coumestan (PCALC36). Our results indicate that PCALC36 oxidizes sulfydryl groups of the enzyme which are essential for its catalytic activity. This mechanism probably explains why this compound also inhibits the other two P-type ATPases studied, in the same range of concentrations.

We had previously described that this compound exhibited a pattern of inhibition different from the classical inhibitor ouabain, since its inhibitory effect was not affected by the nature of the Na^+,K^+-ATPase isoform nor by the K^+ concentration.[7] These results were further supported when we compared the inhibition of rat kidney Na^+,K^+-ATPase produced by the combination of ouabain and 8-methoxycoumestrol *vs.* ouabain and ouabagenin.[16] We also showed that the effect of PCALC36 was irreversible.[7] This is now explained by present results and hypothesis of irreversible oxidation of sulfydryl groups of the enzyme. A direct covalent bond of PCALC36 with SH groups of the enzyme can be discarded according to the negative results showed in Scheme 2. As a final conclusion, we describe here details of the molecular mechanism of action of 2-methoxy-3,8,9-trihydroxy coumestan, representative of a newly synthesized family of coumestans that inhibit Na^+,K^+-ATPase and

Table 1
Effect of different inhibitors and antioxidants on Na^+,K^+-ATPase activity

Treatment (*n*)	Na^+,K^+-ATPase activity (% ±SEM)
Control	100.0
Ouabain 1 mM (10)	11.6 ± 1.8
PCALC36 30 μM (15)	6.9 ± 1.8
β-Mercaptoethanol 500 μM (3)	106.0 ± 1.4
Ouabain 1 mM + β-mercaptoethanol 500 μM (3)	10.5 ± 1.3
PCALC36 30 μM + β-mercaptoethanol 500 μM (3)	81.3 ± 2.7*
Cystein 1 mM (4)	91.8 ± 8.9
Ouabain 1 mM + cystein 1 mM (3)	12.0 ± 1.0
PCALC36 30 μM + cystein 1 mM (4)	75.5 ± 5.9*

* $p < 0.05$, one-way ANOVA followed by Newman–Keuls multiple comparisons analyses in comparison to PCALC36 (30 μM). Numbers into brackets represent the number of experiments performed.

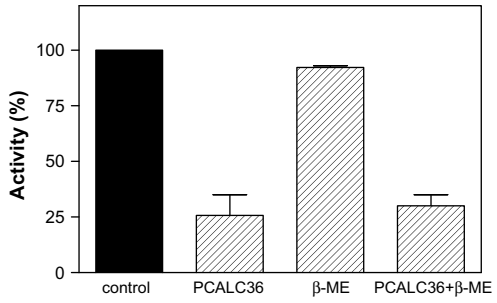


Figure 3. Effect of β-mercaptoethanol on Na^+,K^+-ATPase inhibition after pre-incubation with PCALC36. Na^+,K^+-ATPase was incubated with 30 μM PCALC36 for 30 min (see Section 5). After this time, β-mercaptoethanol was added to the incubation medium and the 2 h incubation completed. Each bar represents mean ± SEM of three experiments performed in triplicate.

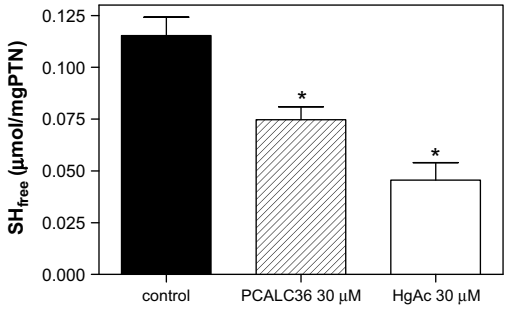


Figure 4. Concentration of free sulfydryl groups in the rat kidney preparation: effect of PCALC36 and mercury acetate (HgAc). Each bar represents mean ± SEM of three experiments performed in triplicate. * $p < 0.05$, one-way ANOVA followed by Dunnett's test.

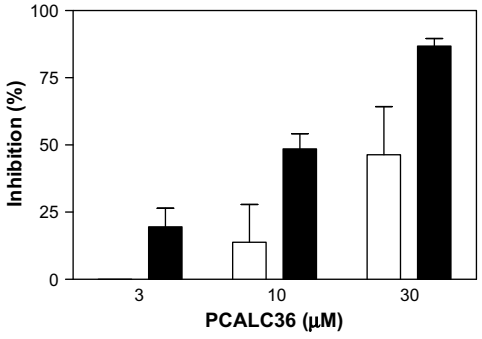


Figure 5. Inhibition of rat gastrocnemius (Ca^{2+} + Mg^{2+})-ATPase (SERCA1,2—filled bars) and stomach H^+,K^+-ATPase (empty bars) by PCALC36. Each bar represents mean ± SEM of at least three experiments performed in triplicate.

could serve as a tool for investigational studies, but probably not for clinical purposes due to its lack of specificity with relation to other P-type ATPases.

5. Experimental

5.1. Coumestan synthesis

The compound PCALC36 (Fig. 1) was prepared according to the methodology previously described.[7] The stock solution of PCALC36 (30 mM in DMSO) was protected from light and stored at −18 °C. Further dilutions were done in water immediately before use and the DMSO final concentration did not exceed 0.1%.

5.2. Tissue preparation

Adult male Wistar rats were killed by decapitation (in accordance to the Institutional Ethical Committee for animal care—Federal University of Rio de Janeiro, Brazil, Process No. DFBC-ICB 002-2007) and their kidneys and *gastrocnemius* muscles were rapidly excised and stored at −80 °C. The stomachs were immediately removed, opened, and washed for the scrap of mucous membrane. Preparation from the scrap was done at the same day.

5.3. Na^+,K^+-ATPase preparation from rat kidney

Preparations enriched in Na^+,K^+-ATPase were obtained by chaotropic treatment with 2 M KI for 1 h and 0.1% sodium deoxycholate over-night, followed by differential centrifugation, as earlier described.[17] The protein concentration was measured according to the method of Lowry using bovine serum albumin as the standard.[18]

5.4. (Ca^{2+} + Mg^{2+})-ATPase preparation from rat *gastrocnemius*

Gastrocnemius muscle preparation was prepared as described previously.[19] Briefly, the muscle was sliced in small pieces and homogenized in an ultraturrax (three times during 30 s at 13,500 rpm) and submitted to differential centrifugation to obtain the microsomal subcellular fraction which sediments at 110,000*g*.

5.5. H^+,K^+-ATPase preparation from rat stomach

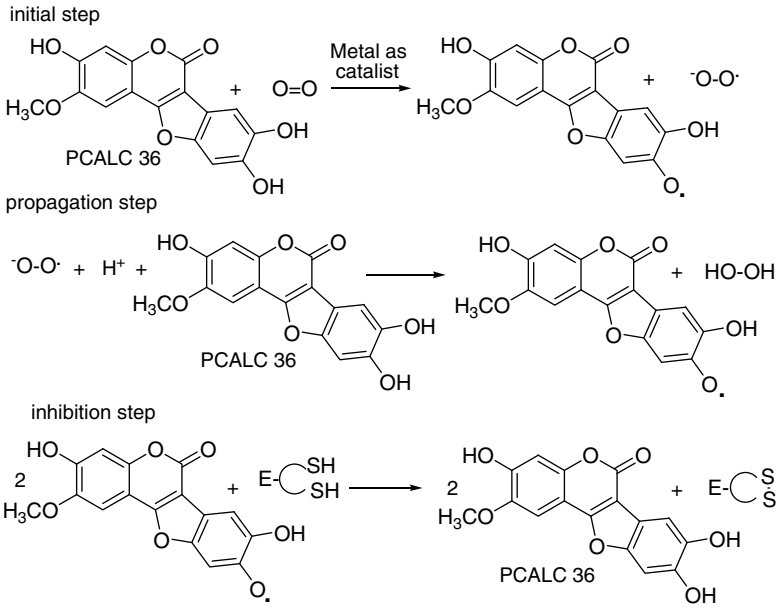
Mucous scrap was homogenized in a solution containing 250 mM sucrose, 5 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 2 mM HEPES–Tris, pH 7.4. The homogenate was submitted to centrifugations for protein recovery as described by Tomiyama et al.[20]

5.6. Inhibition of Na^+,K^+-ATPase activity

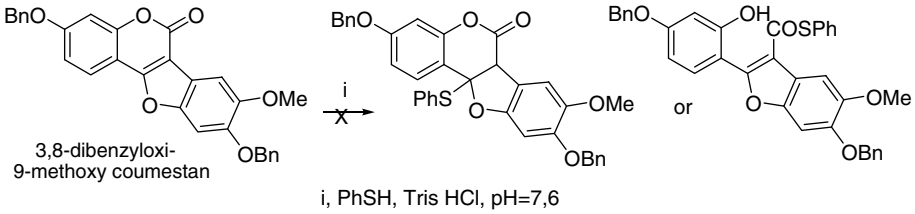
The ATPasic activity was determined by the Fiske and Subbarow method with slight modifications, as described earlier.[21] The specific Na^+,K^+-ATPase activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain. The preparation was incubated at 37 °C for 2 h, in a total volume of 0.5 mL. The incubation was performed in the presence of 84 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1.2 mM $ATPNa_2$, 2.5 mM EGTA, 10 mM sodium azide, and 20 mM maleic acid buffered to pH 7.4 with Tris in the absence or presence of PCALC36, ouabain or antioxidants (details in the figures).

5.7. Inhibition of (Ca^{2+} + Mg^{2+})-ATPase activity

Enzyme incubation was performed for 2 h at 37 °C, in a medium containing 5 mM $ATPNa_2$, 4 mM $MgCl_2$, 100 mM KCl, 0.3 mM EGTA,



Scheme 1. Possible redox reactions involving PCALC36 and Na^+,K^+ATPase.



Scheme 2. Reaction of PCALC36 with thiophenoxide.

10 mM NaN_3, 50 mM HEPES, pH 7.4, and 10 μM free Ca^{2+}. The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the absence of calcium.

5.8. Inhibition of H^+,K^+-ATPase activity

The activity was determined by the Fiske and Subbarow method, with enzyme incubation for 2 h at 37 °C, in a total volume of 0.5 mL of incubation medium containing 2 mM $ATPNa_2$, 2 mM $MgCl_2$, 10 mM KCl, 10 mM NH_4Cl, 5 μg/mL nigericin, 10 mM NaN_3, and 40 mM Tris–HCl buffered to pH 7.4. The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the absence of KCl and NH_4Cl.

5.9. Measurement of free sulfydryl groups

The rat kidney preparation was pre-treated with PCALC36, mercury acetate or water for 1 h at 37 °C in a Mg–ATP–Na medium (3 mM $MgCl_2$, 100 mM NaCl, 1 mM EGTA, 10 mM sodium azide, 3 mM $ATPNa_2$, 20 mM maleic acid, and Tris, pH 7.4). To recover the protein, the preparation was submitted to ultracentrifugation at 100,000g at 4 °C and the pellet was resuspended in sucrose 0.25 M, pH 7.4. The free sulfydryl content was determined by the Ellman's method, as described by Sedlak and Lindsay.[22] Briefly, the colorimetric method is based on the reaction of free sulfydryl groups with 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB, in a final volume of 1 mL medium containing 3 mM EDTA and 30 mM Tris–HCl, pH 8.2, after 30 min of incubation at room temperature. Cystein (5–100 μM) was used as standard.

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