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THE α-2 ISOMER OF THE SODIUM PUMP IS INHIBITED BY CALCIUM AT PHYSIOLOGICAL LEVELS

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The inhibition of the (Na,K)ATPase by calcium was investigated in plasma membrane preparations of rat axolemma, skeletal muscle and kidney outer medulla. Ouabain titration curves demonstrated that physiological calcium (0.08-5μM) inhibited mainly the high affinity α2 isomer. In axolemma all the (Na,K)ATPase had high ouabain affinity and calcium inhibited 40-50% of the activity with a K_i of $1.9\pm0.9\times10^{-7}$M. In skeletal muscle high and low ouabain affinity components were present in equal amounts and calcium inhibited only the high affinity component with a K_i of $1.3\pm0.3\times10^{-7}$M. Kidney enzyme had a low affinity for ouabain and showed very little sensitivity to calcium in the physiological range. It was demonstrated that high calcium levels inhibit the enzyme in a general sense, irrespective of the isomer, with a K_i of $6.5\pm6\times10^{-4}$M for the kidney and $5.9\pm4\times10^{-4}$M for the axolemma enzymes. In axolemma, enzyme activity was studied as a function of sodium concentration. Physiological calcium reduced Vmax while not significantly changing $K_{0.5}$ for sodium binding.

The (Na,K)ATPase) (EC 3.6.1.3) is a plasma membrane protein responsible for pumping Na^+ and K^+ against their concentration gradients. The functional enzyme unit consists of one catalytic α unit, of which there are 3 isomers (termed α1,2&3) and one glycosylated β unit, of which there are 2 isomers with no known function, save that they are required for pump activity (1,2,3). The three isomers of the catalytic unit in the rat have 85% sequence homology (4) but have different affinities for the specific inhibitor, ouabain. The α1 isomer has low affinity for ouabain with a $K_i=10^{-5}$-10^{-4}M and α2&3 have high affinity with $K_i=10^{-7}$-10^{-6}M (5,6,7,8,9). The α2 isomer is regulated by insulin which activates enzyme units possibly by the removal of an inhibitory factor (10). It can be demonstrated experimentally in whole cell preparations that only a fraction of the (Na,K)ATPase units are active in the resting state (11,12,13,14). Hormones such as catecholamines and insulin can be shown to activate these latent units(6,10,15,16,17), and it is of interest to determine what keeps some of the enzyme units inactive prior to the rise in the serum of an activating ligand. Investigators have searched for hypothetical endogenous (Na,K)ATPase inhibitors for some time and removal of calcium inhibition has been suggested as a mechanism to account for norepinephrine, vasopressin and

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angiotensin stimulation of the (Na,K)ATPase (17). Peptide inhibitors have been reported (18) but the effective concentration is orders of magnitude higher than for the calcium effects reported here. As calcium inhibition of the (Na,K)ATPase was recorded by Skou (19) and many other reports have followed (20), a detailed investigation was undertaken, posing calcium as a candidate for the inhibitory factor associated with one or more of the α isomers. In most of the early work the inhibiting levels of calcium were well above those encountered physiologically. There have been some reports of calcium inhibition at lower concentrations (20). However this is the first report of the calcium inhibition over the full physiological range of the ion, and it is the first record of its isomer specificity.

MATERIALS AND METHODS

Three tissues were chosen for the study of the calcium inhibition of the (Na.K)ATPase. Rat axolemma, to provide $\alpha 2$ and $\alpha 3$ (5,8), skeletal muscle for its mixture of $\alpha 1$ and $\alpha 2$ (8), and rat kidney outer medulla as a predominantly $\alpha 1$ tissue (10). Dose response curves to calcium and ouabain titrations were recorded in all three tissues. The effect of calcium on the sodium dependence of (Na,K)ATPase activity was studied in axolemma, which represented a high ouabain affinity tissue with calcium sensitivity.

Plasma membranes were prepared from rat axolemma (21), skeletal muscle (22), and kidney outer medulla (23), and protein content determined according to Peterson (24). A linear enzyme rate was followed over 7 minutes by the coupled assay of Barnett (25). (The assay was verified to be unaffected by calcium by running it with ADP±calcium). The calcium effect on the (Na,K)ATPase was also checked and verified in an independent assay for inorganic phosphate (26). The constant assay conditions were:-Ionic strength=140mM, Temperature=36^0C, pH=7.6(20mM Hepes/Triethylamine), Tris ATP=1mM, $MgCl_2$=2mM, KCl=25mM, EDTA=0.1mM, BAPTA or EGTA=1mM and Na=100mM unless otherwise stated. In experiments with varying sodium the ionic strength was made up using choline as a substitute cation. Free calcium was set using the equilibrium constants for BAPTA and EGTA according to the methods of Harrison and Bers (27) and Tsien (28). BAPTA was used as the chelator in the reported results, and in many additional experiments employing EGTA the same response of the enzyme to calcium was obtained. The free calcium concentration was dominated by the Ca-BAPTA, or Ca-EGTA equilibrium. When the chelating properties of EDTA and ATP for both calcium and magnesium were taken into account the perturbation on calcium concentrations was less than 10% for [Ca] less than 10^{-5}M. 3 different commercial sources of $CaCl_2$ were tested and the water was deionised and distilled. The water source was further checked using Spectroscopically pure water with no change in the calcium response.

Graphing and data analysis

Graphing and data analysis employed the software programme, "Igor" (29). In Figures 1-3 the data comprised calcium dose response curves and ouabain titration curves. One component curves were least squares fitted to the following function:-

c[0]*(1-x/(c[1]+x))+c[2],

where x= calcium or ouabain concentration, c[0]=Vmax, c[1]=$K_{0.5}$, and c[2]= non-inhibitable background activity.

Two component curves were fitted to:-

c[0]*(1-(x/(c[1]+x))*c[2]-(x/(c[3]+x))*(1-c[2]))+c[4],

where x= calcium, sodium or ouabain concentration, c[0]= Total V max, c[1]=1st $K_{0.5}$, c[2]=fraction of 1st component, c[3]=2nd $K_{0.5}$, (1-c[2])=fraction of 2nd component and c[4]=non-inhibitable background activity.

Figure 4 displays enzyme activity in axolemma, as a function of sodium plus or minus calcium. The data was fitted to the following function, representing the consecutive binding of three sodium atoms each with the same Km :-

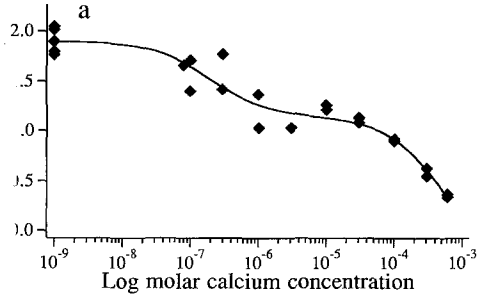
c[0]/(1+c[1]/x+c[1]^2/x^2+c[1]^3/x^3),

where x= sodium concentration, c[0]=Vmax, and c[1]=Km for sodium. It may be shown from this function that $K_{0.5}$=1.84*c[1].

RESULTS AND DISCUSSION

Calcium inhibits the (Na,K)ATPase with a two component curve over the range 10^{-6}M-10^{-3}M (Fig1a and3a). The first plateau of the curve occurs in the micromolar calcium range, which is the the measured range for calcium transients in various cell types (heart (30), oocyte (31), liver (32), Ptk$_1$cells (33)). This physiological range extends from 0.08-5μM. The second component of inhibition occurs at concentrations of calcium greater than 10^{-5}M, when both the high and low affinity (Na,K)ATPase isomers are inhibited to the same degree. The latter phenomenon is explained by:- a) High levels of calcium competing with magnesium for ATP and thus lowering the effective concentration of Mg-ATP, a rate limiting substrate of (Na,K)ATPase (34,35). b) High calcium inhibiting the enzyme directly, by competing with magnesium for the latter ion's binding site (36).

Figures 1a,2a and 3a give the dose response to calcium in the three tissues. The lower part of each Figure:-1b, 2b and 3b, gives the ouabain titration curves plus or minus calcium for that tissue. Here the concentration of calcium in each experiment is set for maximal calcium inhibition of the (Na,K)ATPase in the physiological range of the ion. In axolemma and skeletal muscle, calcium in the physiological range inhibits the enzyme down to 40% and 50% of the original activity respectively (Fig1a and 2a). In axolemma all the enzyme exhibits high affinity for ouabain with a one component titration curve, K_i=1.38±0.2x10-6M. Addition of calcium at 5x10^{-6}M reduces the activity by 40% but the ouabain K_i remains unchanged at 1.98±0.3x10-6M. (Fig1b). Therefore in axolemma at physiological calcium concentrations, approximately



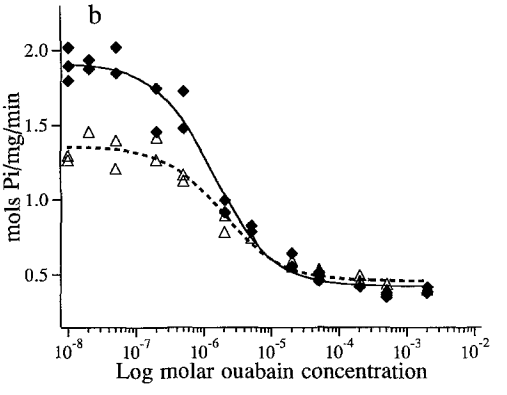


Figure 1. Rat axolemma (Na,K)ATPase activity as:- (1a) a function of calcium (◆--◆), and (1b) as a function of ouabain, control (◆---◆), and + 5x10^{-6}M calcium (Δ---Δ).

Linear rates of plasma membrane (Na,K)ATPase activity (approx 5μg/0.5ml assay) were followed over 7 minutes at 36^{0}C by the coupled assay of Barnett (23), with conditions as described in Materials and Methods. The assay was started by the addition of 5μl of enzyme to the cuvette containing all the other substrates and ions. In all experiments except the ouabain titration curves, at the end of 7 minutes, 2x10^{-3}M ouabain was added to each cuvette and the rate followed for a further 7 minutes. The non-ouabain inhibitable activity was subtracted from the total activity to give the (Na,K)ATPase activity. The non-ouabain inhibitable activity in all three tissues was approximately 10% of the total, and calcium had no sigificant effect on this activity. At each data point for calcium concentration, ouabain concentration, or sodium concentration n=2,3 or 4.

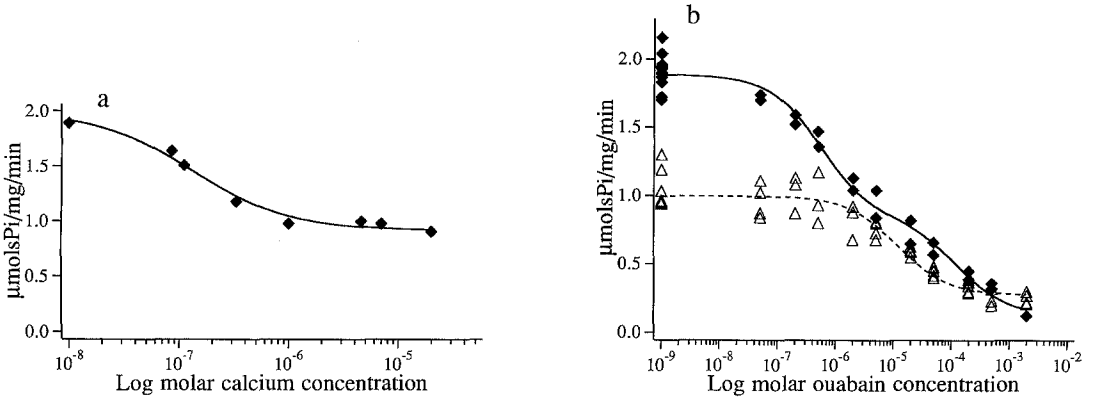


Figure 2. **Rat skeletal muscle (Na,K)ATPase activity as:- (2a) a function of calcium (◆--◆), and (2b) as a function of ouabain, control (◆---◆), and + 5x10^{-6}M calcium (Δ---Δ).**
Conditions as described in Figure 1.

60% of high affinity (Na,K)ATPase is unaffected by the ion. In skeletal muscle the enzyme exhibits a two-component titration curve to ouabain (Fig2b), and the high affinity component is all inhibited by 5x10^{-6}M calcium. The K_i for ouabain of the high affinity component is 5.4±1x10^{-7}M and for the low affinity component is 1.3±0.7x10^{-4}M. In the presence of 5x10^{-6}M calcium all of the high affinity component is inhibited and the remaining component has a K_i of 1.2±0.3x10^{-5}M which is in the low affinity range. Immunoblotting procedures show rat skeletal muscle to contain mainly the α2 (high ouabain affinity) isomer of the (Na,K)ATPase, no α3 (high ouabain affinity) isomer and a varying amount of α1(8) (the latter isomer may be of muscle nerve origin). Thus in muscle, a rise in calcium to the micromolar level inhibits α2, leaving low affinity α1 active. If this muscle result also applies to the α2 component in axolemma then the remaining high affinity axolemma component can be inferred to be α3. The

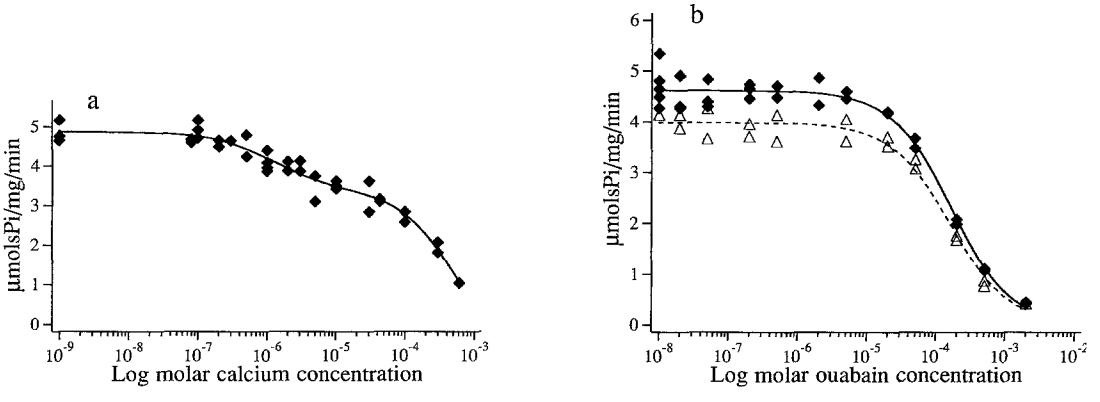


Figure 3. **Rat kidney (Na,K)ATPase activity as:- (3a) a function of calcium (◆--◆), and (3b) as a function of ouabain, control (◆---◆), + 4.x10^{-5}M calcium (Δ---Δ).**
Conditions as described in Figure 1.

phenomenon of calcium inhibition of the (Na,K)ATPase may therefore be a way to discriminate, at the chemical level, between the α2 and α3 isomers. The rat kidney outer medulla contains mainly the low affinity α1 isomer of the (Na,K)ATPase and has a one component titration curve to ouabain, with a K_i=1.65±0.1x10^{-4}M. Addition of 4x10^{-5}M calcium (above physiological but below the level to give competition with Mg^{++}), only reduced the activity to 84% of the original and the K_i was unchanged from the control with a value of 1.62±0.1x10^{-4}M (Fig3b).

A study was performed to look for possible competitive inhibition due to calcium binding to one or more sodium sites of (Na,K)ATPase. Figure 4. shows that the control Vmax is reduced from 2.32±0.03 to 1.82±0.05 μmolsPi/mg/min (21% reduction) at 5x10^{-7}M calcium and to 1.18±0.02 μmolsPi/mg/min (52%) at 5x10^{-6}M calcium. The control $K_{0.5}$ for sodium=5.64±0.3x10^{-3}M and there was no definite change with calcium, although at 5x10^{-6}M calcium the $K_{0.5}$ was slightly higher at 8.63±0.6x10^{-3}M. The inhibition shows some sodium dependence as 5x10^{-7}M calcium does not inhibit the (Na,K)ATPase at 6mM sodium, whereas at 20mM sodium this low calcium concentration inhibits by 21%.

In summary we report the K_i for physiological calcium inhibition of the α2 isomer of the (Na,K)ATPase to be in the region of 160nM in brain axolemma and skeletal muscle. This level of calcium is that found in the cytoplasm in the resting state of the cell, and therefore this ion is a strong candidate for a regulator of the sodium pump. It has been suggested that the mechanism by which hormones like catecholamines and insulin activate the pump may involve the removal of calcium inhibition from the pump protein (17). In our experiments calcium does not compete for the sodium site to any significant degree, and therefore non-competitive binding of calcium to the protein directly, or indirectly via a calcium binding protein is indicated. Work with highly purified α2 (Na,K)ATPase, devoid of all adherent potential calcium binding proteins, will establish whether the inhibition involves (Na,K)ATPase protein alone. Calcium binding proteins frequently have a specific sequence of amino acids termed an EF hand (37). A search

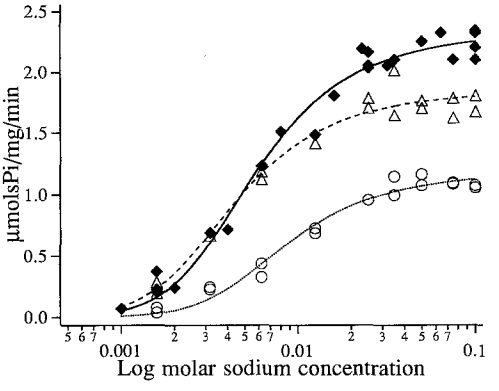


Figure 4. **Rat axolemma (Na,K)ATPase activity as a function of sodium:- control (◆--◆), + 5x10^{-7}M calcium (Δ---Δ), + 5x10^{-6}M calcium (O···O).**
Conditions as described in Figure 1.

(38) for this sequence in the three catalylic sequences of the (Na,K)ATPase (4) proved negative, as expected. However, there exist reports of a calmodulin like molecule termed "calnaktin", associated with calcium inhibition of the (Na,K)ATPase (20). This work used high levels of calcium and the enzyme preparation had a very low specific activity for (Na,K)ATPase, and therefore it would be of interest to reexamine "calnaktin", specifically with regard to its possible involvement in the membrane with $\alpha 2$ (Na,K)ATPase.

There is also some indication that the insulin effects on the pump could involve a calcium binding protein. Insulin was shown to stimulate phosphorylation of calmodulin in adipocytes (39) and the insulin receptor contains a calmodulin binding domain (40) and these findings together with the fact that insulin activates $\alpha 2$(Na,K)ATPase(10) suggest that occupancy of the insulin receptor by insulin could effect a change in the phosphorylation state of a calcium binding protein that results in the release of the calcium inhibition of the (Na,K)ATPase.

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