THE STIMULATORY EFFECT OF CALCIUM ON Na,K-ATPase OF NERVOUS TISSUE

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1. Introduction

On the basis of their different cationic requirements for maximal activity, 4 distinct ATP-phosphohydrolase enzymes associated with the cell membrane have been proposed. While all 4 ATPases apparently require the presence of Mg^{2+}, one needs no other cation (Mg-ATPase) [1], a second is stimulated by the additional presence of Na^+ (Na-ATPase) [2], a third by Ca^{2+} (Ca-ATPase) [3,4] and the fourth by the combined presence of Na^+ and K^+ (Na,K-ATPase) [1,5]. Only Na,K-ATPase is sensitive to inhibition by cardiac glycosides [1,5].

It is widely accepted that the Na,K-ATPase is an enzyme of major importance for the normal functioning of cells and plays the central role in pumping Na^+ and K^+ across the cell membrane [5–7]. This pumping activity is most important in excitable tissues where rapid Na^+ and K^+ fluxes are the hallmark of the action potential and where recovery of the status quo by restoring appropriate Na^+ and K^+ levels across the membrane is necessary to regain excitability. Consequently much attention has been focused on the Na,K-ATPase, particularly with regard to those factors which influence its activity. The effects of Ca^{2+} are of considerable interest because of the central role of Ca^{2+} in the function of excitable cells and its supposed increased mobility and intracellular availability consequent upon increased Na^+- and K^+-flux.

Calcium has been shown to exert a powerful inhibitory effect on Na,K-ATPase of cell membranes [1,3, 8–12]. Although the intracellular $[Ca^{2+}]$ in most tissues under most circumstances is thought to be present at $\leqslant \mu$M levels [3,5,13], most investigators have used much higher levels of Ca^{2+} in their studies. These experiments had been done in the presence of the chelating agents EDTA or EDTA. The present experiments confirm that in the presence of EDTA, Ca^{2+} (10^{-6}–3×10^{-3} mol/l) always exerts an inhibitory effect on Na,K-ATPase. Similarly even in the absence of EDTA, Ca^{2+} at $>10^{-4}$ mol/l is clearly an inhibitor of the enzyme. However, in the absence of EDTA, lower $[Ca^{2+}]$ values have a pronounced stimulatory effect on Na,K-ATPase.

On this basis, it is contended that the normal effect of Ca^{2+} on the Na,K-ATPase in the cell is one of stimulation and that the inhibition reported by others is an artefact occasioned either by the use of chelating agents in the experimental medium or by the use of Ca^{2+} at levels much higher than those which would usually be encountered physiologically.

2. Materials and methods

Wistar rats, of either sex, were decapitated and the whole brain (~2 g) was removed rapidly into ice-cold 0.32 M sucrose solution ± disodium EDTA (200 μM or 5 mM). The tissue was minced coarsely with scissors and rinsed with the above solution to remove blood. The minced brain was homogenised in 15 ml of the same solution using a glass homogeniser fitted with a teflon pestle of clearance 0.2 mm rotating at 600–800 rev./min which was passed vertically 12 times. For ATPase assays, the homogenate was diluted × 20 with 0.32 M sucrose ± EDTA to give 0.25–0.5 mg protein/ml, estimated as in [14]. Aliquots (0.4 ml) of the diluted homogenate were added to buffered media and preincubated for 10 min at 37°C before starting the reaction by addition of vanadium-free Tris–ATP (Sigma A0520; 4 mM final conc.). The media (final vol. 2 ml) contained Mg^{2+} alone (5 mM $MgCl_2$), Mg^{2+} + Na^+ (150 mM NaCl) or Mg^{2+}, Na^+ + K^+ (10 mM KCl) in 50 mM imidazole–HCl (pH 7.4). The reaction was stopped 10 min after ATP addition by adding 1 ml 6% trichloracetic acid and standing the tube on ice.

Enzyme activity was estimated by spectrophotometric determination of P_i in the supernatant remaining after centrifugation at 6650 × *g* for 10 min [15,16]. Relevant blanks were run in which trichloroacetic acid was added to reaction tubes prior to ATP addition. When appropriate, calcium (as $CaCl_2$) was added to the pre-incubation media. In some experiments ($n = 5$), ouabain (10^{-7}–10^{-3} mol/l final conc.) was added to the pre-incubation media 30 s before ATP addition. ATPase activity is expressed as μmol P_i liberated . mg protein^{-1} . h^{-1}. Na,K-ATPase activity was calculated as the difference between total ATPase activity measured in the presence of Na^+,K^+ and Mg^{2+} and that activity determined in the presence of Na^+ and Mg^{2+} only. Mg-ATPase activity is given by the value measured in the presence of Mg^{2+} alone; Na-ATPase activity is given by the difference between this activity and that measured in the presence of Na^+ and Mg^{2+}. All data are expressed as mean values ± SEM. Statistical significance of the difference between control and test values was calculated by Student's *t*-test.

To describe quantitatively the effect of Ca^{2+} per se on ATPase activity it is necessary to estimate what proportion of added Ca^{2+} is free to exert a modifying effect on the enzyme. In the presence of ATP and EDTA, ligands both of which bind divalent cations, the estimation of free [Ca^{2+}] must be based on a calculation taking account of the stability constants of the relevant metal–ligand complexes under the experimental conditions. The capacity of ATP to bind Mg^{2+} and Ca^{2+} was derived from the data in [17] and that of EDTA from [18]. The relevant stability constants ($\log K^1_{app}$) were calculated for pH 7.4 at 37°C with 10 mM K^+, 150 mM Na^+ and an ionic strength of 228 mM. These were for: MgATP, 4.20; CaATP, 3.89; MgEDTA, 5.82; and CaEDTA, 7.72. These values were used in all calculations in which it was necessary to estimate the concentrations of metals, ligands and metal–ligand complexes in the reaction mixture.

3. Results and discussion

In the absence of EDTA, basal Na,K-ATPase activity of rat brain homogenate was low and constituted only a small proportion of total ATPase activity. In the presence of EDTA (4×10^{-5} mol/l in reaction mixture), the activity of Na, K-ATPase was increased by 150% (table 1), that of Mg-ATPase was increased by 65%, whereas that of Na-ATPase was unaffected.

Table 1
Effect of EDTA on ATPase activity of rat brain homogenates (mean ± SEM)

	ATP-phosphohydrolase activity (μmol P_i . mg protein^{-1} . h^{-1})		
	Mg-ATPase	Na-ATPase	Na,K-ATPase
–EDTA ($n = 5$)	24.1 ± 1.81	4.3 ± 0.29	5.9 ± 0.55
+EDTA (4×10^{-5} M) ($n = 5$)	39.8 ± 2.76	4.2 ± 0.40	14.8 ± 1.86

Addition of cardiac glycoside to the incubation media in the presence of EDTA caused a dose-dependent inhibition of Na,K-ATPase activity from 6% at 10^{-7} mol ouabain/l to 99% at 10^{-3} mol ouabain/l. By contrast ATP-phosphohydrolase activity of both Mg-ATPase and of Na-ATPase was unaffected at any ouabain level.

The results obtained with EDTA alone and with ouabain confirm the presence of 3 discrete and independent ATP-phosphohydrolases and enable accurate measurement of them. The activity of the Na,K-ATPase is modified both by EDTA and by ouabain, the Mg-ATPase is modified to an extent by EDTA but is unaffected by ouabain while the Na-ATPase is unaffected by either.

Addition of Ca^{2+} to the incubation media might be expected to have at least two independent effects:

(i) It could influence the activity of any or all of the above 3 ATPases;

(ii) It should stimulate a Ca-ATPase: If Ca-ATPase is present in rat brain homogenates, then the additional phosphohydrolase activity due to stimulation of this enzyme by added Ca^{2+} would appear as an apparent enhancement of Mg-ATPase, Na,K-ATPase and Na-ATPase activities since the media needed for the determination of the activity of each of these 3 enzymes all contain Mg^{2+}, allegedly required for the expression of Ca-ATPase activity.

In the presence of EDTA (1×10^{-3} mol/l), Ca^{2+} added to the media at 3×10^{-5}–10^{-3} mol/l would result in free Ca^{2+} levels of 1.9×10^{-7}–6.8×10^{-5} mol/l (pCa = 6.73 and 4.17, respectively). The balance of the added Ca^{2+} is bound either to ATP or to EDTA: that bound to EDTA would not be expected to par-

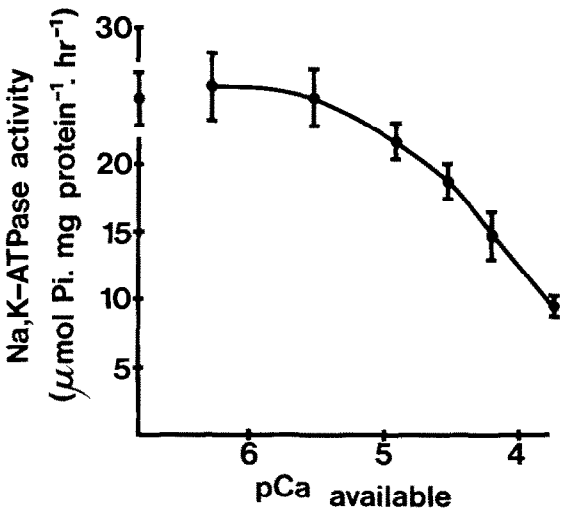

Fig.1. Effects of Ca^{2+} on Na,K-ATPase activity in the presence of EDTA (10^{-3} mol/l). $pCa_{available}$ is the negative logarithm of the $[Ca^{2+}]$ (free Ca^{2+} + Ca-ATP) calculated as in the text. Basal Na,K-ATPase activity measured in the absence of Ca^{2+} is marked on the ordinate. The points show the mean values ± SEM obtained in 5 expt.

ticipate in enzyme-catalysed reactions; that bound to ATP might. The lowest $[Ca^{2+}]$ had no effect on Na,K-ATPase activity, but as the level was increased a dose-dependent inhibition of activity was observed (fig.1). No effect was noted on Mg-ATPase or on Na-ATPase at any $[Ca^{2+}]$; the activities of these enzymes remained at or near basal levels over the entire $[Ca^{2+}]$ range. This finding implies that under the conditions of these experiments Ca-ATPase activity is minimal; if not, an enhancement of phosphohydrolase activity in the media used to estimate Mg-ATPase and Na-ATPase should have been noted. The alternative explanation, that any stimulatory effect of Ca^{2+} on Ca-ATPase is offset by a concomitant inhibitory effect on both Mg-ATPase and on Na-ATPase at all $[Ca^{2+}]$, would be a remarkable coincidence and can be discounted. Therefore, the inhibitory effects noted must be the direct effects of Ca^{2+} on Na,K-ATPase.

In the absence of EDTA, the basal activities of both Na,K-ATPase and Mg-ATPase were lower than in its presence (table 1). Addition of Ca^{2+} to the media in the absence of EDTA caused no change in the activity of Mg-ATPase and a slight, but significant reduction in activity of Na-ATPase at $\geqslant 10^{-3}$ mol Ca^{2+}/l (table 2). However, at low $[Ca^{2+}]$ there was now a marked stimulation of Na,K-ATPase activity, maximal at 10^{-6} mol Ca^{2+}/l and decreasing as $[Ca^{2+}]$ increased. At $\geqslant 10^{-4}$ mol Ca^{2+}/l an inhibition of Na,K-ATPase activity occurred.

Table 2
Effects of calcium on Mg-ATPase, Na-ATPase and Na,K-ATPase activity in the absence of EDTA

Available Ca^{2+} (mol/l)	ATP-phosphohydrolase activity (μmol P_i . mg protein^{-1} . h^{-1})		
	Mg-ATPase[a]	Na-ATPase[a]	Na,K-ATPase[a]
Zero	24.1 ± 1.81	4.3 ± 0.29	5.9 ± 0.55
1×10^{-6}	24.8 ± 2.13	3.2 ± 0.39	8.3 ± 0.90[b]
3×10^{-6}	24.5 ± 1.66	3.8 ± 0.47	7.7 ± 0.77[b]
1×10^{-5}	24.3 ± 1.81	4.3 ± 0.54	6.0 ± 0.59
3×10^{-5}	24.3 ± 1.77	4.0 ± 0.32	6.2 ± 0.61
1×10^{-4}	24.0 ± 1.76	4.5 ± 0.17	4.5 ± 0.47
3×10^{-4}	23.7 ± 1.55	3.4 ± 0.35	3.6 ± 0.53[c]
1×10^{-3}	23.8 ± 1.77	2.4 ± 0.30[c]	2.3 ± 0.31[c]
3×10^{-3}	22.4 ± 1.48	1.8 ± 0.14[c]	1.1 ± 0.13[c]

[a] Mean (n = 5) ± SEM
[b] Value significantly greater ($P < 0.01$) than that measured in absence of Ca^{2+}
[c] Value significantly less ($P < 0.01$) than that measured in absence of Ca^{2+}

The contrast between the effects of low $[Ca^{2+}]$ on Na,K-ATPase activity in the presence and absence of EDTA are highlighted in fig.2.

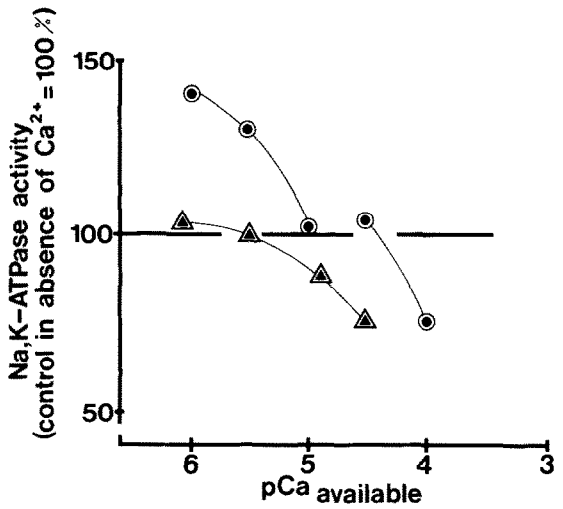

Fig.2. Effects of Ca^{2+} on Na,K-ATPase activity in the absence (•) n = 5) and presence of EDTA (10^{-3} mol/l (△) n = 5). In each case basal Na,K-ATPase activity measured in the absence of added Ca^{2+} is given as 100%.

Any result obtained in the absence of EDTA or other chelating agents might be considered as a result which reflects more closely the physiological situation. One could also more confidently propose a physiological role for an effect that can be evoked with in vivo Ca^{2+} levels. Those effects seen at mM Ca^{2+} levels may not be seen in the cell except, perhaps, transiently.

Some of the effects of Ca^{2+} on the function of excitable tissues may be brought about not by free Ca^{2+} alone but through the mediation of the heat-stable regulator protein, calmodulin [13,19,20]. It may be the Ca^{2+}–calmodulin complex that is biologically active. Removal of an endogenous heat-stable protein fraction from synaptic vesicle preparations [21] abolished the facilitation of neurotransmitter release and protein phosphorylation by Ca^{2+}, but the effects of Ca^{2+} on the vesicle system could be restored when protein vesicle extract or highly purified calmodulin isolated from rat brain were re-admitted to the treated vesicles.

In [21] the synaptic vesicles could be washed repeatedly in the standard EDTA-free experimental medium without significant change in protein pattern or loss of Ca^{2+}-dependent noradrenaline release or protein phosphorylation. However, after treatment of the vesicles with EDTA, the ability of calcium to elicit these effects was lost. Analogous findings have been reported with EGTA-pretreated preparations in [4].

From the above stimulation of Na,K-ATPase by low levels of Ca^{2+}, it can be hypothesed that:

Under normal circumstances Ca^{2+} in combination with the regulator protein calmodulin constitutes an active complex which is able to stimulate Na,K-ATPase. This may be the physiological effect of calcium on the enzyme. When $[Ca^{2+}]$ is high, the cellular availability of calmodulin may be exceeded, and the inhibition of Na,K-ATPase reflect the direct effects of Ca^{2+} on the enzyme. When EDTA has been added, calmodulin is prohibited from forming an effective combination with added Ca^{2+}. Consequently, there is no stimulation of Na,K-ATPase by Ca^{2+}–calmodulin and only inhibition of the enzyme by free Ca^{2+} is observed.

Summary: A stimulation of Na,K-ATPase by Ca^{2+} is reported and it is suggested that this effect is the major physiological action of the cation on the enzyme.

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