Biochemical Pharmacology, Vol. 32, No. 7, pp. 1219–1227, 1983.
Printed in Great Britain.

0006-2952/83/071219-09 $03.00/0

STIMULATION OF NEURONAL Na^+,K^+-ATPase BY CALCIUM

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(*Received* 1 *June* 1982; *accepted* 17 *September* 1982)

Abstract—The effect of calcium on ATP-phosphohydrolase activity of rat brain homogenates has been investigated. In both the presence and absence of the chelating agent EDTA, free calcium within the concentration range 1.2×10^{-7} to 5.0×10^{-4} moles/l consistently affected only the activity of Na^+,K^+-ATPase; the activities of Mg^{2+}-ATPase and Na^+-ATPase were essentially unchanged by Ca^{2+}; Ca^{2+}-ATPase could not be demonstrated. In either the presence or absence of EDTA, concentrations of free-Ca^{2+} above 3×10^{-6} moles/l caused an inhibition of Na^+,K^+-ATPase activity. In the presence of EDTA, concentrations of free-Ca^{2+} below 3×10^{-6} moles/l were ineffective at altering Na^+,K^+-ATPase activity but, in the absence of EDTA, free-Ca^{2+} in this concentration range caused a marked stimulation of the enzyme. Evidence is presented to show that the stimulation of Na^+,K^+-ATPase by calcium is modulated by the regulatory protein calmodulin. Since the stimulation occurs over the range of concentrations at which calcium would be expected to be encountered within the cell, it is suggested that this is the major physiological effect of calcium on Na^+,K^+-ATPase.

During the last 25 years, calcium has been shown repeatedly and consistently to exert a powerful inhibitory effect on the sodium-potassium stimulated ATP-phosphohydrolase (Na^+,K^+-ATPase) of cell membranes [1–7].

Skou [1] was one of the first investigators to demonstrate an inhibition of the activity of the enzyme, but the lowest concentration of Ca^{2+} used in his experiments was 1 mmole/l, a concentration far in excess of that which would be encountered intracellularly under normal operating conditions. Subsequent investigators [2–7] have used a range of concentrations of Ca^{2+} encompassing those that would be encountered in the cell, but most have used also the chelating agents EDTA or ethyleneglycol bis (amino-ethylether) tetra-acetate (EGTA) in their experimental protocols. These agents were presumably used both to produce a Ca^{2+} buffer system and also to remove traces of inhibitory heavy metal contaminants from the membrane preparations and thereby promote a higher basal level of Na^+,K^+-ATPase activity suitable for subsequent experimentation. Although the enhanced level of Na^+,K^+-ATPase activity in the presence of a chelating agent allows better differentiation between control and test levels of ATP-phosphohydrolase activity when evaluating a presumed enzyme inhibitor, the presence of such chelators is nonphysiological.

The purpose of these experiments was to investigate in the absence of such cation chelators the effects of Ca^{2+} in physiological concentrations on Na^+,K^+-ATPase of cell membranes derived from nervous tissue.

Preliminary accounts of part of this work have appeared elesewhere [8, 9].

MATERIALS AND METHODS

Wistar rats, of either sex, were decapitated and the whole brain (*ca.* 2 g) was removed rapidly into ice-cold 0.32 mole/l sucrose solution ± disodium EDTA (0.01, 0.2, 1.0 and 5.0×10^{-3} moles/l). The tissue was minced coarsely with scissors and rinsed with the appropriate solution above to remove blood. The minced brain was homogenized in 15 ml of the same solution using a glass homogenizer fitted with a teflon pestle, of clearance 0.2 mm, rotating at 600–800 rpm which was passed vertically twelve times. For ATPase assays, the homogenate was diluted × 20 with 0.32 mole/l sucrose ± EDTA to give a protein concentration (estimated by the method of Lowry *et al.* [10]) of 0.25 to 0.5 mg/ml. Aliquots (0.4 ml) of the diluted homogenate were added to buffered media and pre-incubated for 10 min at 37° before starting the reaction by addition of vanadium-free Tris–ATP (Sigma A0520; 4×10^{-3} moles/l final concentration). The media (final volume 2 ml after all additions) contained Mg^{2+} alone (5 mmoles/l $MgCl_2$), Mg^{2+} plus Na^+ (150 mmoles/l NaCl), or Mg^{2+}, Na^+ plus K^+ (10 mmoles/l KCl) in 50 mmoles/l imidazole: HCl, pH 7.4. The reaction was stopped 10 min after ATP addition by adding 1 ml of 6% trichloroacetic acid (TCA), mixing, and standing the tube on ice. Enzyme activity was estimated by spectrophotometric determination of inorganic phosphate in the supernatant fraction remaining after centrifugation at 6650 *g* for 10 min [11, 12]. Relevant blanks were run in which TCA was added to reaction tubes prior to ATP addition.

ATP-phosphohydrolase activity is expressed in all cases as μmoles of inorganic phosphate (P_i) liberated per mg protein per hr. Na^+,K^+-ATPase activity was calculated as the difference between total ATPase activity measured in the presence of Na^+, K^+ and

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Mg^{2+} and that activity determined in the presence of Na^+ and Mg^{2+} only. Mg^{2+}-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+}.

In most experiments, various additional compounds were added to the buffered media. These were added prior to (calcium, calmodulin, fluphenazine) or after (ouabain) the addition of brain homogenate. All such compounds were contained in 0.1 ml imidazole:HCl buffer. An equivalent volume of this buffer was omitted from each medium to maintain constant volume. Calcium was added as $CaCl_2 \cdot 2H_2O$. Calmodulin (calcium-dependent modulator protein) was prepared by the method of Watterson *et al.* [13], characterized by its electrophoretic mobility on polyacrylamide gels and the ability to stimulate phosphodiesterase activity, and stored in solid form. Prior to use, the solid was dissolved in imidazole:HCl buffer. Each 0.1 ml addition to the buffered experimental media contained 150 μg of the solid preparation. Expressed in terms of its phosphodiesterase activating potential, this quantity of calmodulin was determined to be approximately equivalent to 40 units of the commercial calmodulin preparation (Sigma P0270). Fluphenazine hydrochloride (Squibb) was added where appropriate to give a final concentration in the experimental medium of 20 μmoles/l. In those instances where calcium was present also, the fluphenazine was added first. Ouabain octahydrate was added during preincubation of the homogenate 30 sec before ATP addition.

Except where stated otherwise, all data are expressed as mean values ± S.E.M. Statistical significance of the difference between control and test values was calculated by Student's *t*-test.

Effects of calcium

To describe quantitatively the effects of Ca^{2+} *per se* on ATPase activity, it is necessary to estimate what proportion of the ion added 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+} concentration must be based on a calculation taking account of the association constants of the relevant metal–ligand complexes under the prevailing experimental conditions. In this regard, the capacity of ATP to bind Mg^{2+} and Ca^{2+} was derived from the data of O'Sullivan and Smithers [14] and that of EDTA was obtained from O'Sullivan [15]. The relevant association constants [logK^1 apparent] were calculated for pH 7.4 at a reaction temperature of 37° with [K^+] 10 mM, [Na^+] 150 mM, and with a prevailing ionic strength of 230 mmoles/l. These were for Mg^{2+}ATP, 4.20, Ca^{2+}ATP, 3.89, Mg^{2+}EDTA, 5.82; and Ca^{2+}EDTA, 7.72. In those calculations in which these values were used to estimate the concentrations of metals, ligands and metal–ligand complexes in the reaction mixture, no account was taken of the relatively small amounts of endogenous Ca^{2+} and Mg^{2+} deriving from the homogenate.

Table 1. ATP-phosphohydrolase basal activity in rat brain homogenates

	ATP-phosphohydrolase specific activity [μmoles $P_i \cdot$ (mg protein)$^{-1} \cdot$ hr^{-1}]
Total ATPase	21.4 ± 4.48*
Mg^{2+}-ATPase	10.8 ± 2.66
Na^+,K^+-ATPase	6.8 ± 2.77
Na^+-ATPase	3.8 ± 1.71

* Mean ± S.D.; N = 16 experiments.

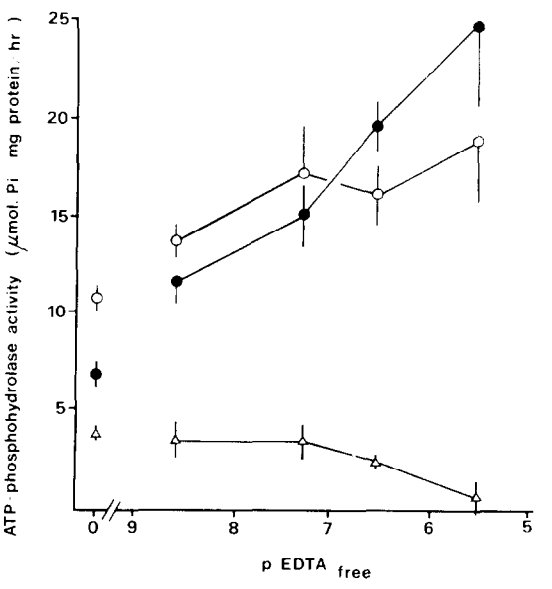

Fig. 1. Effect of EDTA on ATP-phosphohydrolase activity of rat brain homogenates. Key: (○) Mg^{2+}-ATPase, (●) Na^+,K^+-ATPase, and (△) Na^+-ATPase. Each point is the mean of at least seven determinations. The vertical bars indicate the S.E. of the mean. In each determination of ATPase activity, the buffered media contained Na^+ (150 mmoles/l), K^+ (10 mmoles/l), Mg^{2+} (5 mmoles/l) and, except for the determinations of basal ATPase activity, increasing concentrations of disodium EDTA.

RESULTS

Basal ATP-phosphohydrolase activity of rat brain homogenates

Values for ATP-phosphohydrolase activity of rat brain homogenates were obtained in media containing Mg^{2+} alone, Mg^{2+} plus Na^+ or Mg^{2+}, Na^+ plus K^+. These values are given in Table 1.

Effects of EDTA on ATP-phosphohydrolase activity

In some experiments disodium EDTA was added to the sucrose media to give final concentrations in the range 2×10^{-6} moles/l to 1×10^{-3} moles/l. After making allowance (see Materials and Methods) for the EDTA which is effectively removed by virtue of chelating the Mg^{2+} in the experimental media, the concentration range of free EDTA was calculated to be 2.63×10^{-9}–3.19×10^{-6} moles/l.

Total ATPase activity was increased by EDTA in a concentration-dependent manner. The increase was due predominantly to raised Na^+,K^+-ATPase

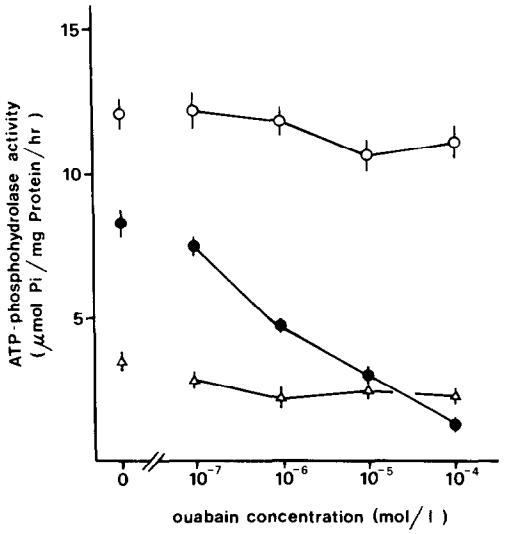

Fig. 2. Effect of ouabain on ATP-phosphohydrolase activity of rat brain homogenates. Key: (○) Mg^{2+}-ATPase, (●) Na^+,K^+-ATPase, and (△) Na^+-ATPase. Each point is the mean of five determinations. The vertical bars indicate the S.E. of the mean. In each determination, the buffered media contained Na^+ (150 mmoles/l), K^+ (10 mmoles/l) and Mg^{2+} (5 mmoles/l). No Ca^{2+} or EDTA was present.

activity (Fig. 1). Mg^{2+}-ATPase activity was also raised; Na^+-ATPase activity was reduced.

It is unlikely that these changes were due to variation in the availability of Mg^{2+} or Mg^{2+}ATP produced by the EDTA; our calculations show that the concentrations of these species were not markedly affected. For example, in the absence of EDTA, free-Mg^{2+} and Mg^{2+}-ATP concentrations would have been 1.20×10^{-3} and 3.80×10^{-3} moles/l respectively; in the presence of EDTA (2×10^{-4} moles/l) the free-Mg^{2+} and Mg^{2+}-ATP concentrations would have been 1.03×10^{-3} and 3.77×10^{-3} moles/l respectively.

Effects of altered Mg^{2+} concentration on ATP-phosphohydrolase activity

In four experiments, aliquots of brain homogenate were added to sucrose media containing either Mg^{2+} in the usual concentration of 5 mmoles/l or Mg^{2+} at 4 mmoles/l.

In the media containing Mg^{2+} at 5 mmoles/l, free-Mg^{2+} and Mg^{2+}ATP concentrations were calculated to be 1.20×10^{-3} and 3.80×10^{-3} moles/l respectively; in those containing Mg^{2+} at 4 mmoles/l and free Mg^{2+} and Mg^{2+}ATP concentrations were 0.47×10^{-3} and 3.53×10^{-3} moles/l respectively.

Despite these differences in Mg^{2+} and Mg^{2+}ATP availability, Mg^{2+}-, Na^+,K^+- and Na^+-ATPase activities in the two situations were very similar.

Effects of ouabain on ATP-phosphohydrolase activity

Addition of ouabain (10^{-7}–10^{-4} moles/l) to the sucrose media caused a dose-dependent inhibition of Na^+,K^+-ATPase activity but no significant change in either Mg^{2+}-ATPase or Na^+-ATPase activity (Fig. 2). With EDTA (2×10^{-4} moles/l) in the sucrose media ($pEDTA_{free}$ 6.53), Na^+,K^+-ATPase activity was higher than in its absence. Despite the higher initial activity of the enzyme, however, ouabain in the presence of EDTA was equieffective at all concentrations in inhibiting Na^+,K^+-ATPase (Fig. 3).

Effects of Ca^{2+} on ATP-phosphohydrolase activity

In the presence of EDTA. ATP-phosphohydrolase activity was measured in sucrose media containing EDTA at total concentrations of 4×10^{-5}, 2×10^{-4} and 1×10^{-3} moles/l. At each concentration of EDTA, calcium was added to give a range of total concentrations between 3×10^{-6} and 1×10^{-3} moles/l. However, after taking into account the fact that added calcium along with the magnesium present associates with both EDTA and with ATP in the reaction mixture to an extent predicted by the association constants given in Materials and Methods, the concentration range of ionic calcium in these experiments was 1.66×10^{-7} to 4.75×10^{-4} moles/l (pCa_{free} 6.780–3.323).

Over this range calcium exerted no consistent effect on either Mg^{2+}-ATPase or Na^+-ATPase, and at all concentrations it exerted only an inhibitory effect on Na^+,K^+-ATPase (Fig. 4).

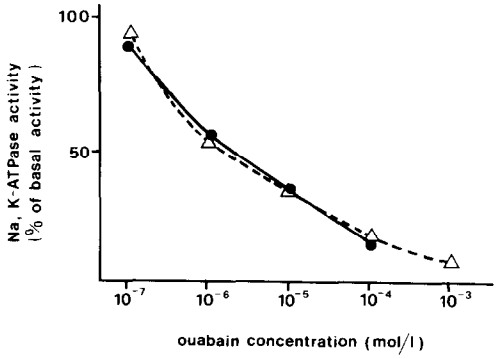

Fig. 3. Effect of ouabain on Na^+,K^+-ATPase activity in the absence of EDTA (●——●) and in its presence (2×10^{-4} moles/l, △----△). The ordinate shows Na^+,K^+-ATPase activity as a percentage of basal activity; in the absence of EDTA this was 8.3 ± 0.52 μmoles P_i per mg protein per hr, N = 5; in the presence of EDTA this was 23.8 ± 2.68 μmoles/l P_i per mg protein per hr, N = 5. K_i for ouabain in the absence of EDTA is 2.1×10^{-6}; in the presence of EDTA K_i for ouabain is 1.5×10^{-6}.

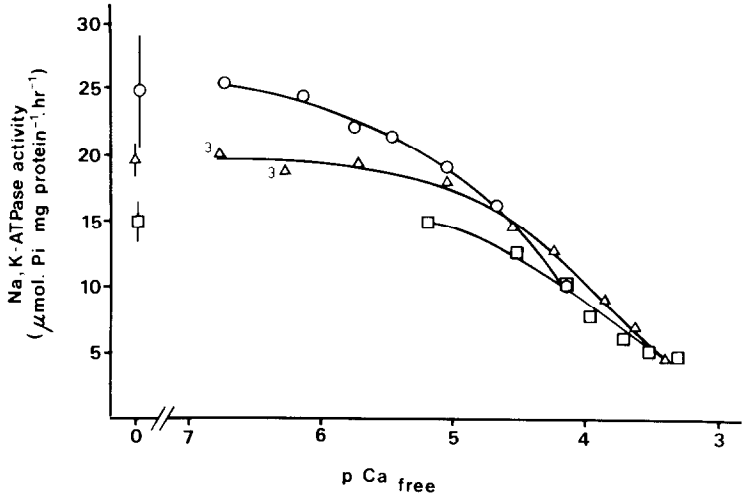

Fig. 4. Effect of Ca^{2+} on Na^+,K^+-ATPase activity in the presence of different concentrations of EDTA. Key: (□) EDTA, 4×10^{-5} moles/l; (△) EDTA, 2×10^{-4} moles/l; and (○) EDTA, 1×10^{-3} moles/l. The basal Na^+,K^+-ATPase activity which prevailed at each concentration of EDTA and in the absence of Ca^{2-} is shown at point O on the abscissa. Each point is the mean of seven determinations except where stated. Vertical bars indicate S.E. of the mean.

In the absence of EDTA. As noted above, the basal activity of Mg^{2+}-ATPase and particularly of Na^+,K^+-ATPase was lower in the absence of EDTA than in its presence. Addition of calcium, 3×10^{-7}–1×10^{-3} moles/l ($[Ca]_{free}$ 1.18×10^{-7}–4.97×10^{-4} moles/l), had no effect on Mg^{2+}-ATPase activity and exerted an inhibitory effect on Na^+-ATPase activity only a high concentration ($pCa_{free} < 3.30$). On Na^+,K^+-ATPase, calcium had a biphasic effect; at low concentration ($< 3 \times 10^{-6}$ moles/l, $pCa_{free} > 5.5$ approx.) calcium caused stimulation, whereas at higher concentrations an inhibition was measured (Fig. 5).

The maximum stimulation of Na^+,K^+-ATPase activity was obtained at pCa_{free} 6.41 which represented a free calcium concentration of 3.9×10^{-7} moles/l.

Effect of fluphenazine on calcium stimulation of ATP-phosphohydrolase activity

In five experiments conducted on rat brain homogenates in the absence of EDTA, the mean basal Na^+,K^+-ATPase activity was 5.8 ± 1.2 μmoles P_i per mg protein per hr. In these experiments, addition of Ca^{2+} (3×10^{-7} moles/l; pCa_{free} 6.93) caused a small (+26%) but significant ($P < 0.01$) increase in

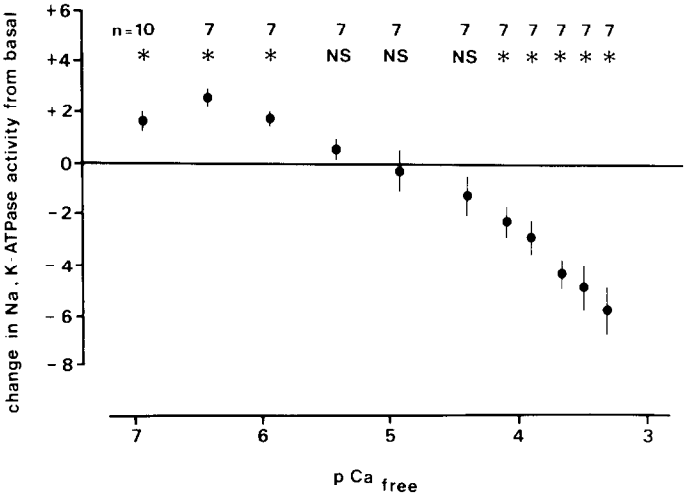

Fig. 5. Effect of Ca^{2+} on Na^+,K^+-ATPase activity in the absence of EDTA. Each point is the mean (± S.E.M.) of N determinations and is shown as the change (+ or −) from Na^+,K^+-ATPase activity measured in the absence of Ca^{2+}. An asterisk (*) indicates a difference statistically significant at the level $P < 0.0025$. Basal Na^+,K^+-ATPase specific activity in different experiments fell within the range 6.5 to 8.7 μmoles P_i per mg protein per hr. Basal Mg^{2+}-ATPase activity and Na^+-ATPase activity were within the range 9.3 to 12.2 μmoles P_i per mg protein per hr and 2.2 to 5.0 μmoles P_i per mg protein per hr respectively.

Na^+,K^+-ATPase activity of 1.5 ± 0.33 μmoles P_i per mg protein per hr. Fluphenazine (20 μmoles/l) itself caused no significant alteration in basal Na^+,K^+-ATPase activity (+0.3 ± 0.76 μmole P_i per mg protein per hr) but the compound attenuated significantly the stimulation of the enzyme by calcium. After fluphenazine, calcium evoked an increase in Na^+,K^+-ATPase activity of 0.4 ± 0.87 μmole P_i per mg protein per hr (+7%; NS).

Neither Mg^{2+}-ATPase nor Na^+-ATPase activity was altered consistently by fluphenazine or by calcium in the presence or absence of fluphenazine.

Effect of calmodulin on calcium stimulation of ATP-phosphohydrolase activity

In six experiments with rat brain homogenates in the absence of EDTA, calcium (3×10^{-7} moles/l; pCa_{free} 6.93) caused a stimulation of Na^+, K^+-ATPase activity from 5.4 ± 0.58 to 6.7 ± 0.56 μmoles P_i per mg protein per hr (+ 24%; $P < 0.005$). In these experiments, Mg^{2+}-ATPase activity was unchanged by Ca^{2+} (8.9 ± 1.02 to 9.5 ± 1.17 μmoles P_i per mg protein per hr) but there was a reduction in Na^+-ATPase activity (3.2 ± 0.64 to 1.9 ± 0.76 μmoles P_i per mg protein per hr; $P < 0.005$). When calmodulin (*ca.* 40 units) was added to the homogenates together with Ca^{2+} (3×10^{-7} moles/l), Na^+,K^+-ATPase activity was increased to 9.1 ± 0.71 μmoles P_i per mg protein per hr or to 169% of control activity ($P < 0.001$). Neither Mg^{2+}-ATPase activity nor Na^+-ATPase activity was further altered by the addition of calmodulin.

Effects of ouabain on calcium-stimulated ATP-phosphohydrolase

In four experiments, ouabain (10^{-3} moles/l) inhibited the activity of Na^+,K^+-ATPase from 5.0 ± 0.78 to 1.7 ± 0.42 μmoles P_i per mg protein per hr. Neither Mg^{2+}-ATPase nor Na^+-ATPase activity was altered by the ouabain (Fig. 6). When calcium (3×10^{-7} moles/l; pCa_{free} 6.93) was present, although resultant Na^+,K^+-ATPase activity was higher than in its absence (6.6 ± 0.87 μmoles P_i per mg protein per hr), ouabain caused a reduction to a level similar to that recorded in the absence of calcium (1.1 ± 0.40 μmoles P_i per mg protein per hr). The same result was obtained in the simultaneous presence of calcium and calmodulin. Under these conditions ouabain (10^{-3} moles/l) reduced Na^+,K^+-ATPase activity from 9.1 ± 1.10 to 0.53 ± 0.48 μmoles P_i per mg protein per hr and had no consistent effect on either Mg^{2+}-ATPase or Na^+-ATPase activity (Fig. 6).

Effect of calcium on ATP-phosphohydrolase activity revealed in the presence of Mg^{2+} and K^+

In twelve determinations in three experiments Na^+ was omitted from the experimental media. The cation was replaced with sucrose to maintain tonicity. In these determinations, ATPase activity measured in the presence of Mg^{2+} and K^+ together was slightly, but not statistically significantly, greater than that measured in the presence of Mg^{2+} alone (10.7 ± 0.98 compared with 9.4 ± 0.81 μmoles P_i per mg protein per hr).

Addition of Ca^{2+} (1×10^{-6} moles/l; pCa_{free} 6.41) to the media containing Mg^{2+} and K^+ together revealed no further ATPase activity; in twelve determinations, ATPase activity revealed in the presence of Mg^{2+}, K^+ and Ca^{2+} was 10.5 ± 0.84 μmoles P_i per mg protein per hr.

DISCUSSION

The observations recorded above show that in the presence of EDTA calcium at all effective concentrations inhibited Na^+,K^+-ATPase activity. In the absence of EDTA, calcium at concentrations below 3 μmoles/l apparently stimulated Na^+,K^+-ATPase, but at higher concentrations it inhibited the activity of the enzyme. If this stimulation of Na^+,K^+-ATPase is real, the finding would be of profound physio-

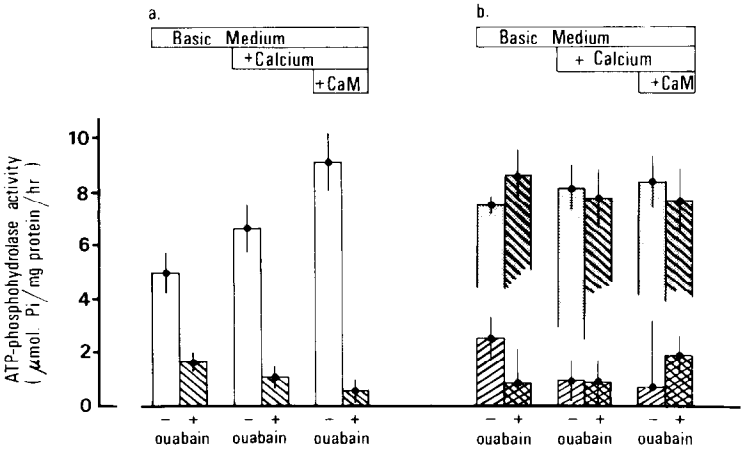

Fig. 6. (a) Effect of ouabain (1×10^{-3} moles/l) on basal activity of Na^+,K^+-ATPase and on that activity modified either by the presence of calcium (pCa_{free} 6.93) or by the simultaneous presence of Ca^{2+} + calmodulin (approximately 40 units). (b) Effect of ouabain on activity of Mg^{2+}-ATPase (□) and Na^+-ATPase (▨) under the same conditions as in (a). The data in both parts (a) and (b) were derived from the same experiments. No EDTA was present in the experimental media. Mean values ± S.E.M. are shown; N = 4.

logical significance in view of the importance of the enzyme in cellular sodium and potassium homeostasis and the fact that the effect was observed over the range of Ca^{2+} likely to be encountered in the cell throughout the course of its normal function [5, 16, 17].

Two facts need to be established. The first is that the ATP-phosphohydrolase activity stimulated by Ca^{2+} was Na^+,K^+-ATPase and not another ATPase erroneously identified as Na^+,K^+-ATPase. The second is that the stimulation of the enzyme is a physiological phenomenon that could be expected *in vivo* and not merely in artifact observed as a result of the *in vitro* study methods.

Is the stimulation by Ca^{2+} of ATP-phosphohydrolase activity due to its action of Na^+,K^+-ATPase?

On the basis of their particular cation requirements for expression of activity, four distinct ATP-phosphohydrolase enzymes associated with biological membranes have been described. While all four ATPases apparently require the presence of Mg^{2+}, one needs no other cation (Mg^{2+}-ATPase) [1], a second is stimulated by the additional presence of Na^+ (Na^+-ATPase) [18], a third by Ca^{2+} (Ca^{2+}-ATPase) [5, 19] and the fourth by the combined presence of Na^+ and K^+ (Na^+,K^+-ATPase) [1, 16]. Hence, Na^+,K^+-ATPase activity is here defined as that which is revealed in the presence of Mg^{2+}, Na^+ and K^+ over and above that revealed in the presence of Mg^{2+} and Na^+. Likewise Na^+-ATPase activity would be that measured with Mg^{2+} and Na^+ together less that activity (Mg^{2+}-ATPase) measured in the presence of Mg^{2+} alone.

Addition of Ca^{2+} to each of the incubation media would be expected to have at least two independent effects: (i) it could influence the activity of any or all of the three ATPases discriminated as above; and (ii) it should stimulate Ca^{2+}-ATPase. If Ca^{2+}-ATPase is present in rat brain homogenates, then additional phosphohydrolase activity due to stimulation of this enzyme by Ca^{2+} would be detected in each of the media used to discriminate the other three ATPases since all of these contain Mg^{2+} allegedly essential for expression of Ca^{2+}-ATPase activity (e.g. Ref. 20). Any Ca^{2+}-ATPase activity would appear as a numerically equal enhancement of those activities attributed to Mg^{2+}-ATPase, Na^+-ATPase or Na^+,K^+-ATPase.

In no experiment did calcium consistently produce an increase in that ATP-phosphohydrolase activity revealed in the presence of either Mg^{2+} alone or Mg^{2+} and Na^+ together; ATPase activity revealed by these cations remained at or near basal levels over the entire range of Ca^{2+} concentrations tested. This finding indicated not only that Ca^{2+} has no effect on either Mg^{2+}-ATPase or Na^+-ATPase activity but also that under the conditions of these experiments Ca^{2+}-ATPase activity is minimal. The possibility that any stimulatory effect of Ca^{2+} on a Ca^{2+}-ATPase was offset exactly by a concomitant inhibitory effect on both Mg^{2+}-ATPase and Na^+-ATPase would be a remarkable coincidence and can be discounted.

Whereas Ca^{2+} had no effect on Mg^{2+}-ATPase or Na^+-ATPase, it did have a pronounced stimulatory effect on that ATPase activity discriminated in the presence of Na^+, Mg^{2+} and K^+ over and above that measured with Na^+ and Mg^{2+} only. This finding can be explained in two ways, either that Ca^{2+} stimulated Na^+,K^+-ATPase, or that Ca^{2+} stimulated another ATPase which requires K^+ [21–24]. The second possibility is excluded by the experimental results obtained with Mg^{2+} and K^+ in the presence or absence of Ca^{2+}. Ca^{2+} did not stimulate further the ATPase activity revealed by Mg^{2+} and K^+ together.

The effects of the glycoside ouabain on the ATPase activity measured in the presence of Na^+, K^+, $Mg^{2+} \pm Ca^{2+}$ are crucial to the hypothesis that the ATPase stimulated by Ca^{2+} is Na^+,K^+-ATPase.

The present experiments show clearly, and previous reports [4, 16, 21, 25] leave little doubt, that the only ATPase affected by cardiac glycosides is Na^+,K^+-ATPase; in these experiments a 93% inhibition of Na^+,K^+-ATPase activity but little change in Mg^{2+} or Na^+-ATPase activity was produced by ouabain (10^{-3} moles/l). If the ATPase that is stimulated by Ca^{2+} is Na^+,K^+-ATPase, then the additional activity that is revealed by this cation should be inhibited by ouabain, whereas if the Ca^{2+} in reality stimulated an ATPase that is independent of Na^+,K^+-ATPase but revealed in the presence of Na^+, K^+ and Mg^{2+} then the ouabain would not inhibit the additional activity. The present experiments (Fig. 6) demonstrate that the additional ATP-phosphohydrolase activity revealed by Ca^{2+} was inhibited by ouabain, and this signifies that Na^+,K^+-ATPase is the enzyme which is stimulated by Ca^{2+}.

Is the stimulation of Na^+,K^+-ATPase by Ca^{2+} a physiological phenomenon?

To determine if the stimulation by Ca^{2+} is a physiological phenomenon rather than an artifact occasioned by the *in vitro* study methods, it is necessary to describe further the mechanism of the stimulation. Answers to the following questions are pertinent.

Could the stimulation of Na^+,K^+-ATPase in these experiments be the effect not of Ca^{2+} on the enzyme but, rather, on the availability of substrate in the reaction mixture? Although Mg^{2+}-ATP is the physiological substrate for Na^+,K^+-ATPase, the ratio between Mg^{2+} and ATP is of some importance in determining Na^+,K^+-ATPase activity; an excess of either Mg^{2+} or ATP in the reaction medium has been reported to inhibit the enzyme [26]. It is possible to argue that in our experiments the Mg^{2+}:ATP ratio was suboptimal, with the consequence that Na^+,K^+-ATPase activity, was, to an extent, inhibited. Added Ca^{2+}, by competing with Mg^{2+} for ATP, would, then, have increased the ratio between Mg^{2+} and ATP and, as a consequence, reduced Na^+,K^+-ATPase inhibition. The reduction of inhibition would be interpreted as a stimulation by Ca^{2+}. This explanation may be discounted. Calculation shows that the free-Mg^{2+}:free-ATP ratio of 6:1 which prevailed in the experimental media would not have been changed significantly by any addition of Ca^{2+} within the concentration range in which stimulation of Na^+,K^+-ATPase was observed. Furthermore, under the conditions of the present experiments, the relative insensitivity of Na^+,K^+-ATPase to alterations in Mg^{2+}:ATP ratio is indicated by the data

obtained when Mg^{2+} 4 moles/l was substituted for Mg^{2+} 5 mmoles/l in the experimental media. Under these conditions, the Mg^{2+}:ATP ratio would have been reduced to 1:1 but even a change in ratio of this magnitude had no effect on Na^+,K^+-ATPase activity.

Why does Ca^{2+} not stimulate Na^+,K^+-ATPase in the presence of EDTA? The following suggestions can be made: (a) Since EDTA itself stimulates Na^+,K^+-ATPase activity (Fig. 1), the subsequent addition of Ca^{2+} can cause no further enhancement. This suggestion is unlikely. Even at an EDTA concentration of 2×10^{-4} moles/l (i.e. $pEDTA_{free}$ 6.53), calcium in all effective concentrations did not stimulate Na^+,K^+-ATPase activity (Fig. 4) even though the potential activity that could have been revealed (Fig. 1) was much greater than that which pertained at EDTA 2×10^{-4} moles/l.

An alternative suggestion can be stated as follows: (b) EDTA chelates added Ca^{2+} and, as a consequence, the free EDTA in the experimental medium falls. Na^+,K^+-ATPase activity falls due to reduction of EDTA-induced stimulation. Any direct stimulatory effect that Ca^{2+} might have had on Na^+,K^+-ATPase is thus obscured.

Figure 7 is offered to allow the quantitative evaluation of this contention. The effects of free EDTA on Na^+,K^+-ATPase activity in the absence of Ca^{2+} (data from Fig. 1) and in its presence (data from Fig. 4) are shown. Together the data enable calculation of those effects of Ca^{2+} on Na^+,K^+-ATPase activity *that are independent* of EDTA. Such effects are derived by subtracting the Na^+,K^+-ATPase activity measured in the presence of Ca^{2+} from that in its absence at constant pEDTA. Figure 8 shows the independent effects of Ca^{2+} on Na^+,K^+-ATPase activity derived in this way. It is noteworthy that all data points obtained in experiments where EDTA had been present in total concentrations of 4×10^{-5} moles/l and 2×10^{-4} moles/l lie on a single curve. Moreover, at $pCa < 5$ this curve is superimposed on that obtained from those experiments that had been conducted in the absence of EDTA (data from Fig. 5). This is taken to indicate that the inhibitory effects on Na^+,K^+-ATPase activity of higher concentrations of free Ca^{2+} are independent of EDTA. At pCa free > 5, however, the curve obtained in those experiments in which EDTA *was never* present is different from that from the experiments where EDTA had been present. This analysis, by eliminating those effects on Na^+,K^+-ATPase caused by EDTA alone, indicates clearly that the failure of Ca^{2+} to cause a stimulaton of Na^+,K^+-ATPase was a consequence of the presence of EDTA. It was not, however, due simply to the stimulatory effect of Ca^{2+} being obscured by the simultaneous reduction of the EDTA-induced stimulation.

Hence the suggestion may be made that: (c) EDTA affects the disposition of the calcium binding protein; calmodulin, and inhibits formation of the calcium:calmodulin complex, a normal effect of which is to stimulate Na^+,K^+-ATPase. It is being suggested with increasing frequency that many of the effects of Ca^{2+} on the function of excitable tissues are brought about not by free Ca^{2+} alone but through

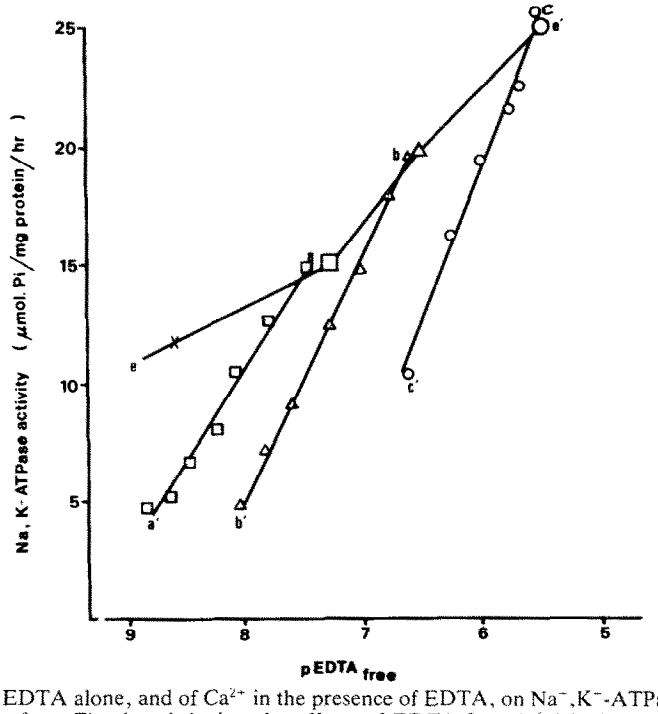

Fig. 7. Effect of EDTA alone, and of Ca^{2+} in the presence of EDTA, on Na^+,K^+-ATPase activity. The line e-e' is taken from Fig. 1 and depicts the effects of EDTA 2×10^{-6} (x), 4×10^{-5} (□), 2×10^{-4} (△) and 1×10^{-3} moles/l (○). The lines a-a', b-b' and c-c' (data from Fig. 4) depict Na^+,K^+-ATPase activity as a function of the EDTA that remains at increasing concentrations of Ca^{2+}. The approximate effect of Ca^{2+} on Na^+,K^+-ATPase that is independent of EDTA is given by the vertical difference between lines e-e' and a-a', b-b' and c-c' respectively. (Also see text.)

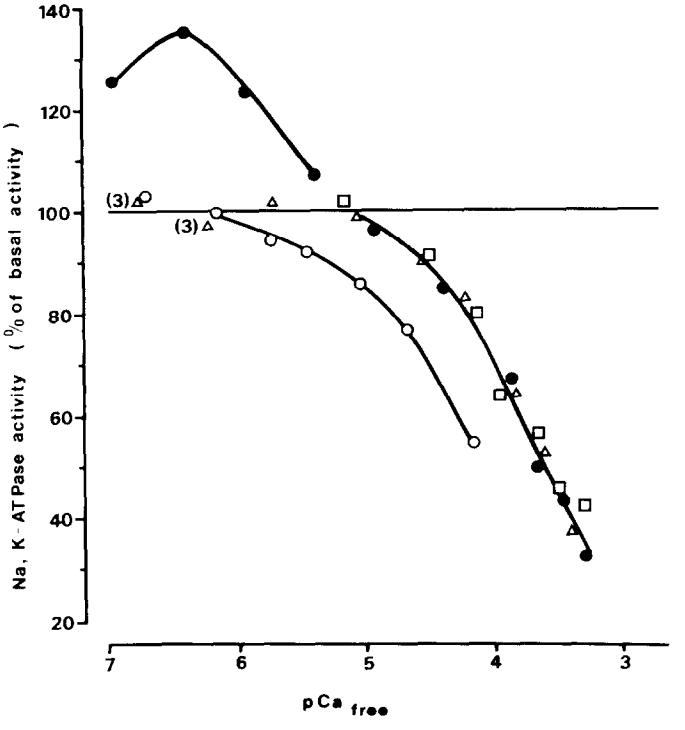

Fig. 8. Effect of Ca^{2+} alone on Na^+,K^+-ATPase activity. The direct effects of EDTA *per se* on Na^+,K^+-ATPase activity have been allowed for as explained in the text and in Fig. 7. Key: (●) effect of Ca^{2+} on Na^+,K^+-ATPase in those experiments where EDTA was never present (data from Fig. 5); (□, △ and ○) effect of Ca^{2+} on Na^+,K^+-ATPase in those experiments where EDTA had been present at concentrations of 4×10^{-5}, 2×10^{-4} and 1×10^{-3} moles/l respectively. Each point is the mean of at least seven determinations except where so marked.

the mediation of the heat stable regulator protein, calmodulin [17, 27–29]. It has been noted elsewhere that the effects of Ca^{2+} in biological tissues can be modified by the chelating agents EDTA or EGTA. de Lorenzo *et al.* [30] reported that Ca^{2+}-dependent protein phosphorylation and noradrenaline release from synaptic vesicles were lost following treatment of the vesicles with EDTA; Sulakhe and St. Louis [19] have reported analogous findings with EGTA.

Accordingly, the following hypothesis is offered to explain the experimental findings. Ca^{2+}, added to rat brain homogenates, binds to calmodulin, and the active moiety produced has the capacity to stimulate Na^+,K^+-ATPase activity. EDTA interferes with this association [19, 30, 31] and thereby prevents Na^+,K^+-ATPase stimulation.

The data presented are entirely consistent with the suggestion above. For example, while low concentrations of added Ca^{2+} cause stimulation of Na^+,K^+-ATPase, the addition of exogenous calmodulin along with Ca^{2+} leads to a greater stimulation of the enzyme. The stimulation seen in the absence of added calmodulin is presumably due to association between Ca^{2+} and endogenous calmodulin in view of the attenuation of the stimulation by the Ca/calmodulin binding inhibitor fluphenazine.

Based on the findings presented above, it is suggested that the stimulation by Ca^{2+} of Na^+,K^+-ATPase is a physiological phenomenon consequent upon the combination of Ca^{2+} with the calcium regulating protein calmodulin.

Acknowledgements—The generous support of the NH & MRC of Australia is gratefully acknowledged. Grateful thanks are also offered to Professor Philip Kuchel for his assistance with the calculations to determine the concentrations of metal–ligand complexes in the experimental media and to Drs Peter Dunkley, Keith Lyne-Smith and John Rostas for their time devoted to helpful discussion.

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