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Detection of a highly ouabain sensitive isoform of rat brainstem Na,K-ATPase

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The present work provides evidence for the existence in rat brainstem of a form of the Na,K-ATPase catalytic subunit that displays a high affinity for ouabain (K_d about 10^{-9} M). Its kinetic identification was made out from studies on dose response curves of ouabain inhibition of Na,K-ATPase activity, ouabain inhibition of Na^+-dependent phosphorylation from ATP and ouabain stabilized phosphoenzyme formation from inorganic phosphate (P_i). In all these studies this isoform comprises around 11 percent of the total Na,K-ATPase enzyme. The PAGE electrophoretic mobility of its phosphoprotein obtained from P_i in the presence of ouabain is lower than that of the alpha-1 form but it cannot be distinguished from that of alpha-2. Whether this highly ouabain sensitive form corresponds to the alpha-3 isoenzyme or represents the translational product of one of the additional genes described for the large catalytic subunit remains at the moment an open question.

Introduction

The heterogeneity of the alpha subunit of the Na,K-ATPase in many tissues and species is now well established (see Ref. 1 for references). The existence of two forms: alpha and alpha(+) (now 1 and 2, respectively) was first reported by Sweadner [2] on the basis of their differences in gel mobility and sensitivity to cardiotonic steroids. Cloning studies showed that these forms are the products of separate genes [3]; in addition, these studies predicted the existence of a third isozyme, termed alpha-3. Furthermore, the finding of other genes encoding the catalytic subunit raised the possibility of the existence of more isozymes for the Na,K-ATPase [4,5].

An alpha-3 like protein has been expressed from its cDNA in BALB/c 3T3 cells [6], whereas the presence of the alpha-3 isozyme has been recognized in axolemma membranes by means of polyclonal [7] and monoclonal antibodies [8,9]. Besides, there is evidence indicating that alpha-3 has a ouabain sensitivity similar to, or a slightly higher than, alpha-2 [6,9]. In a previous paper, on the basis of strophanthidin inhibition of ATP hydrolysis, we suggested the existence of at least three isoforms of the Na,K-ATPase in rat hippocampus, one of which had a K_d for the inhibitor around 10^{-9} M [10]. This coincides with a report of a third isoform in brain with a dissociation constant for ouabain about 10^{-8} M [11]. The present report is an attempt to its identification in rat brainstem by means of the ouabain inhibition of ATP hydrolysis and E-P formation from ATP and of the ouabain stimulation of enzyme phosphorylation from inorganic phosphate.

Materials and Methods

Microsomes were prepared from brainstem of 6-month-old rats according to the procedure described in Ref. 10. Partial purification of the enzyme was performed following the method of Jørgensen [12] with modifications: (i) the ratio protein/SDS was 2.5 : 0.8, and (ii) the enzyme fraction was collected at the 15%–25% interface of 10%, 15% and 25% (w/v) discontinuous sucrose gradients after centrifugation at 180 000 × *g* for 150 min. The usual specific activity was 4–6 μmol $P_i \cdot mg^{-1} \cdot min^{-1}$ which remained stable for months when stored at −70 °C in 25 mM imidazole (pH 7.5), 2 mM EDTA-Tris, 10% sucrose. Protein was determined by a modification [13] of the method of Lowry et al. [14].

ATPase activity was estimated from the P_i released according to Fiske and SubbaRow [15], using the amidol

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reagent, or from the release of [^{32}P]P_i from [gamma-^{32}P]ATP after 20 min incubation at 37 °C. The incubation solutions (0.5 ml or 1.0 ml final volume) contained 3 mM $MgCl_2$, 120 mM NaCl, 30 mM KCl, 30 mM imidazole (pH 7.5) and 3 mM ATP. At non-limiting ligand concentrations the ATP hydrolysis resistant to 1 mM ouabain was identical to the hydrolysis found with no ouabain in the absence of both Na^+ and K^+. To obtain the dose-response curve for ouabain, the enzyme was preincubated at 37 °C in the final reaction medium without the substrate in the presence of different concentrations of the inhibitor. The preincubation times depended on the ouabain concentration used: 20 or 180 min in the cases of 10^{-8} M or higher and 180 min below 10^{-8} M. The preincubation procedure did not affect the enzyme activity but allowed to attain, or be close enough to, the equilibrium state of the enzyme-inhibitor reaction at the lowest concentrations of the steroid (see Results). Reactions began with the addition of ATP. Similar preincubation procedures were followed in the experiments of phosphorylation from ATP and inorganic phosphate.

For ATP phosphorylation aliquots of 25 μg protein were incubated for 15 s at 0 °C in media containing 3 mM $MgCl_2$, 150 mM NaCl, 30 mM imidazole (pH 7.5), 10 μM [gamma-^{32}P]ATP and different ouabain concentrations in a final volume of 0.50 ml or 1.0 ml. The reaction was started by the addition of [gamma-^{32}P]ATP and terminated by the addition of 2.5 ml or 5.0 ml of an ice-cold solution containing 10% perchloric acid, 1 mM ATP and 10% P_i. The mixture was allowed to stand for 15 min at 0 °C and the denatured protein was collected and washed in Whatman fiber glass filters as indicated previously [16]. For phosphorylation from inorganic phosphate enzyme aliquots of 50 μg protein were used, the media contained 3 mM $MgCl_2$ and 150 mM Tris-HCl (pH 7.4 at 37 °C) and the temperature was 37 °C. The concentration of [^{32}P]P_i was 1 mM in a final volume of 1.0 ml. After 10 min phosphorylation, the samples were placed in an ice bath to slow the release of P_i from the ouabain-E-P complex. Once temperature equilibration was achieved (3 min) dephosphorylation was allowed to proceed for 2 s by chelating free Mg^{2+} with 20 mM EDTA-Tris; the reaction was then stopped with 3.5 ml of ice-cold solution containing 12% perchloric acid, 50 mM cold P_i, 10% polyphosphates and 20 mM pyrophosphate. After 15 min at 0 °C the denatured protein was either assayed for radioactivity or used for LDS gels. In the first instance the samples were collected and washed in glass fiber filters as described before; in these cases the radioactivity incorporated without ouabain was 1–2% of the maximum values obtained in the presence of 10^{-3} M ouabain and was subtracted from the samples containing different steroid concentrations. Before applied to the LDS gels, the other samples were centrifuged for 5 min at 9000 × g; the pellets were then rinsed with 0.5 ml of ice-cold 5% trichloroacetic acid and 50 mM P_i and dissolved in LDS-buffer.

LDS-polyacrylamide gel electrophoreses were run as described by Litchtner and Wolf [17]. Aliquots of 40–60 μg protein were applied on a 4.5% running gel, which had been prerun for 2 h at 15 mA (pH 2.4 at 5 °C). Runs were carried out at 30 mA, 5 °C and pH 2.4. Phosphorylase (M_r 94000) and bovine serum albumin (M_r 68000) were used as standard molecular weights. The gels were cut with a gel slicer and the radioactivity of the 2 mm slices was assayed in a scintillation counter using a Triton X-100 toluene-based scintillator. The scanned areas were measured with an area-reading attachment adapted to an Apple II computer. For autoradiography gels were dried onto filter paper and exposed to X-ray film at −70 °C overnight.

All solutions were made with bidistilled deionized water. NaCl and KCl were Baker Ultrex. ATP (vanadium free), ouabain, Tris and imidazole were purchased from Sigma Chemical Co., U.S.A. All other chemicals were reagent grade. Carrier-free [^{32}P]P_i, provided by the Comisión Nacional de Energía Atómica of Argentina, was purified following De Meis [18]. [Gamma-^{32}P]ATP was labelled according to Glynn and Chappel [19].

Duplicate or triplicate samples were run in each experiment. The symbols on the figures are the mean of two to four different experiments; in the cases they represent more than two, the S.E. values are included as vertical bars. Curve fitting was performed with a non-linear regression computing program using the chi-squared criterion (SCoPfit, National Biomedical Simulation Resource, Duke University Medical Center, U.S.A.). The usual fitting evaluation considered the eventualities of two or three isoforms As shown in the figures, in all cases the best fit was achieved by employing an equation which anticipated the existence of three enzymatic isoforms with different affinities for ouabain. When the enzyme concentration is high enough, as is the case for the phosphorylation experiments, ouabain binding reduces its free concentration in the solution and [total ouabain] > [free ouabain]. This becomes relevant in the case of the high ouabain affinity form (K_i about 10^{-9} M) for concentrations of the inhibitor around or below 10^{-9} M. This ouabain depletion was taken into account by replacing [total ouabain] with the expression leading to [free ouabain] obtained from the equation for fractional occupancy of binding sites by a ligand.

Results

Before obtaining the dose response curves for ouabain, it was necessary to asses what preincubation time was required in order that the binding of the

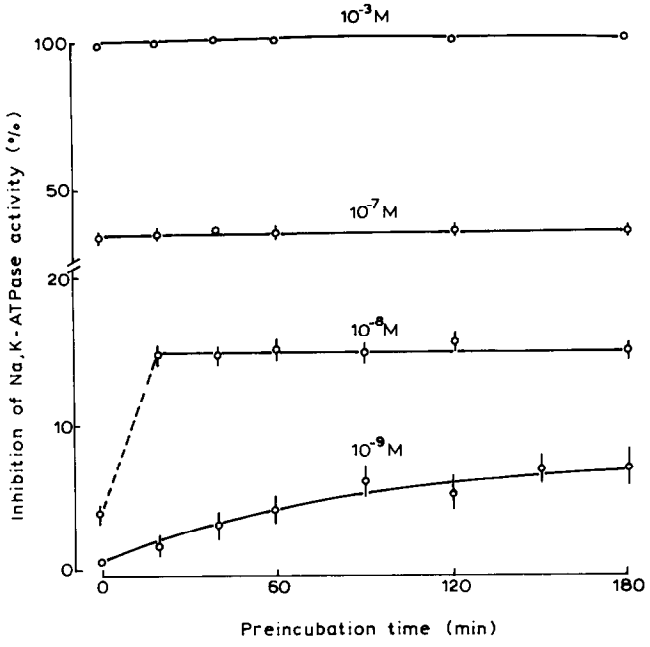

Fig. 1. Effect of preincubation time and ouabain concentration on the ouabain inhibition of rat brainstem Na,K-ATPase activity. Aliquots of 1 μg total protein were preincubated for different times (0 to 180 min) at 37° C in 0.5 ml of media containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4 at 37° C) and different ouabain concentrations. The reaction was started with the addition of ATP (final concentration 3 mM); the reaction time lasted 20 min at the same temperature. The results are expressed as the percentage of inhibition of the Na,K-ATPase activity observed in the absence of the inhibitor. Each point is the mean ± S.E. of a single experiment carried out in triplicate. The absence of vertical bars indicate that the S.E. value falls within the circle. Note: (i) at 10^{-7} M ouabain or above there is no detectable lag time for inhibition; (ii) at 10^{-8} M ouabain there is a lag time which is already over at 20 min; (iii) 150 min of preincubation time are more than enough to bring about all the inhibition due to 10^{-9} M ouabain; (iv) preincubation did not affect the Na,K-ATPase activity in the absence of ouabain (not shown).

inhibitor to the enzyme had reached equilibrium. This was particularly important for the lowest ouabain concentrations, where the pseudo-first-order rate constant for the on reaction would be expected to be rather low. We estimated it indirectly on the basis of the percentage of Na,K-ATPase inhibition as a function of the preincubation time with the steroid. That exposure took place at 37° C in the standard reaction mixture were the assays were carried out (see Methods) except for the absence of ATP. The results of one of these experiments are plotted in Fig. 1. Not included in the figure are the control activities in the absence of the inhibitor which remained unaffected. A ouabain concentration as low as 10^{-7} M developed its effect without any delay. With 10^{-8} M, although inhibition was lower without than with preincubation, the amount of inhibited activity remained constant from 20 min onwards. Finally, when 10^{-9} M ouabain was used a longer time lag existed; within the resolution of the method that lag could have been as short as 90 min but surely no more than 150 min. In view of this behavior, in all experiments described below the preincubation time was routinely 180 min for ouabain concentrations below 10^{-8} M and either 20 or 180 min for 10^{-8} M or higher.

The first indication of the presence of various forms of rat brain Na,K-ATPase is seen in the dose-response curve for ouabain inhibition of ATP hydrolysis under non-limiting ligand concentrations (Fig. 2) where all values are expressed as the percentage of activity in the absence of the inhibitor. The experimental points (a total of 54) are best fitted to an equation that assumes the existence of three (1.8 percent fitting error) rather than two (5.5 percent error) enzyme populations with different affinities for the inhibitor. An analysis of the data with the Snedecor's F test ($F = 129$, df = 50 and 48, $P < 0.01$) showed that the results were not due simply to an increased flexibility of the equation with a larger number of parameters. In addition, the best fit obtained with the three isoforms assumption is most conspicuous in the region of the lowest ouabain concentrations. Although not illustrated in the figure, the assumption of four isoforms did not increase the accuracy of the fitting. For three isoforms, the calculated ouabain-enzyme dissociation constants were $1.1 \cdot 10^{-4}$ M, $0.52 \cdot 10^{-6}$ M and $1.1 \cdot 10^{-9}$ M. The first two values agree with those reported for the alpha-1 and alpha-2 isoforms [1,2] whereas the third would represent an

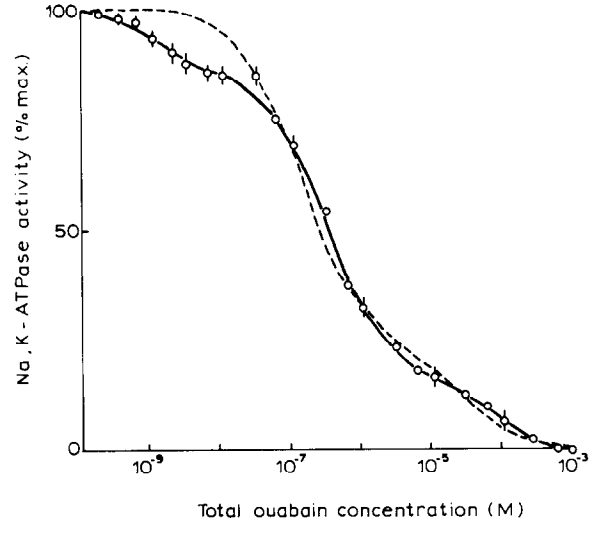

Fig. 2. Ouabain inhibition of rat brainstem Na,K-ATPase activity. Samples of 1 μg total protein were preincubated for 20 or 180 min (10^{-8} M ouabain or above) and 180 min (below 10^{-8} M ouabain) at 37° C. The reaction was started with the addition of ATP (final concentration 3 mM). Symbols represent the mean ± S.E. (vertical bars) of 2–4 different experiments. The curves are the best fits corresponding to heterogeneous enzyme populations of three (———) or two (— — —) isoforms with different affinities for ouabain. The fitting errors were 1.8 percent and 5.5 percent for three and two forms, respectively. The figures on the horizontal axis refer to total (free + bound) ouabain concentration. See Methods for details.

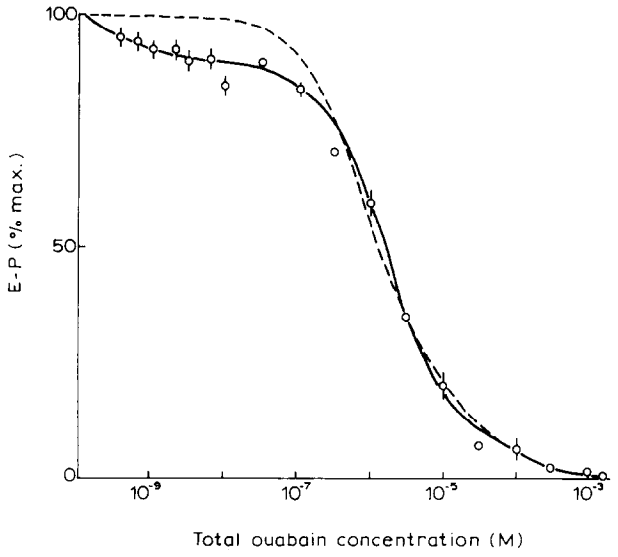

Fig. 3. Ouabain inhibition of phosphorylation of rat brainstem Na,K-ATPase from ATP. Aliquots of 25 μg total protein were preincubated for 20 or 180 min (10^{-8} M ouabain or above) and 180 min (below 10^{-8} M ouabain) at 37 °C. Phosphorylation was performed for 15 s at 0 °C starting the reaction with the addition of [γ-^{32}P]ATP (final concentration 10 μM). Symbols represent the mean ± S.E. (vertical bars) of 2–4 different experiments. The curves are the best fits corresponding to heterogeneous enzyme populations of three (———) or two (— — —) isoforms with different affinities for ouabain. The fitting errors were 3.0 percent and 7.2 percent for three and two forms, respectively. The figures on the horizontal axis refer to total (free + bound) ouabain concentration. For details see Methods.

additional highly ouabain sensitive form of the enzyme. Their estimated fractional concentrations were 17%, 69% and 14%, respectively.

The three Na,K-ATPase populations could be also exposed following the dose-response curve for ouabain inhibition of the Na^+-dependent steady-state phosphorylation from ATP (Fig. 3). From each value the phosphorylation obtained with $2 \cdot 10^{-3}$ M ouabain was subtracted and these results are given as a percentage of the maximal ouabain inhibitable phosphorylation. Again, a much better fitting of the experimental points (a total of 45) was achieved by employing a three (3.0 percent error) rather than a two (7.2 percent error) components model (Snedecor's F test: $F = 79$, df = 41 and 39, $P < 0.01$). Again, the difference between the two fits was more noticeable in the region below 10^{-8} M ouabain. For three populations, the estimated ouabain-enzyme dissociation constants in this case were $1.3 \cdot 10^{-4}$ M, $1.5 \cdot 10^{-6}$ M and $0.53 \cdot 10^{-9}$ M which corresponded to a fractional composition of 10%, 80% and 10%, respectively.

The two sets of experiments described so far are a kinetic demonstration of the existence of a high ouabain affinity isoform of the brainstem Na,K-ATPase. Any attempt to further characterize that form required to isolate it from the others. We intended to do that on the basis of their ouabain-stimulated E-P formation from inorganic phosphate. In preliminary experiments (not shown) we found that E-P formation with 1 mM P_i in the absence of Na^+, K^+ and ouabain was rather small in rat brain enzyme (about 5 percent of the maximal phosphorylation acquired with ATP); this phosphoenzyme was about 50 percent lost after 2–3 seconds at 0 °C following Mg^{2+} chelation with EDTA. On the other hand, in the presence of ouabain the P_i incorporation was higher, almost equal to the maximal obtained with ATP, and much more stable, with a half-time for breakdown near 50 s at 0 °C. This behavior justifies the procedure described in Methods: phosphorylate at 37 °C for 10 min, cool down the reaction mixture to 0 °C, chelate free Mg^{2+} with 20 mM EDTA and 2 s later stop the reaction with acid. In a first group of experiments we determined the dose-response curve for the ouabain-dependent E-P formation following the interaction of brainstem Na,K-ATPase with inorganic phosphate in the presence of Mg^{2+} ions. The results are shown in Fig. 4, which for all practical purposes is a mirror image of Figs. 2 and 3, where they are presented as percentage of the phosphorylation obtained with 10^{-3} M ouabain. Once more, the best fit of the data points (a total of 44) favored the existence of three (3.8% fitting error) rather than two (5.8% error) iso-

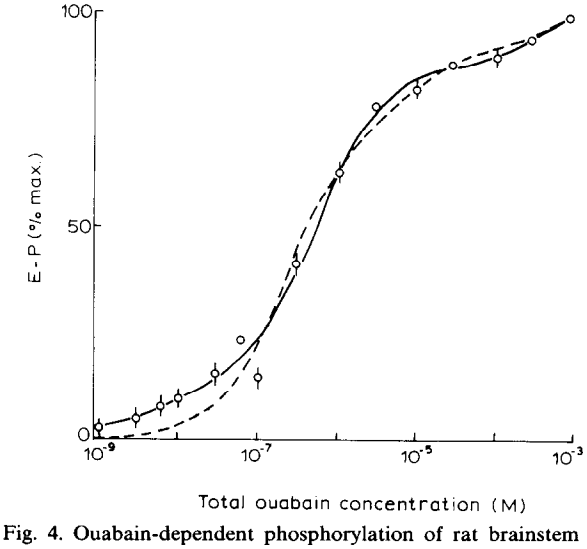

Fig. 4. Ouabain-dependent phosphorylation of rat brainstem Na,K-ATPase from inorganic phosphate. Aliquots of 50 μg total protein were preincubated for 20 or 180 min (10^{-8} M ouabain or above) and 180 min (below 10^{-8} M ouabain) at 37 °C in 1.0 ml of media. Phosphorylation was carried for 10 min at 37 °C. The reaction was started by addition of [^{32}P]P_i at a final concentration of 1 mM. Symbols represent the mean ± S.E. (vertical bars) of 2–4 different experiments. The curves are the best fits corresponding to heterogeneous enzyme populations of three (———) or two (— — —) isoforms with different affinities for ouabain. The fitting errors were 3.8 percent and 5.8 percent for three and two forms, respectively. The figures on the horizontal axis refer to total (free + bound) ouabain concentration. See Methods for more details.

forms (Snedecor's F test: $F = 23$, df = 40 and 38, $P < 0.01$). For three Na,K-ATPase populations, the computed ouabain-enzyme dissociation constants were $2.6 \cdot 10^{-4}$ M, $0.47 \cdot 10^{-6}$ M and $1.6 \cdot 10^{-9}$ M, and their respective amounts 16%, 74% and 10%, respectively).

Considering that the ouabain-dependent phosphorylation from P_i reflects only the presence of Na,K-ATPase, further experiments can be performed to actually isolate the high ouabain affinity isoenzyme once it has been phosphorylated. From the kinetic parameters obtained from Fig. 4, a ouabain concentration of 10^{-8} M should almost exclusively stimulate P_i labelling of the high-affinity isoform (with about 1.5 percent contamination with the intermediate-affinity form); on the other hand, 10^{-5} M ouabain would stimulate phosphorylation of both high- and intermediate-affinity enzymes and 10^{-3} M ouabain should lead to phosphorylation of all of them. Phosphorylation from [^{32}P]P_i was carried out without and with ouabain (at the concentrations given above) as described in Methods after 20 min preincubation time. This was followed by duplicate gel electrophoresis for autoradiography and slicing and counting. The results of one of such experiments are illustrated in Fig. 5. The main panel shows the areas of the gel under the peak of phosphoproteins normalized for equal amounts of protein (40 μg) in each slab; the inside panel is an autoradiogram of the duplicate gel. The first point to be noticed is that whereas there is no phosphorylation in the absence of ouabain a small but readily detectable peak appears with 10^{-8} M concentration of the inhibitor. Since the areas below each peak were normalized, the subtraction of the area corresponding to the lowest from that of the middle and the middle from that of the highest concentration of inhibitor would allow the estimation of the relative contribution of each form to the total area (that obtained with 10^{-3} M ouabain). The results average 11%, 66% and 23% for the high-, intermediate- and low-affinity forms; these values do not differ greatly from those obtained from the fitting of the dose-response curves for ouabain inhibition of ATPase activity and ATP-dependent phosphorylation and for ouabain stimulation of E-P formation from inorganic phosphate (Figs. 2, 3 and 4). Looking at the inside panel of Fig. 5 it is obvious that the diffuse nature of autoradiography of isotopes such as [^{32}P]P_i do not allow to resolve the phosphorylated regions into discrete bands. Nevertheless, two properties of the migration patterns can be observed: (i) whereas all brain bands begin at the same distance, the high- and the mixture of high- plus intermediate-affinity forms (B and C) stop shorter than that obtained with 10^{-3} M ouabain (D), where all isoforms are phosphorylated; (ii) the band corresponding to phosphoenzyme obtained with pig kidney Na,K-ATPase, which is known to con-

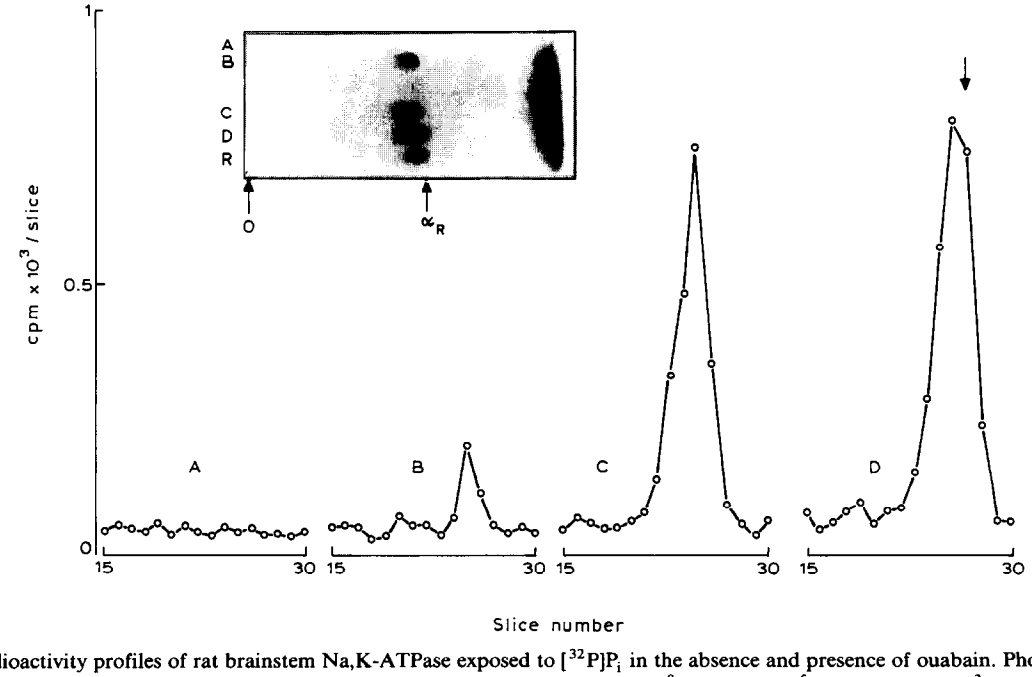

Fig. 5. PAGE radioactivity profiles of rat brainstem Na,K-ATPase exposed to [^{32}P]P_i in the absence and presence of ouabain. Phosphorylation was carried out as explained under Methods in the absence (A) and the presence of 10^{-8} M (B), 10^{-5} M (C) and 10^{-3} M (D) total ouabain concentration. After enzyme denaturation with acid, 40–60 μg of protein samples were subjected to LDS-polyacrylamide electrophoresis. Each gel was sliced in 2-mm sections which were counted in a liquid scintillation counter. The areas under the peaks of phosphorylation, normalized to 40 μg protein, are shown in the figure. Note that no phosphorylation was detected in the absence of ouabain. The inside panel is an autoradiogram of the phosphorylated intermediates obtained under the conditions indicated above. In this case the amounts of protein per sample were: A: 40 μg, B: 56 μg, C: 60.5 μg, D: 57.8 μg and R: (pig kidney Na,K-ATPase) 10 μg. The arrow points to the position of the phosphorylated intermediate obtained from pig kidney Na,K-ATPase in the presence of 10^{-3} M ouabain.

tain only the alpha-1 form (R) [1], begins after all brain phosphoenzyme mixtures but stops at the same distance as the brain E-P formed in the presence of 10^{-3} M ouabain.

Discussion

The data presented in this work indicate the existence of a functionally competent isoform of rat brainstem Na,K-ATPase extremely sensitive to ouabain. The dissociation constant of this high ouabain affinity form ranged from $0.53 \cdot 10^{-9}$ M to $1.6 \cdot 10^{-9}$ M. As a whole, and for all isoforms, there is not much difference between the K_i and fractional abundance values obtained with the three methods used (inhibition of ATP hydrolysis, inhibition of ATP phosphorylation and stabilization of P_i phosphorylation). Despite the different experimental conditions and the fact that exact K_d values for the less abundant sites are difficult to obtain by curve fitting, the results appear internally consistent. Thus, an average K_d of $1.08(\pm 0.31) \cdot 10^{-9}$ M for alpha-h is clearly distant from those observed for the alpha-2 ($0.83(\pm 0.34) \cdot 10^{-6}$ M) and alpha-1 ($1.67(\pm 0.47) \cdot 10^{-4}$ M) (see also Refs. 1 and 2). Actually, this feature served us to isolate the ouabain-stabilized phosphorylated form obtained from inorganic phosphate and to compare its PAGE mobility with those of the other isoform separated in the same way. The PAGE migration was similar to that of what is considered the alpha-2 subunit but slightly slower than that of the alpha-1 form, both from the same brain preparation and from pig kidney enzyme. The fractional abundance averaged 11.3 ± 1.3, 74.3 ± 3.2 and 14.3 ± 2.2 for alpha-h, alpha-2 and alpha-1, respectively. Similar values for the fractional amounts of alpha-h were obtained in the hippocampus of prenatal and adult rats on the basis of the ouabain inhibition of the Na^+,K^+-dependent ATP hydrolysis [10]. This suggests that this form is probably expressed at early stages of development and it is perhaps widely distributed in the nervous system (see also Ref. 8), although at present there is no information about its functional consequences, if there is any.

Another question that arises is whether this high ouabain affinity form represents the alpha-3 subunit of Na,K-ATPase expected from the cDNA studies of Shull et al. [3]. The available experimental information on the matter is conflicting. On the one hand, Urayama and Sweadner [9] reported the same ouabain affinity for the alpha-2 and alpha-3 isozymes (K_d about 10^{-7} M). However, in their experiments alpha-3 was isolated by trypsin inactivation of a pool of isoenzymes (alpha-3 was the most resistant); therefore the possibility that trypsin might have modified the ouabain affinity without largely affecting the catalytic power of the enzyme cannot be ruled out. On the other hand, Hara et al. [6] obtained a K_d of about $8 \cdot 10^{-8}$ M for an alpha-3 expressed in vitro, while Schwartz et al. [11] reported a K_d of 10^{-8} M for a third Na,K-ATPase isoform in brain. Furthermore, Lowndes et al. [20] have identified a high-affinity Na,K-ATPase subunit in brain microsomal membranes by using glycoside photolabels at concentrations as low as 1 nM. The similar electrophoretic mobility of the high- and moderate-ouabain affinity forms we described here would concur with the findings of Schneider et al. [21] who stated that alpha-3 protein synthesized in vitro has the same gel mobility as alpha-2; however, labelling the Na,K-ATPase isoforms with monoclonal antibodies, Urayama et al. [8] detected that the alpha-3 subunit has a slightly lower electrophoretic mobility than alpha-2. An interesting alternative regarding our findings, is that the third isoform we have encountered does not represent alpha-3 but it is the translational product (alpha-4?) encoded by one of the additional genes that have recently been described for the catalytic subunit of Na,K-ATPase [4,5].

Acknowledgements

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