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Kinetics of Na^+, K^+-ATPase Inhibition by an Endogenous Modulator (II-A)*

A. Reinés,[1] C. Peña,[2] and G. Rodríguez de Lores Arnaiz[1,3]

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We have previously reported the isolation by gel filtration and anionic exchange HPLC of two brain Na^+, K^+-ATPase inhibitors, II-A and II-E, and kinetics of enzyme interaction with the latter. In the present study we evaluated the kinetics of synaptosomal membrane Na^+, K^+-ATPase with II-A and found that inhibitory activity was independent of ATP (2–8 mM), Na^+ (3.1–100 mM), or K^+ (2.5–40 mM) concentration. Hanes-Woolf plots showed that II-A decreases V_{max} in all cases; K_M value decreased for ATP but remained unaltered for Na^+ and K^+, indicating respectively uncompetitive and noncompetitive interaction. However, II-A became a stimulator at 0.3 mM K^+ concentration. It is postulated that brain endogenous factor II-A may behave as a sodium pump modulator at the synaptic region, an action which depends on K^+ concentration.

KEY WORDS: Synaptosomal membranes; Na^+, K^+-ATPase; enzyme inhibitor; endogenous modulator; brain soluble factor.

INTRODUCTION

It is well known that an adequate balance between Na^+ and K^+ concentration is essential to propagate nervous impulse, to prevent osmotic cell rupture and to maintain cation homeostasis. In the maintenance of Na^+ and K^+ gradients, the participation of Na^+, K^+-ATPase is required (1–4). This enzyme is highly concentrated in nerve ending membranes (5) where it plays an essential role in neurotransmission. Therefore, regulatory mechanisms controlling the Na^+, K^+-ATPase activity are of great interest.

Previous work from our laboratory has shown that filtration of the brain soluble fraction through a Sephadex G-50 column led to the isolation of two fractions, peaks I and II, which behave respectively as a stimulator and an inhibitor of Na^+, K^+-ATPase activity (6). Peak II exerts ouabain-like properties, inhibiting the activity of Na^+, K^+-ATPase (but failing to affect other synaptosomal membrane enzymes), favouring neurotransmitter release, blocking ^{3}H-ouabain binding and inducing diuresis and natriuresis (see 7). Together with kinetic studies, these findings led us to propose the term *endobain* (8).

In order to achieve partial purification and characterization of the endogenous inhibitor(s), peak II was fractionated through anionic exchange HPLC performed in a Synchropak column, allowing the isolation of eight fractions (II-A to II-H), two exerting inhibitory activity (II-A and II-E) (9). II-E fraction is an anionic factor, neither peptidic nor lipidic in nature, while II-A fraction presents properties indicative of a peptidic molecule (9).

II-E fraction behaves as an uncompetitive Na^+, K^+-ATPase inhibitor within a wide Na^+, K^+ and ATP

[1] Instituto de Biología Celular y Neurociencias "Prof. Eduardo De Robertis", PROBICENE-CONICET, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121-Buenos Aires, Argentina.

[2] IQUIFIB, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113-Buenos Aires, Argentina.

[3] Address reprint requests to: Dra. Georgina Rodríguez de Lores Arnaiz, Instituto de Biología Celular y Neurociencias "Prof. Eduardo De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, 1121-Buenos Aires, Argentina. Phone: 54-11-4508-3675; FAX: 54-11-4508-3645; E-mail: grodrig@ffyb.uba.ar

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concentration range, but as an enzyme stimulator at suboptimal ATP concentration (10).

In the present study we determined synaptosomal membrane Na^+, K^+-ATPase activity in the presence of II-A at variable ATP, Na^+ and K^+ concentrations in order to elucidate the nature of the interaction between such factor with the enzyme. II-A proved to be an enzyme inhibitor over a wide concentration range of ATP and Na^+, and K^+ above 2.5 mM, but a stimulator at 0.3 mM K^+ concentration.

EXPERIMENTAL PROCEDURE

Animals and Drugs. Adult male Wistar rats weighing 100–150 g were employed. Reagents were analytical grade. Ouabain, disodium ATP (grade I, prepared by phosphorylation of adenosine) and Sephadex G-10 were from Sigma Chemical Co., St Louis, MO, USA; Sephadex G-50 (fine grade) was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Preparation of Synaptosomal Membranes. Synaptosomal membranes were prepared following the method developed in our laboratory (5). Accordingly, for each preparation, cerebral cortices from five rats were pooled and homogenized at 10% (w/v, original tissue) in 0.32 M sucrose (neutralized to pH 7.0 with Tris base) using a Teflon glass homogenizer of the Potter-Elvehjem type. The homogenate was processed by differential centrifugation to separate nuclear and mitochondrial fractions. The crude mitochondrial pellet was resuspended at 10% (w/v) in water (pH 7.0 with Tris base) and homogenized during 2 min for osmotic shock. A pellet containing mitochondria, nerve ending membranes and myelin was separated by centrifugation at 20,000 *g* for 30 min, then resuspended in 0.32 M sucrose and layered on top of a gradient containing 0.8, 0.9, 1.0 and 1.2 M sucrose. The gradient was centrifuged at 50,000 *g* for 2 h in a SW 28 rotor of an L8 Beckman ultracentrifuge; the fraction at 1.0 M sucrose level was separated, diluted with 0.16 M sucrose to reach 0.32 M sucrose and spun down at 100,000 *g* for 30 min.

The pellet was stored at –70°C and, prior to enzyme assay, resuspended by brief homogenization in bidistilled water to reach a final concentration of 9–11 mg protein per ml, stored frozen and used for three weeks without appreciable change in enzyme activity.

Preparation of II-A Fraction. Peak I and II fractions from rat cerebral cortex were prepared as previously described (6, 9). Thus, for each preparation, cerebral cortices from five rats were pooled, homogenized at 25% (w/v) in bidistilled water, and centrifuged at 100,000 *g* for 30 min in a 70.1 rotor of an L8 Beckman ultracentrifuge. A 5-ml supernatant sample (brain soluble fraction) was taken to pH 7.4 with 100 mM NH_4HCO_3 and loaded on a Sephadex G-10 column (1 × 20 cm); the eluate from 8 to 20 ml was collected and then applied to a column packed with Sephadex G-50 (1.8 × 25 cm). For gel equilibration and elution, 10 mM NH_4HCO_3 was used. Fractions of 1.4 ml each at a flow rate of 0.3 ml per min were collected in an LKB Ultrorac Fraction Collector 7000. The absorbance profile was recorded at 280 nm.

Peaks I and II were made up with the fractions presenting maximal UV absorbance. Peak I was made up with fractions 19–23 but not used in this study; peak II was made up with fractions 48–52. Peak II was taken to pH 2.0 with 2 N HCl, lyophilized and stored at –20°C overnight; peak II samples, each equivalent to 0.6 g fresh cerebral cortex were processed by anionic exchange HPLC performed in a Synchropak AX-300 column, 250 × 4.6 mm (Synchrom Inc., Lafayette, IN, USA), and eluted at a flow rate of 0.5 ml per min with a 20-min gradient from 1 to 10 mM NH_4HCO_3 to separate fractions II-A to II-H. Fractions were collected by monitoring absorbance at 230 nm; II-A was collected at 5–7 min, then lyophilized and stored at –20°C. The whole preparation procedure lasted 2 days after tissue harvesting.

II-A Sample Preparation for Enzyme Assays. Lyophilized II-A samples were dissolved in 0.006 N HCl at a concentration of 60 mg original tissue per µl, neutralized with Tris base (which reached 0.02 M final concentration) and assayed immediately after for their effect on Na^+, K^+-ATPase activity.

Effect of Variable Preincubation Time. Before performing ATPase assay, samples of synaptosomal membranes were preincubated with 0.2 M Tris-HCl buffer (pH 7.4) and II-A at 37°C for 3, 5, 10 and 15 min prior to Na^+, K^+-ATPase assay.

Enzyme Assays. ATPase activity was measured as described by Albers *et al.* (11). Total ATPase activity was assayed in a medium containing 100 mM NaCl, 20 mM KCl, 3 mM $MgCl_2$, 0.16 M Tris-HCl (pH 7.4) and 4 mM ATP, unless otherwise stated. Mg^{2+}-ATPase activity was determined in a similar medium with 3 mM $MgCl_2$, 0.16 M Tris-HCl (pH 7.4) and 4 mM ATP, but with no Na^+ and K^+ added, and containing 1 mM ouabain. The difference between activities was taken to correspond to Na^+, K^+-ATPase. In kinetic studies, the medium contained variable concentrations of ATP (2.0–8.0 mM ATP), or Na^+ (3.1—100 mM NaCl), or K^+ (2.5–40 mM KCl); when indicated, a low K^+ concentration (0.3 mM KCl) was also employed.

Before performing ATPase assay, samples of synaptosomal membranes were preincubated with 0.2 M Tris-HCl buffer (pH 7.4) and II-A at 37°C for 10 min (unless otherwise stated). Incubation volume (µl) was 20:5:15 for buffer:membranes:II-A fraction. Samples of the preincubated fractions (3 µl) were distributed in two series of microtubes containing 40 µl of medium for the assay of total- and Mg^{2+}-ATPase activities, and incubated at 37°C for 30 min or 20 min (kinetics). II-A concentration during enzyme assay was 3 mg original tissue per µl.

The reaction was stopped with 30% trichloroacetic acid solution. ATPase activity was monitored by colorimetric determination of released orthophosphate (12). In all enzyme assays, tubes containing enzyme preparations and assay media maintained at 0°C throughout the incubation period were used as blanks.

Protein Content Determination. Protein concentration in synaptosomal membrane samples was determined by the method of Lowry *et al.* (13) using bovine serum albumin as standard.

Expression of Results. Results are expressed as µmol P_i released per mg protein per hour or as percentage enzyme activity taking as 100% values obtained in the absence of II-A fraction. Results were taken from a single experiment chosen to illustrate findings in a series of 3–7 runs or are means ± SD from 3–12 experiments. Statistical analysis was performed using the two-tailed Student's *t*-test for nonpaired observations.

RESULTS

Absolute values for Na^+, K^+- and Mg^{2+}-ATPase activities in synaptosomal membranes was 40.9 ± 10.3 and 14.9 ± 3.2 µmol P_i released per mg protein per hour, respectively (means ± SD, n = 10). Since preliminary experiments indicated that II-A inhibitory activ-

ity quickly disappears over time, aging of this fraction was evaluated. At different times of its preparation, lyophilized II-A samples were dissolved in 0.006 N HCl (pH 2) and neutralized with Tris. At 2–3 days of tissue harvesting, II-A fraction inhibited 37% but had no effect as from 4 days. II-A failed to modify Mg^{2+}-dependent ATPase activity in either case (Fig. 1), in agreement with previous findings (9).

In order to determine whether the inhibitory effect of II-A fraction changes with preincubation time, membrane samples plus II-A were preincubated for 3, 5, 10 and 15 min. It was observed that the inhibition of Na^+, K^+-ATPase activity became evident after 5 min preincubation, and leveled off thereafter (data not shown). Thus, a 10 min preincubation time was employed for further enzyme assays.

As a function of ATP concentration enzyme activity was inhibited by II-A over the 2–8 mM range (Fig. 2). In order to study the kinetics of Na^+, K^+-ATPase inhibition by II-A, results were plotted according to Hanes-Woolf. It was observed that the slope of the plot [S] / v *versus* [S] was increased by II-A and that the lines crossed quite near the vertical axis at 0 mM ATP (Fig. 2, inset).

In the presence of variable Na^+ concentrations, the extent of enzyme inhibition remained unaltered over the 3.1–100 mM range (Fig. 3). The evaluation of

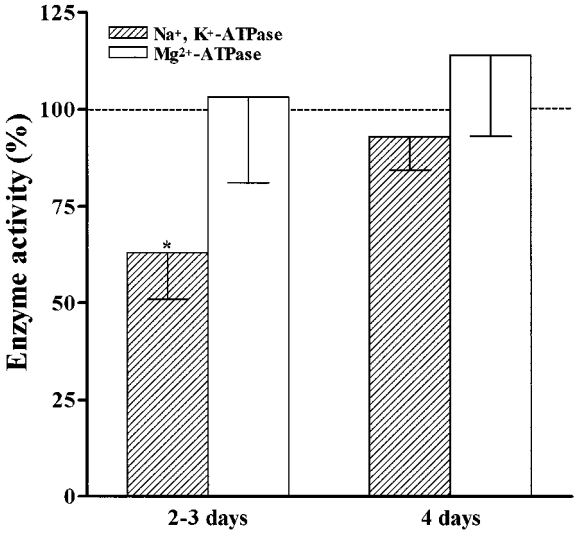

Fig. 1. Synaptosomal membrane Na^+, K^+-ATPase activity in the presence of II-A fraction at 2–3 and 4 days after tissue harvesting. During enzyme assay, II-A concentration was 3 mg original tissue per µl. Results are expressed as percentage enzyme activity taking as 100% values obtained in the absence of II-A. Data are means ± SD from 3–5 experiments.
* $P < 0.05$ with respect to data obtained in the absence of II-A (Student's-*t*-test).

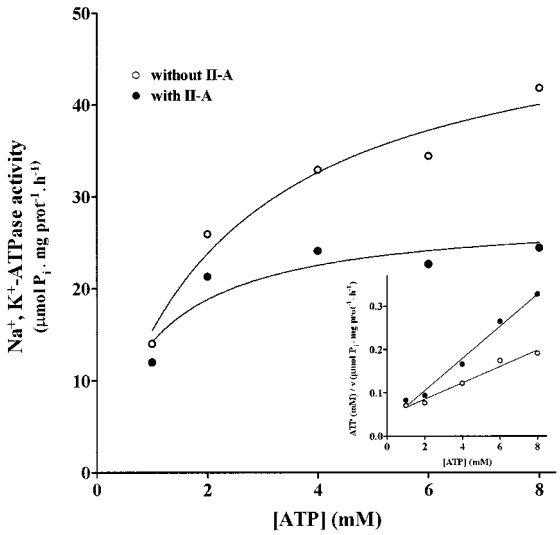

Fig. 2. Na^+, K^+-ATPase activity of synaptosomal membranes as a function of ATP concentration in the absence and presence of II-A fraction. Results of a single experiment are presented as µmole P_i released per mg protein per hour and as Hanes-Woolf plot (inset).

Hanes-Woolf plot indicated an increase in the slope by II-A and that the lines intercepted horizontal axis at the same point (Fig. 3, inset).

At variable K^+ concentration II-A inhibited enzyme activity over the 2.5–40 mM range (Fig. 4). In this case, Hanes-Woolf plot showed increased slope by II-A and the lines intercepted horizontal axis at the same point (Fig. 4, inset).

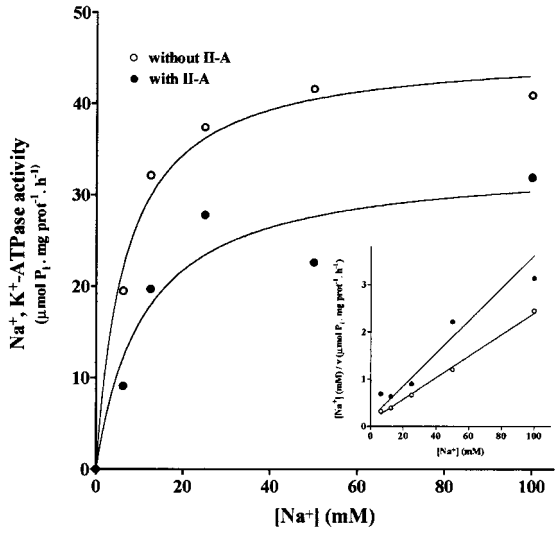

Fig. 3. Na^+, K^+-ATPase activity of synaptosomal membranes as a function of Na^+ concentration in the absence and presence of II-A fraction. Results of a single experiment are presented as µmol P_i released per mg protein per hour and as Hanes-Woolf plot (inset).

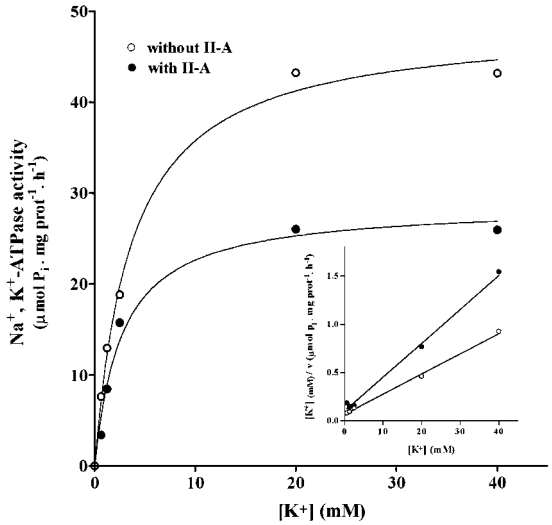

Fig. 4. Na^+, K^+-ATPase activity of synaptosomal membranes as a function of K^+ concentration in the absence and presence of II-A fraction. Results of a single experiment are presented as μmol P_i released per mg protein per hour and as Hanes-Woolf plot (inset).

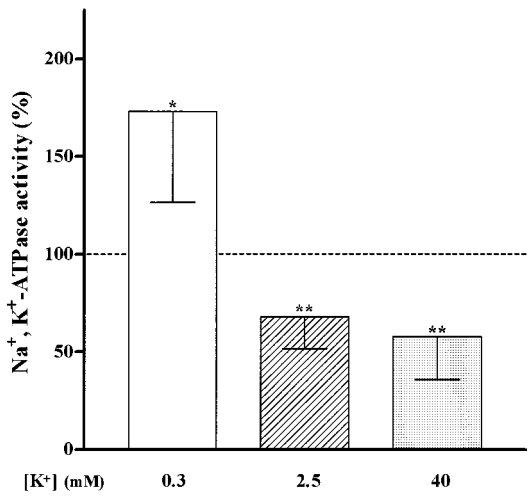

Fig. 5. The effect of K^+ concentration on Na^+, K^+-ATPase modulation by II-A. Results are expressed as percentage enzyme activity with respect to control values recorded in the absence of II-A. Data are means ± SD from 3–12 experiments.
* $P < 0.05$; ** $P < 0.01$ with respect to data obtained in the absence of II-A (Student's-*t*-test).

The inhibitor decreased V_{max} values from 48 and 45 to 25 and 33 μmol per mg protein per h *versus* ATP and Na^+, respectively; K_M value was reduced or unchanged by II-A *versus* ATP and Na^+, respectively.

Within the 2.5–40 mM K^+ concentration range, II-A decreased V_{max} value from 48 to 27 μmol per mg protein per h but failed to alter K_M value.

These results indicated that II-A shows uncompetitive inhibition with respect to ATP but noncompetitive inhibition *versus* Na^+ and K^+ (within the 2.5–40 mM concentration range). However, II-A became a stimulator at 0.3 mM K^+ (Fig. 5, first column), thus contrasting with inhibitory effect shown with 2.5 and 40 mM as illustrated in Fig. 5.

DISCUSSION

In previous studies we described the isolation by gel filtration of a brain soluble fraction (peak II) which highly inhibits synaptosomal membrane Na^+, K^+-ATPase activity (6).

Regarding central nervous system Na^+, K^+-ATPase isoforms, it is known that α3 and α1 respectively predominate in neurons and glial cells (14). In several brain areas peak II blocks high affinity ^{3}H-ouabain binding, corresponding to α3 enzyme isoform (15). On the other hand, peak II does not alter kidney Na^+, K^+-ATPase (16) where α1 isoform predominates (17). Taken jointly, this indicates that in our studies we are mainly dealing with the α3 isoform and felt justified in disregarding the weight of the Na^+, K^+-ATPase low affinity isoform (α1). Given the interest to identify a specific neuronal Na^+, K^+-ATPase modulator, by resorting to the biological properties of peak II, we focused on such brain fraction. Thus, further purification of peak II by anionic exchange HPLC led to the isolation of two inhibitory factors, II-A and II-E (9).

We observed that a 10 min preincubation period of II-A with the enzyme is required for its optimal inhibitory effect, a finding attributable to the fraction's hydrophilic nature (9) requiring longer interaction time with the enzyme. This suggests that an optimal period seems necessary to observe II-A effect, though some oxidative and / or proteolytic processes might also be involved.

Kinetics of the interaction between II-A and synaptosomal membrane Na^+, K^+-ATPase was characterized in this study. Substrate and cofactors concentration curves indicated that II-A decreases V_{max} in all cases and K_M value only *versus* ATP, behaving as an uncompetitive inhibitor *versus* this substrate but as a noncompetitive inhibitor *versus* Na^+ and K^+ (the latter within the 2.5–40 mM concentration range). Interestingly, in the presence of 0.3 mM K^+, II-A increased Na^+, K^+-ATPase activity.

Na^+, K^+-ATPase has been recognized as the receptor for cardiac glycosides and several attempts have been made to disclose the endogenous regulators of Na^+, K^+-ATPase activity. Thus, diverse inhibitory factors have been purified from several tissues as well as from biological fluids (7, 18). Some of those factors are chemically and chromatographically identical or very similar to ouabain (19–23). Moreover, other endogenous inhibitors such as fatty acids and hydrocarbons (24) or bufodienolides (25) have also been isolated.

Although II-A fraction inhibits Na^+, K^+-ATPase activity like cardiac glycosides and behaves similar to ouabain in TLC (9), their mechanisms seem to differ according to preincubation period requirement, kinetics and HPLC behaviour. Membrane preincubation with the inhibitor was necessary for II-A fraction (data not shown) whereas it has no effect for ouabain action (26) on Na^+, K^+-ATPase activity. Whereas inhibitory potency of II-A was unchanged by increasing K^+ concentration, ouabain inhibition is competitive *versus* K^+ (27). Moreover, II-A HPLC behaviour is quite dissimilar to that of ouabain since the former is not retained in a C-18 column, indicating its hydrophilic nature, whereas ouabain presents a 26-min retention time in reverse phase HPLC (28).

Initially, we studied the kinetics of Na^+, K^+-ATPase inhibition by peak II (8), followed by evaluation of the more purified fractions II-E (10) and II-A (present findings). Synaptosomal membrane Na^+, K^+-ATPase inhibition in no case proved competitive either with II-E (10) or II-A. However, enzyme interaction with II-A differs from that recorded for II-E since this factor exerted inhibitory activity regardless of Na^+ and K^+ concentration or ATP within the 2–8 mM range but became a stimulator in the presence of low ATP concentration (10). Present results indicated a dissimilar response of Na^+, K^+-ATPase to II-A according to K^+ concentration present during enzyme assay. Although II-A behaved as an inhibitor in the presence of 2.5–40 mM K^+, remarkably enough it acted as an enzyme stimulator at 0.3 mM K^+. A further difference between these two factors is their stability at −20°C. Whereas II-E potency is detectable up to 20 days (26), II-A inhibitory activity was lost within 4 days after tissue harvesting.

We have previously reported that peak II impairs Na^+, K^+-ATPase stimulation by K^+ (8). As described for endobain E (10), the inhibitory potency of II-A against Na^+, K^+-ATPase activity was not reverted by increasing K^+ concentration, so that all three fractions differ from cardiac glycosides (27, 29–31), from a factor purified from urine (32) and from an endogenous digitalis-like factor obtained from newborn plasma (33).

Present results show that K^+ concentration conditions II-A effect on enzyme activity. At low K^+ concentration, II-A is not only unable to inhibit the enzyme but becomes an enzyme stimulator, suggesting a biphasic action.

The measurement of endogenous digitalis-like factors (EDLF) in plasma or tissue is complicated by potential nonspecific interference resulting from ions such as vanadate or K^+, among others (18). Since II-A is highly hydrophilic, the possibility that any contaminant cation is present should be taken into account. Vanadium salts may be ruled out as in previous studies we described the absence of vanadium in the original peak II (8), the source for II-A. Since II-A is unstable over time, its effect is hardly attributable to any cation, i.e. K^+ itself, even though present in II-A.

Diverse changes in Na^+, K^+-ATPase abundance either due to induced in vivo hypokalemia (34–36) or to in vitro exposure to 0.25 mM K^+ concentration (35) have been reported. Interestingly, such K^+ concentration is similar to that (0.3 mM) which led to enzyme stimulation by II-A, thus differing from those which induce Na^+, K^+-ATPase inhibition (2.5−40 mM). It may be postulated that K^+ concentration changes in the nerve ending membrane microenviroment determine differential effects of II-A on sodium pump functioning.

Endogenous Na^+, K^+-ATPase modulators may be relevant to normal physiology in the control and regulation of Na^+/K^+ gradients, as well as in pathophysiological states, such as hypertension (37, 38) and myocardial ischemia (25,39). Several problems were encountered during EDLF extraction and purification, including a loss of EDLF related to its unstability during isolation and artifactual interference by purification products. Starting materials are accumulatively stored for several months before processing. However, the stability of EDLF has not been thoroughly examined, so that storing or processing could have reduced the amount of EDLF originally present in vivo and/or generated unreliable results (18). We observed that II-A is highly unstable over time since it became inactive after 4 days of tissue harvesting. Thus, in our case, an extra step would be required to avoid II-A degradation during processing to achieve its further purification.

In summary, present findings indicate that II-A invariably inhibits Na^+, K^+-ATPase activity at several Na^+ and ATP concentrations and that K^+ levels determine the type of effect on the enzyme. Dual behavior

of II-A (according to K^+ concentration) may suggest a critical role for this endogenous factor as a sodium pump modulator at the synaptic region.

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