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CATECHOLAMINES AND THE BRAIN MICROSOMAL Na, K-ADENOSINETRIPHOSPHATASE—II. THE MECHANISM OF ACTION

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Abstract—In the presence of ATP and an endogenous inhibitory system, presumably ascorbic acid and Fe^{2+} cation, the brain microsomal Na, K-ATPase is slowly inhibited. The inhibition rate is diminished in the presence of catecholamines and EDTA. This effect is not mediated by any specific adrenergic receptor. At present, the slow type of inhibition appears to be best explained by the lipoperoxidative degradation of the unsaturated phospholipid component of Na, K-ATPase. In the absence of ATP, the fast type of inhibition of Na, K-ATPase occurs. The inhibition could also be restrained by catecholamines but it is not directly related to the lipoperoxidation. The underlying mechanism is largely unknown but could be partially explained by divalent metal inhibition of Na, K-ATPase.

Evidence has been presented [1, 2] that the brain microsomal Na, K-ATPase is readily inactivated by an endogenous inhibitory system comprised of the membrane-bound iron and ascorbic acid. Addition of adrenaline and/or EDTA blocked this inhibitory system. It was also found [3] that the Na, K-ATPase inhibition closely correlated with the induction of lipoperoxidation of the brain subcellular fraction and that the adrenaline and EDTA inhibited the peroxidation and thus protected the Na, K-ATPase activity.

Therefore, the mechanism of Na, K-ATPase inhibition was explained in terms of peroxidative degradation of unsaturated phospholipids essential for the Na, K-ATPase activity. The effect of adrenaline and EDTA was related to the properties of these agents to bind free Fe^{2+} cations [4], the ortho-catechol structure of the catecholamine molecule being responsible for this effect.

The present study was aimed at:

(a) the effect of cyclic AMP and various alfa or beta adrenergic blockers or agonists which should reveal whether the catecholamine effect on the Na, K-ATPase is mediated by a specific adrenergic receptor; and

(b) the time-dependency studies of lipoperoxidation and the Na, K-ATPase inhibition that should define the interrelationship of both processes from the kinetic point of view.

MATERIALS AND METHODS

Preparation of the microsomal fractions from the rat cerebral cortex. White rats of the Wistar strain (140–220 g) were killed by decapitation and the cerebral cortex was separated from the white matter. The 10% homogenate in 0.32 M sucrose, 0.05 M Tris–HCl, pH 7.4 was prepared using a Elvehjm–Potter homogeniser (teflon–glass, clearance 0.03 mm, 2000 rpm). The brain subcellular fractions were prepared similarly as described by De Robertis *et al.* [5, 6]. The 10% homogenate was centrifuged first for 10 min at 900 *g* followed by centrifugation for 20 min at 12,000 *g*. The microsomal membrane particles were sedimented from the 12,000 *g* supernatant by centrifugation for 60 min at 100,000 *g*. The resulting 100,000 *g* sediments were divided in the two identical portions (based on the same volume of supernatant 12,000 *g* and the content of protein). One portion of the 100,000 *g* sediment was suspended with rehomogenization in the 0.32 M sucrose ('microsomes'), the second portion was suspended in the 100,000 *g* supernatant ('microsomes plus cytosol'). The same volume of 0.32 M sucrose or cytosol fraction (supernatant 100,000 *g*) were used. Thus, the microsomes and microsomal plus cytosol fractions contained the same amount of the membrane protein.

Measurement of enzyme activities. The activity of the sodium plus potassium activated, magnesium dependent adenosinetriphosphatase (Na, K-ATPase) was determined as inorganic phosphorus production as described in details in the preceding paper [3]. The microsomal fractions (0.03–0.05 mg membrane protein per ml) were preincubated in 2 ml of medium A (100 mM NaCl, 20 mM KCl, 100 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$) and medium B (120 mM NaCl, 100 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$ 2×10^{-4} M ouabain). The enzyme reaction was started by addition of ATP (final concentration 2.5 mM), continued for 15 min at 37° and terminated by addition of 0.5 ml 1M $HClO_4$. The precipitated protein was removed by centrifugation and the inorganic phosphate produced in the sample was assayed according to Taussky and Shorr [7]. All chemicals tested (cAMP, adrenergic blocking agents, catecholamines) were present in the incubation media during the assay of the enzyme activities. The Na, K-ATPase activity was expressed as the inorganic phosphate produced per hour per mg of the membrane protein in medium A minus medium B.

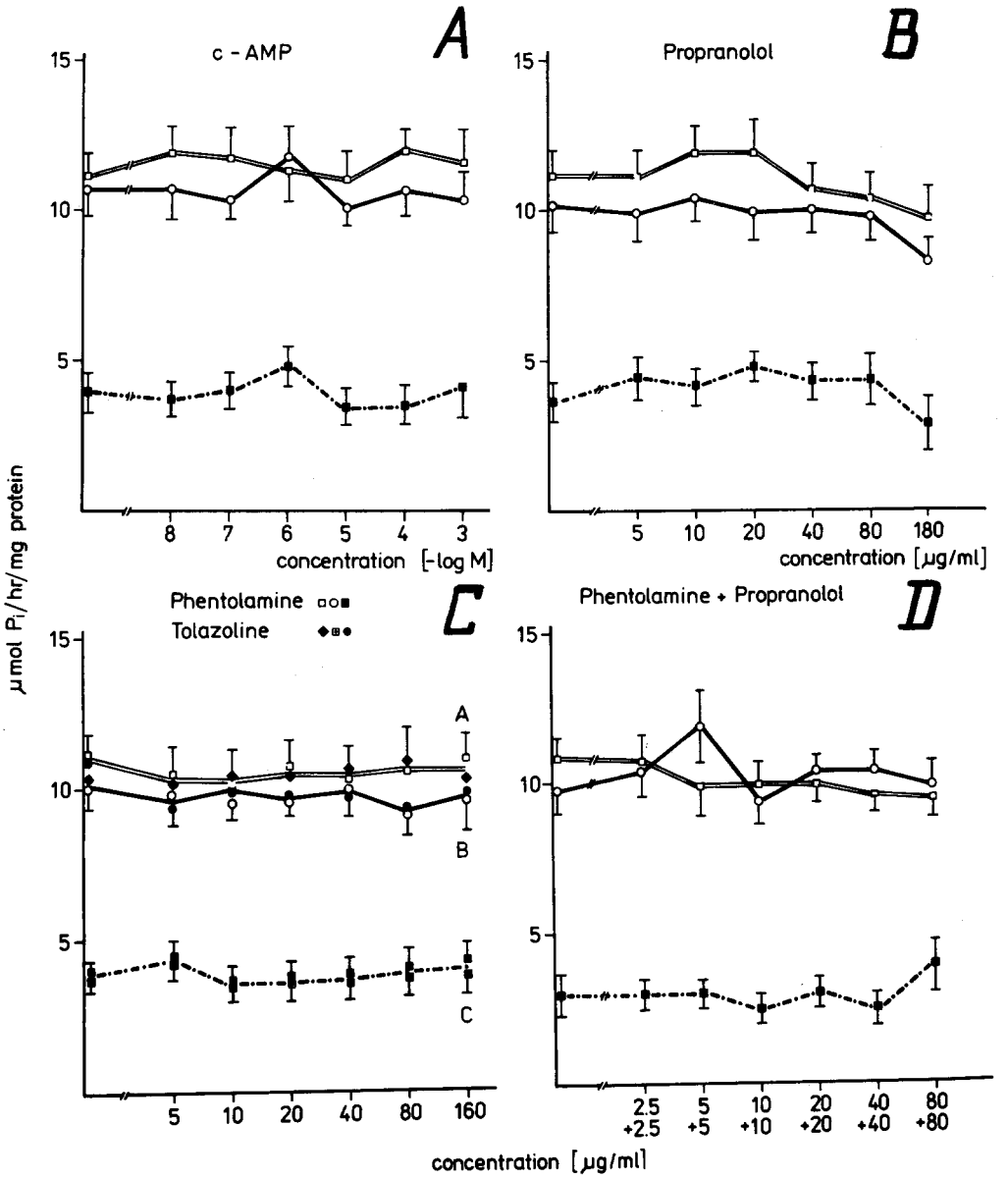

Fig. 1. The effect of cyclic adenosine monophosphate (cAMP) and of adrenergic blocking agents on the brain microsomal Na, K-ATPase activity. The microsomes or microsomes plus cytosol fraction were preincubated with increasing concentrations of cAMP (panel A), propranolol (panel B), phentolamine or tolazoline (panel C), propranolol plus phentolamine (panel D) and the Na, K-ATPase activities were determined as described in the Methods. The effect of the above mentioned substances was measured using microsomes (□——□) of microsomes plus cytosol fraction. The microsomes plus cytosol fraction was preincubated, in addition to the above substances, either with (○——○) or without (■——■) 5.4×10^{-5} M adrenaline.

In the case of panel C, where the effect of phentolamine and tolazoline is demonstrated, diffferent symbols were used for tolazoline: (◆——◆) microsomes; (●——●) microsomes plus cytosol fraction with 5.4×10^{-5} M adrenaline; (□——□) microsomes plus cytosol fraction.

The ouabain independent Mg-ATPase was defined at the inorganic phosphate produced per hour per mg of the membrane protein.

Measurement of lipid peroxidation. Lipid peroxidation was measured using the thiobarbituric acid colour reaction [14]. The microsomal fractions were incubated at 37° in 0.2 M phosphate buffer pH 5.9 or in the 'ATPase reaction medium A' either with of without 2.5 mM ATP. A 1 ml aliquot of the incubation mixture was added to 1 ml of 10% trichloracetic acid and the colour reaction was developed after treating the solution with the thiobarbituric acid reagent and boiling the sample for 10 min. The protein precipitate was centrifuged and the colour was read at 530 nm. The amount of malonyldialdehyde formed was determined using $e_{530} = 1.56 \times 10^5$ cm/nmole. The lipoperoxidative capacity of the brain subcellular fractions was expressed as nmoles malonyldialdehyde (MDA) produced per minute per mg of the membrane protein.

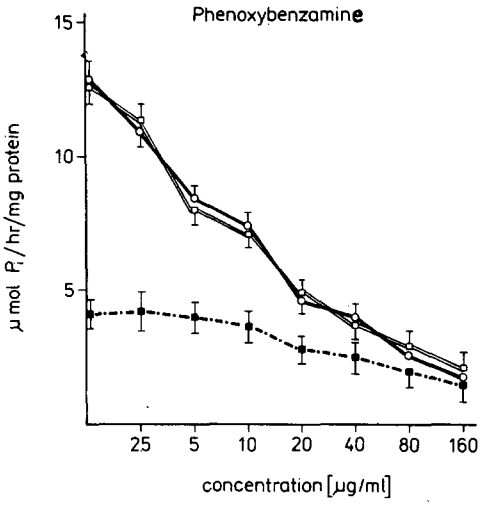

Fig. 2. Inhibition of the brain microsomal Na, K-ATPase activity by phenoxybenzamine. The microsomes (□——□) or microsomes plus cytosol fraction (○——○, ■——■) were preincubated with increasing concentrations of phenoxybenzamine and the Na, K-ATPase activities were assayed as described in Methods. The microsomes plus cytosol fraction was preincubated with phenoxybenzamine and either with (○——○) or with (■——■) 5.4×10^{-5} M adrenaline.

RESULTS

The effect of cyclic adenosinemonophosphate and alfa and beta adrenergic blocking agents on the Na, K-ATPase activity

As is presented in Figure 1, the microsomes suspended in 0.32 M sucrose (microsomes) exhibited a high Na, K-ATPase activity = 10–12 μmoles P_i/hr/mg protein. Addition of the cytosol fraction (supernatant 100,000 *g*) resulted in a marked inactivation of the enzyme activity manifestant in the microsomes plus cytosol fraction = 3–4 μmoles P_i/hr/mg protein. Adrenaline (5.4×10^{-5}M) completely abolished the cytosol induced inhibition of the Na, K-ATPase activity.

The cyclic adenosinemonophosphate (cAMP) and the alfa or beta adrenergic blocking agents were tested in order to demonstrate a possible interference with the catecholamine effect on the Na, K-ATPase activity. Therefore, the effect of these substances was followed during the preincubation of the microsomes plus cytosol fraction with 5.4×10^{-5} M adrenaline. To test the influence of cAMP and of the adrenergic blocking agents on the basal levels of the Na, K-ATPase activity, the microsomes and microsomes plus cytosol fractions were also measured. The microsomes represented the fully active Na, K-ATPase, while the microsomes plus cytosol fraction represented its inhibited levels.

The results presented in Fig. 1(A) show that increasing concentrations of cAMP exhibited no effect under any of the above mentioned experimental conditions.

As shown in Fig. 1(B) and 1(C), both reversible beta (Fig.1(B)) and alfa (Fig. 1(C)) adrenolytics (propranolol, phentolamine and tolazoline) were completely ineffective in competition with adrenaline in its protective effect on the Na, K-ATPase. Also the control levels of Na, K-ATPase measured in the microsomes or microsomes plus cytosol fraction were not influenced by these agents. Similar negative results were found with the combination of both alfa and beta adrenergic blocking agents, propranolol plus phentolamine.

The only exception from these negative data was the slight inhibitory effect of very high propranolol concentrations (see Fig. 1(B)). However, this inhibition was found not only in the presence of adrenaline but also in the control samples without adrenaline.

As compared with the results obtained with the reversible adrenergic blockers, phenoxybenzamine, an irreversible alfa adrenergic blocking agent, affected the brain Na, K-ATPase very pronouncedly. This substance inhibited Na, K-ATPase of the microsomal plus cytosol fraction preincubated with 5.4×10^{-5} M adrenaline at a half dose of 10 μg per ml. However, this effect resulted probably not from the alfa adrenergic blockade, because the same type of inhibition was observed in the control samples of the microsomes incubated without adrenaline. The Na, K-ATPase inhibition was irreversible as it could not be removed by repeated washings of the microsomes with the incubation media.

There was only a slight inhibition of the Na, K-ATPase activity measured in the microsomes plus cytosol fraction, where the Na, K-ATPase was already inhibited by the endogenous inhibitory system present in the cytosol.

The effect of catecholamines with different alfa or beta adrenergic specificity on the Na, K-ATPase activity

The effect of increasing concentrations of adrenaline, noradrenaline and isoprenaline (isoproterenol) on the Na, K-ATPase activity was measured in the brain microsomes plus cytosol fraction (Fig. 3). The results were presented as a difference, μmoles P_i/hr/mg protein, between the catecholamine affected and basal level of the Na, K-ATPase activity.

The dose–response curves of these catecholamines showed that all three agents were equally effective in protection of enzyme activities against the cytosol induced inhibition. No difference could be found with respect to different alfa or beta adrenergic specificity.

Comparison of the time course of malonyldialdehyde production and the Na, K-*ATPase inhibition in the microsomes plus cytosol fraction*

According to the working hypothesis that the lipoperoxidative degradation is responsible for the inhibition of Na, K-ATPase activities, the malonyldialdehyde production should be temporarily related to the inhibition of Na, K-ATPase activities. Therefore, the time–course of the Na, K-ATPase reaction was studied in the presence of the endogenous inhibition system and compared with the lipoperoxidation.

As demonstrated in Fig. 4, the malonyldialdehyde production in the microsomes plus cytosol fraction is a time-dependent process: the first significant tri-

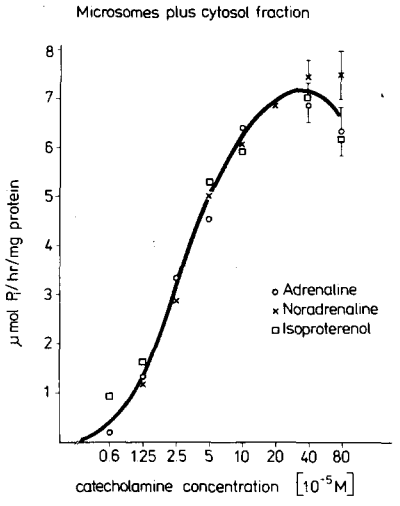

Fig. 3. Comparison of the ability of catecholamines with different alfa or beta adrenergic specificity to protect the Na, K-ATPase activity. Microsomes plus cytosol fraction was preincubated 5 min with increasing concentrations of isoproterenol (□) adrenaline (○) or noradrenaline (×). The ATPase reaction was initiated than by the addition of ATP (see Methods). μmoles P (hr) mg prot. = difference between the catecholamine affected and basal level of the Na, K-ATPase activity.

methine colour was detected after 5 min of the incubation. The highest rate of MDA production was detected between the 5th and 20th minute of the incubation being followed by a slower phase between the 20th and 30th minute. The same MDA production was obtained in =0.2 M phosphate buffer pH 7.4 and in the 'ATPase reaction medium A' = 100 mM NaCl, 20 mM KCl, 100 mM Tris–HCl pH 7.4, 5 mM $MgCl_2$ and 2.5 mM ATP. Omission of

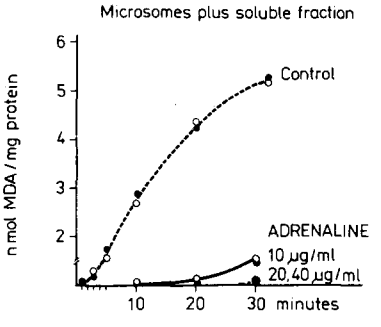

Fig. 4. Malonyldialdehyde (MDA) production in the microsomes plus cytosol fraction. Blockade of the lipoperoxidation by increasing concentrations of adrenaline. Microsomes plus cytosol fraction was incubated at 37° in either 0.2 M phosphate buffer, pH 7.4 (○——○) or in the 'ATPase reaction medium A' = 120 mM NaCl, 20 mM KCl, 5 mM MgCl, 100 mM Tris–HCl pH 7.4, 2.5 mM ATP (●——●). At indicated time interval, an aliquot of the incubation mixture was removed and the malonyldialdehyde produced in the sample was determined as described in the Methods. The MDA production was inhibited by adding increasing concentrations of adrenaline (10, 20 and 40 μg per ml) to the incubation media.

ATP from medium A did not influence the lipoperoxidation (not shown).

As also demonstrated in Fig. 4, the lipoperoxidation was almost diminished by adrenaline at a concentration of 10 μg per ml (5.4×10^{-5} M). There was no detectable MDA produced during the incubation with higher adrenaline concentrations (20 μg and 40 μg per ml).

In the previous experiments and in the results presented in Fig. 5, the ATPase reaction was started by adding ATP after a 5 min preincubation of the enzyme (microsomes plus cytosol fraction). The inorganic phosphate production (ATP breakdown) was linear under these conditions and in the presence of adrenaline (10^{-4} M) or EDTA (5×10^{-5} M) within the whole time interval studied. On the other hand, the control reaction rate without adrenaline or EDTA steadily declined to a minimum at the 15th min of the incubation. The activity of the control and the adrenaline or EDTA treated enzymes markedly differed from the very beginning of the reaction. The P_i production measured at lower concentrations of adrenaline (10^{-5} M, 5×10^{-5} M) and EDTA (5×10^{-6} M, 10^{-5} M) was intermediate between the values obtained with 10^{-4} M adrenaline or 5×10^{-5} M EDTA and the control levels without any additions.

A different picture was obtained when the preincubation period was excluded and the ATPase reaction was started by addition of the enzyme (microsomes plus cytosol fraction). The Na, K-ATPase reaction in the presence of adrenaline and EDTA did not significantly differ from the control up to the 5th min of the incubation (see Fig. 6(A) and (B)). Afterwards, the Na, K-ATPase reaction catalyzed by the adrenaline or EDTA affected enzyme proceeded with an unchanged initial rate, whereas the control enzyme activity began to decrease up to the 15th min of the incubation, when the phosphate production almost stopped. Thus, the preincubated and the non-preincubated microsomal plus cytosol fraction differed in the time of the beginning of the inhibition. This difference required a further clarification.

Therefore, the effect of the time of preincubation of the microsomal plus cytosol fraction (without ATP) on the Na, K-ATPase activity was measured. The enzyme suspension was preincubated at 37° for 1, 2, 3, 4 and 5 min. The Na, K-ATPase reaction was started by adding ATP and the Na, K-ATPase was assayed routinely after a 15 min incubation at 37° (see Fig. 7, measurement at 37°). The enzyme activity was expressed as a percentage of the Na, K-ATPase activity determined in the non-preincubated sample. As much as 50 per cent of the initial Na, K-ATPase activity was lost during the first minute of the preincubation. The 90 per cent inhibition was reached after 5 min of preincubation. The inhibition showed two phases. The first rapid phase occurring until the 2nd min, was followed by a slower phase between the 2nd and 5th min of preincubation.

The inhibition of the Na, K-ATPase activity was a temperature dependent process (see Fig. 7, measurement at 25° and 0°). The maximum rate of the inhibition was found at 37°, it decreased at 25° and the preincubation of the microsomal plus cytosol

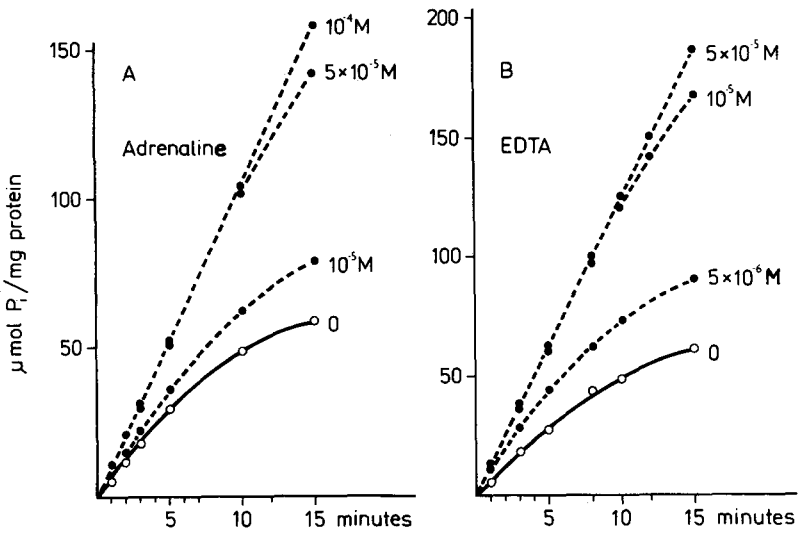

Fig. 5. The time course of the Na, K-ATPase reaction catalysed by the microsomes plus cytosol fraction. The effect of adrenaline and EDTA on the preincubated samples. The microsomes plus cytosol fraction was preincubated for 5 min with increasing concentrations of adrenaline or EDTA before starting the enzyme reaction by the addition of ATP. The basal level of the Na, K-ATPase activity was determined after a 5 min preincubation without adrenaline or EDTA. At indicated time intervals aliquots of the reaction media were removed and the inorganic phosphate was assayed as described in Methods. The inorganic phosphate production was related to mg of the membrane protein present in the samples.

fraction at 0° did not affect the Na, K-ATPase activity at all.

As shown in Fig. 8, the inhibition of the Na, K-ATPase activity during the preincubation without ATP could be prevented by adding 5.4×10^{-5}M adrenaline or 2×10^{-5}M EDTA (not shown). This holds for the first 5 min of the preincubation and only 14 per cent of the enzyme activity were lost at the end of the 10th min, in comparison with the non-preincubated samples.

When summarising the results presented in Figs 5 – 8, two types of inhibition of the Na, K-ATPase could be distinguished from the kinetic point of view: the slow type of inhibition was demonstrated during incubation of the microsomes plus cytosol fraction in the presence of 2.5 mM ATP (see Fig. 6). The fast type of inhibition was detected during preincubation of the enzyme without ATP (Fig. 7). Adrenaline and EDTA protected the Na, K-ATPase activities against both types of inhibition.

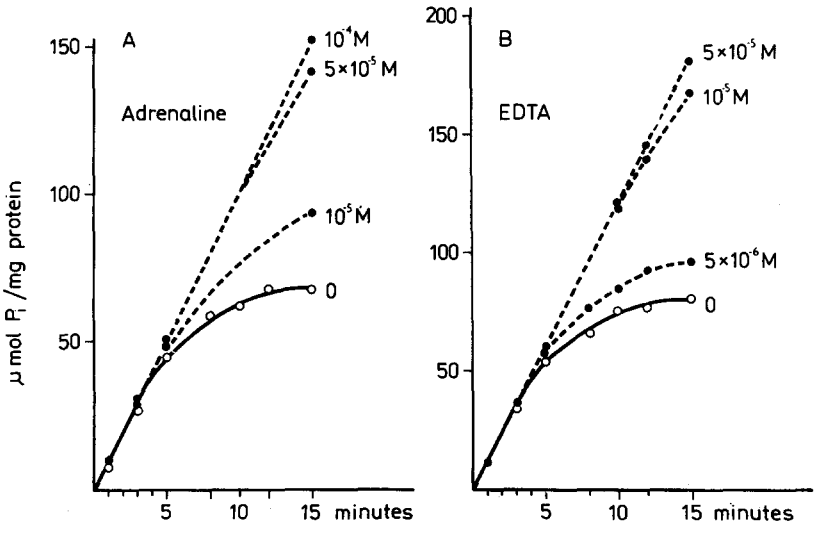

Fig. 6. The time course of the Na, K-ATPase reaction catalysed by the microsomes plus cytosol fraction. The effect of adrenaline and EDTA on the non-preincubated samples. In contrast to the results in Fig. 5, the preincubation of the microsomes plus cytosol fraction was excluded and the ATPase reaction was started by addition of the enzyme. ATP was added 10 sec before the enzyme (microsomes plus cytosol fraction). Increasing concentrations of adrenaline or EDTA were present during the enzyme assay. The inorganic phosphate was determined in aliquots of the reaction mixture (see Methods) and related to mg of the membrane protein.

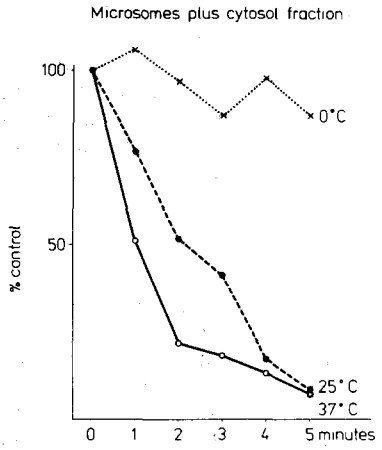

Fig. 7. The fast type of inhibition of the brain microsomal Na, K-ATPase during the preincubation of the microsomes plus cytosol fraction without ATP. Temperature dependency of the inhibition. The brain microsomes plus cytosol fraction (0.01 – 0.025 mg membrane protein per ml) was preincubated for 1, 2, 3, 4 and 5 min in the ATPase reaction media A and B before starting the reaction by the addition of ATP. The samples were preincubated at 37°, 25° or 0°. In the case of incubations at 0° and 25°, tubes with the reaction mixture were transferred, simultaneously with the addition at ATP to another incubation bath equilibrated at 37°. The Na, K-ATPase activity was then assayed identically in all preincubated samples as described in Materials and Methods.

The control, i.e., 100% level of the Na, K-ATPase activity was determined in non-preincubated samples. In this case the Na, K-ATPse reaction was started by the addition of the enzyme (microsomes plus cytosol fraction) 10 sec after ATP. The results were expressed as percentage of the non-preincubated samples.

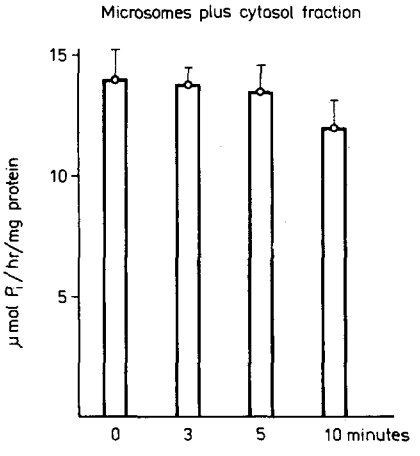

Fig. 8. The microsomes plus cytosol fraction was preincubated at 37° for 3.5 and 10 min in the presence of 5.4×10^{-5} M adrenaline before starting the reaction by the addition of ATP. The Na, K-ATPase was determined identically in all preincubated samples (see Methods) by 15 min incubation at 37°. In the case of the non-preincubated sample (time = 0), the Na, K-ATPase reaction was started by addition of the enzyme (microsmes plus cytosol) 10 sec after ATP. The results are presented as μmoles P_i (hr) mg protein.

DISCUSSION

It was the aim of the first part of this work to find out, whether the antilipoperoxidative effect of catecholamines on the brain Na, K-ATPase is mediated by a specific adrenergic receptor and/or adenylate cyclase system. As described in the previous report [3], the microsomes suspended in 0.32 M sucrose contained a fully active Na, K-ATPase. The inhibitory lipoperoxidative system is absent under these conditions because of the absence of ascorbic acid. The addition of the cytosol fraction containing endogenous ascorbic acid (100,000 *g* supernatant) resulted in a marked inactivation of the enzyme activity and, at the same time, induced lipoperoxidation [2, 3]. Adrenaline (5.4×10^{-5} M) and EDTA (2×10^{-5} M) completely abolished the lipoperoxidation and protected Na, K-ATPase against the inactivation [3]. Therefore, the influence of cyclic AMP and adrenergic blocking agents on Na, K-ATPase activity was determined under three experimental conditions (see Figs 1 and 2).

(a) The microsomal plus soluble fraction was preincubated with 5.4×10^{-5} M adrenaline which blocked the inhibitory system.

(b) The microsomes were suspended in 0.32 M sucrose and, thus, the inhibitory system was eliminated because of the absence of the cytosol fraction.

(c) The microsomes plus cytosol fraction was incubated without any addition and the lipoperoxidative activation and inhibition of Na, K-ATPase took place.

The Na, K-ATPase activities measured according to the experimental protocols (b) and (c) are either low (c) or high (b) and represent the enzyme controls without catecholamine.

As demonstrated in Fig. 1, cAMP, propranolol, phentolamine and tolazoline were not able to compete with adrenaline in its protective action on Na, K-ATPase. Also the control levels of the enzyme activity were not effected by these agents.

On the other hand phenoxybenzamine, a compound of the dibenzamine class of irreversible alfa adrenergic blockers, inhibited not only the adrenaline-protected but also control enzyme activities in agreement with the results of Rougofalis and Belleau [9]. Therefore, the phenoxybenzamine action could not be related to the specific adrenergic receptor.

Similarly, isoprenaline, adrenaline and noradrenaline were equally potent in protection of Na, K-ATPase activities against the cytosol induced inhibition. No difference could be found with respect of different alfa or beta adrenergic specificity.

The absence of the phenomena of reversibility and specificity in the effect of catecholamines on the brain Na, K-ATPase activity seems to reflect the assumed mechanism of action of these compounds (antilipoperoxidative or chelatory capacities), which should differ from the specific receptor [10, 11]. These results and conclusions differ from those of Hexum [12], who was able to demonstrate both the blockade by propanolol of the isoproterenol action on the brain Na, K-ATPase and the beta-adrenergic specificity in the action of catecholamines on this enzyme.

In the second part of this work, the reaction rate of the brain microsomal Na, K-ATPase was measured under various conditions and two types of inhibition could be distinguished from the kinetic point of view:

(1) The slow type started after a 5-min incubation in the presence of ATP.

(2) The fast type of inhibition was detected during the preincubation without ATP. More than 50 per cent of Na, K-ATPase activity was lost during the first minute and the remaining activity disappeared up to 7 – 8 min of the preincubation.

The results were obtained with the simple inorganic phosphate release method. It should be mentioned that the Na, K-ATPase activity was also determined by the coupled assay using pyruvatekinase and lactatedehydrogenase in some experiments. No difference was found between this type of assay and the phosphate release method. As it is possible that some of a wide variety of substances or inhibitory factors tested in this work could interfere with certain components of the coupled ATPase assay, the more complicated methodology was abandoned. Furthermore, the phosphate production was a linear function of time on the condition that the cytosol induced inhibition was prevented.

The slow type of inhibition was closely associated with the development of the lipoperoxidative damage of the brain microsomal fraction suggesting a damage of the phospholipid moiety of Na, K-ATPase.

On the other hand, the fast type of inactivation cannot be interpreted simply as being due to the lipoperoxidative degradation of unsaturated Na, K-ATPase phospholipids. It was possible (see Figs 4 and 7) to obtain a distinction between the onset of the Na, K-ATPase inhibition and the first production of malonyldialdehyde. Na, K-ATPase was significantly inhibited within the first min of the preincubation (Fig. 7) whereas a substantial malonyldialdehyde production was not observed before 3 – 5 min (Fig. 4). The fast type of inhibition was temperature dependent, it declined at 25° and even more at 0°.

A possible mechanism of the fast type of the Na, K-ATPase inhibition may be explained as a direct inhibition by divalent cations, as described before by Schaeffer *et al.* [1, 2], Godfraind [13] and Hexum [12]. However, in the preceding paper [3] it was found that the iron content in the rat brain subcellular fractions is not sufficient to account for the pronounced inhibition observed in the microsomal plus cytosol fraction. Accordingly, the inhibition by the cytosol fraction can hardly be explained by the effect of Ca^{2+} ions as the Ca^{2+} concentration is nearly equal in the cytosol and 0.32 M sucrose, the latter medium not being inhibitory.

It may be visualized that all mechanisms, i.e., the direct inhibition by endogenous Fe^{2+} and Ca^{2+} [1, 2, 12, 13] cations and the lipoperoxidative degradation induced by Fe^{2+} plus ascorbic acid cooperate in the effect on the Na, K-ATPase. The difference between the slow and fast type of inhibition may be explained by assuming that ATP plays a role here. The substrate of the ATPase reaction may protect the enzyme and simultaneously decrease free inhibitory concentrations of Fe^{2+}, Ca^{2+} or other divalent cations.

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