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REGULATION OF RAT BRAIN ($Na^+ + K^+$)-ATPase ACTIVITY BY CYCLIC AMP

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The interaction between the ($Na^+ + K^+$)-ATPase and the adenylate cyclase enzyme systems was examined. Cyclic AMP, but not 5′-AMP, cyclic GMP or 5′-GMP, could inhibit the ($Na^+ + K^+$)-ATPase enzyme present in crude rat brain plasma membranes. On the other hand, the cyclic AMP inhibition could not be observed with purified preparations of ($Na^+ + K^+$)-ATPase enzyme. Rat brain synaptosomal membranes were prepared and treated with either NaCl or cyclic AMP plus NaCl as described by Corbin, J., Sugden, P., Lincoln, T. and Keely, S. ((1977) J. Biol. Chem. 252, 3854–3861). This resulted in the dissociation and removal of the catalytic subunit of a membrane-bound cyclic AMP-dependent protein kinase. The decrease in cyclic AMP-dependent protein kinase activity was accompanied by an increase in ($Na^+ + K^+$)-ATPase activity. Exposure of synaptosomal membranes containing the cyclic AMP-dependent protein kinase holoenzyme to a specific cyclic AMP-dependent protein kinase inhibitor resulted in an increase in ($Na^+ + K^+$)-ATPase enzyme activity. Synaptosomal membranes lacking the catalytic subunit of the cyclic-AMP-dependent protein kinase did not show this effect. Reconstitution of the solubilized membrane-bound cyclic AMP-dependent protein kinase, in the presence of a neuronal membrane substrate protein for the activated protein kinase, with a purified preparation of ($Na^+ + K^+$)-ATPase, resulted in a decrease in overall ($Na^+ + K^+$)-ATPase activity in the presence of cyclic AMP. Reconstitution of the protein kinase alone or the substrate protein alone, with the ($Na^+ + K^+$)-ATPase has no effect on ($Na^+ + K^+$)-ATPase activity in the absence or presence of cyclic AMP. Preliminary experiments indicate that, when the activated protein kinase and the substrate protein were reconstituted with the ($Na^+ + K^+$)-ATPase enzyme, there appeared to be a decrease in the Na^+-dependent phosphorylation of the Na^+-ATPase enzyme, while the K^+-dependent dephosphorylation of the ($Na^+ + K^+$)-ATPase was unaffected.

Introduction

The sodium-potassium adenosine triphosphatase (($Na^+ + K^+$)-ATPase) is the biochemical manifestation of the sodium pump. The ($Na^+ + K^+$)-ATPase enzyme regulates the active transport of sodium (Na^+) and potassium (K^+) across the cell membrane (for references, see Refs. 1 and 2). In addition, the sodium pump is involved, either directly or indirectly, in several physiological processes. These include (i) the release and uptake of neurotransmitters [3,4]; (ii) the generation of the Na^+ and K^+ gradients across the cell membrane necessary for the maintenance of the cell membrane resting potential; (iii) the control of vascular [5] and visceral [6] smooth muscle tone; (iv) the transport of glucose across cell membranes [7]; and (v) the secretion of fluid in several epithelial tissues [8]. The activity of the sodium pump

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is also affected by ethanol [9], as well as by insulin [10] and thyroid hormone [11].

It is clear that changes in $(Na^+ + K^+)$-ATPase activity will affect several physiological processes and it is apparent that cellular mechanisms must exist which can closely regulate $(Na^+ + K^+)$-ATPase activity.

Despite the fact that the enzymatic properties of the $(Na^+ + K^+)$-ATPase are known in considerable detail, little is known about the cellular mechanisms that regulate enzyme activity. Evidence from this [12,13] and other laboratories [14,15], indicate that an important form of control resides in the cellular mechanisms that regulate protein synthesis and degradation. Changes in Na^+ and K^+ transport that occur as a result of changes in enzyme content would occur rather slowly and would not be an efficient mechanism for producing rapid changes in transport rates. Therefore, it is possible that other cellular mechanisms must exist that are capable of producing a rapid change in the turn-over rate of the $(Na^+ + K^+)$-ATPase enzyme. It is now recognized that cyclic nucleotides can modulate the activities of several enzyme systems (for references, see Ref. 16). The effects of cyclic nucleotides in mammalian cells are mediated through protein kinases.

Mammalian tissues contain cyclic AMP-dependent protein kinases. There are two principal soluble enzymes, namely protein kinases I and II (for references, see Ref. 16). The protein kinase isozymes can be readily distinguished from each other by standard biochemical techniques (for references, see Ref. 17). In several tissues, including brain [18], human erythrocytes [19] and rabbit heart [20], approx. 50% of the total cyclic AMP-dependent protein kinase activity is found in particulate subcellular fractions. This membrane-bound cyclic AMP-dependent protein kinase activity is similar to the soluble type II protein kinase [21] and may be important in the cyclic AMP-regulated phosphorylation of specific membrane proteins (for references, see Ref. 22). The phosphorylation of specific membrane proteins has been correlated with changes in membrane function [24] and in particular, with the transport of ions across the cell membrane [23].

In a previous communication we have reported that the $(Na^+ + K^+)$-ATPase enzyme prepared from rat brain could be inhibited approx. 30% by cyclic AMP [25]. In this communication we report that the mechanism of cyclic AMP inhibition is mediated through a cyclic AMP-dependent protein kinase which phosphorylates a substrate protein. Phosphorylation of the neuronal substrate protein leads to a decrease in the overall $(Na^+ + K^+)$-ATPase activity. The decrease in $(Na^+ + K^+)$-ATPase activity appears to be due to inhibition of the Na^+-dependent phosphorylation step of the $(Na^+ + K^+)$-ATPase enzyme.

Materials

[γ-^{32}P]ATP (>200 Ci/mmol) was purchased from ICN (Montreal, Quebec) as the sodium salt. Cyclic AMP, cyclic AMP-dependent protein kinase inhibitor, Na_2-ATP (vanadium free), benzamidine, histone type II S and dithiothreitol were obtained from Sigma. All other chemicals were of reagent grade.

Methods

(a) Preparation of synaptosomal membranes with and without the catalytic subunit of the cyclic AMP-dependent protein kinase

Synaptosomes were prepared from the brains of male Wistar rats (180–200 g) by standard techniques of differential and density gradient centrifugation [26]. The synaptosomal membranes were prepared by lysing the synaptosomes in an hypotonic medium containing 10 mM Tris-HCl, pH 7.4, followed by centrifugation at $39000 \times g$ for 20 min. The pellet was resuspended in a volume of 10 mM Tris-HCl, pH 7.4, such that the protein concentration was approx. 3–4 mg/ml and divided into two portions. One portion was mixed with 2 vol. of 20 mM benzamidine, 1.0 mM Na_2EDTA, 10 mM Tris-HCl, pH 7.4, while the other portion was mixed with 2 vol. of the same solution containing in addition 10 μM cyclic AMP. The tubes were vortexed and centrifuged as before. The supernatants from each tube were poured off and kept on ice while the pellets were resuspended in 10 mM Tris-HCl, pH 7.4.

(b) Purification of $(Na^+ + K^+)$-ATPase

Rat brain microsomes were obtained as

described by Sweadner [27]. The microsomes were exposed to 0.055% sodium dodecyl sulphate (SDS), in the presence of 3 mM Na_2ATP, according to the procedure of Jørgensen [28]. Following slow stirring at room temperature for 30 min, the SDS-treated microsomes were layered on a discontinuous sucrose density gradient consisting of 10, 15, 20 and 50% sucrose in 5.0 mM imidazole-HCl, 1.0 mM Na_2EDTA, pH 7.6. Following centrifugation at $105000 \times g$ for 3 h, the gradient was fractionated at each interface. The $(Na^+ + K^+)$-ATPase activity was found at the 35% sucrose interface (between 20 and 50%) and at the 17.5% sucrose interface (between 15 and 20%). The enzyme specific activities at the 35% and 17.5% sucrose interfaces were 575 and 325 μmol P_i/mg per h, respectively. These preparations of $(Na^+ + K^+)$-ATPase did not contain any cyclic AMP-dependent protein kinase activity.

(c) Solubilization of the membrane-bound cyclic AMP-dependent protein kinase

The membrane-bound cyclic AMP-dependent protein kinase was solubilized, from the crude synaptosomal fraction, with 0.2% Triton X-100 in the presence of 20 mM Tris-HCl, 4.0 mM Na_2EDTA, and 0.1 mM dithiothreitol pH 7.4, essentially as described by Rubin et al. [21]. The solubilized cyclic AMP-dependent protein kinase was placed on a DEAE-Sephacel column (1.6×20 cm) which has been equilibrated with 20 mM Tris-HCl, 4.0 mM Na_2EDTA and 0.1 mM dithiothreitol, pH 7.4. The column was washed with the same buffer until all the unbound protein had been removed. The cyclic AMP-dependent protein kinase activity was eluted with the above buffer containing 350 mM NaCl. The peak of cyclic AMP-dependent protein kinase activity was pooled and concentrated with 36.9% (w/v) solid ammonium sulphate. The precipitated protein was collected by centrifugation, resuspended in 20 mM Tris-HCl, 4.0 mM Na_2EDTA and 0.1 mM dithiothreitol, pH 7.4, and dialyzed overnight against 1000 vol. of the same buffer. This preparation of cyclic AMP-dependent protein kinase did not contain any $(Na^+ + K^+)$-ATPase activity.

(d) Preparation of the substrate protein

A neuronal membrane protein (substrate protein) was isolated from a crude synaptosomal fraction essentially as described by Ueda and Greengard [29]. Following the acid and base extraction procedures, the suspension was fractionated with 80% saturated ammonium sulphate. The precipitated protein was collected by centrifugation, resuspended in 20 mM Tris-HCl, 4.0 mM Na_2EDTA, and 0.1 mM dithiothreitol, pH 7.4 and dialyzed overnight against 1000 vol. of the same buffer. This fraction could be phosphorylated by the solubilized cyclic AMP-dependent protein kinase as reported by Ueda and Greengard [29]. This fraction did not contain any $(Na^+ + K^+)$-ATPase or cyclic AMP-dependent protein kinase activities. The cyclic AMP-dependent protein kinase and substrate protein was reconstituted with the $(Na^+ + K^+)$-ATPase enzyme as described in the legend to Table III.

$(Na^+ + K^+)$-ATPase and cyclic AMP-dependent protein kinase activities were assayed as described by Post and Sen [30] and Grant et al. [19], respectively. The substrate protein was phosphorylated and visualized as described by Ueda and Greengard [29]. Protein content was determined as described by Lowry et al. [31] using bovine serum albumin as a standard.

Statistical analysis, when performed, was done according to the Student's *t*-test. The correlation coefficients and *P* values in Fig. 2 were generated by performing a linear regression analysis.

Results

We have previously reported that the $(Na^+ + K^+)$-ATPase enzyme prepared from rat brain could be inhibited by low levels of cyclic AMP [25]. In this investigation we attempted to elucidate the mechanism of the cyclic AMP-mediated inhibition.

It is generally accepted that the effects of cyclic AMP are mediated through soluble and membrane-bound protein kinases. We posulated that the cyclic AMP-mediated inhibition of the $(Na^+ + K^+)$-ATPase enzyme initially proceeded through activation of a membrane-bound cyclic AMP-dependent protein kinase. We believed that cyclic AMP would have little or no effect on $(Na^+ + K^+)$-ATPase activity if the enzyme had been

prepared in the presence of activating agents such as deoxycholate or SDS.

To examine this possibility we prepared membranes, containing the $(Na^+ + K^+)$-ATPase enzyme, in the absence and presence of activating agents, and monitored the effects of cyclic AMP and 5′-AMP on $(Na^+ + K^+)$-ATPase activity. The results (shown in Table I) indicate that cyclic AMP could decrease the $(Na^+ + K^+)$-ATPase activity in membranes prepared in the absence of SDS. The metabolite, 5′-AMP, also produced a small decrease in $(Na^+ + K^+)$-ATPase activity; however, the effect of 5′-AMP was not as pronounced as that of cyclic AMP.

Table I also shows that purification of the $(Na^+ + K^+)$-ATPase enzyme with SDS resulted in high levels of enzyme activity being obtained. Cyclic AMP and 5′-AMP had no effect on the $(Na^+ + K^+)$-ATPase activity. In addition, several other nucleotides, such as cyclic GMP, 5′-GMP, cyclic CMP, 5′-CMP and cyclic TMP, had no effect on the $(Na^+ + K^+)$-ATPase activity (unpublished observations).

We also monitored the protein kinase activity in the absence and presence of cyclic AMP. The results indicated that crude plasma membranes, in contrast to SDS-treated membranes, contained an highly active cyclic AMP-dependent protein kinase. The activity in the former membranes was 21.1, in the absence, and 199.8 pmol ^{32}P transferred per mg per min in the presence of cyclic AMP. There was no detectable protein kinase activity associated with the SDS-treated membranes. We concluded from these preliminary results that the cyclic AMP-mediated inhibition of the $(Na^+ + K^+)$-ATPase enzyme reported in a previous [25] and this (Table I) communication initially proceeded through activation of a membrane-bound cyclic AMP-dependent protein kinase.

The next step in this investigation was to demonstrate a more direct relationship between the $(Na^+ + K^+)$-ATPase enzyme and the cyclic AMP-dependent protein kinase. To this end, we utilized a technique originally described by Corbin et al. [20]. These investigators reported that it was possible to specifically remove the catalytic subunit of the cyclic AMP-dependent protein kinase from rabbit heart membranes by treating the membranes with cyclic AMP and hypertonic NaCl, but not with NaCl alone. We theorized that removal of the catalytic subunit of the cyclic AMP-dependent protein kinase should result in an increase in $(Na^+ + K^+)$-ATPase activity.

Synaptosomal membranes were prepared and treated as described in the Methods. The effects of the two treatments on total protein kinase activity

TABLE I

THE EFFECT OF CYCLIC AMP AND 5′-AMP ON $(Na^+ + K^+)$-ATPase ACTIVITY IN RAT BRAIN PLASMA MEMBRANES

Conc. (M)	$(Na^+ + K^+)$-ATPase activity (% of control)			
	Crude plasma membranes [a]		Membranes treated with SDS [b]	
	Cyclic AMP	5′-AMP	Cyclic AMP	5′-AMP
0	100 [c]	100 [c]	100 [d]	100 [d]
10^{-9}	83.2 ± 6.8	91.9 ± 5.9	107.8	95.4
10^{-8}	89.0 ± 5.0	91.3 ± 0.8	102.9	105.4
10^{-7}	86.8 ± 5.6	90.9 ± 2.5	108.3	99.2
10^{-6}	89.7 ± 6.5	95.1 ± 3.1	107.0	96.9
10^{-5}	91.3 ± 3.4	94.2 ± 1.7	95.4	93.8
10^{-4}	78.8 ± 5.6	92.1 ± 4.1	102.1	102.3

[a] Membranes were prepared, in the absence of detergents, as described by Post and Sen [30].

[b] Membranes were prepared as described by Sweadner [27].

[c] The absolute enzyme activity ranged from 55 to 75 μmol P_i/mg per h in three separate experiments. Values are mean ± S.E.

[d] The absolute enzyme activity was 574.9 μmol P_i/mg per h. Values are the means of two determinations done in duplicate. $(Na^+ + K^+)$-ATPase activity was assayed as described by Post and Sen [30].

present in the soluble or particulate fractions are shown in Fig. 1. Following the NaCl treatment alone, a small amount of protein kinase activity appeared in the supernatant (Fig. 1, group A). Approx. 30% of the total protein kinase activity in this fraction was cyclic AMP-dependent. The presence of this cyclic AMP-dependent protein kinase activity in the NaCl-soluble fraction can be explained either by the fact that NaCl was capable of removing a proportion of the cyclic AMP-dependent protein kinase holoenzyme from the membrane or that the centrifugal force and the time it was applied to sediment the membranes were insufficient. In keeping with the latter point it was observed that there was $(Na^+ + K^+)$-ATPase activity (12.6 ± 4.4 μmol P_i/mg per h) present in the NaCl-soluble fraction. The particulate fraction (Fig. 1, group B) displayed significant levels of protein kinase activity of which approx. 43% was cyclic AMP-dependent.

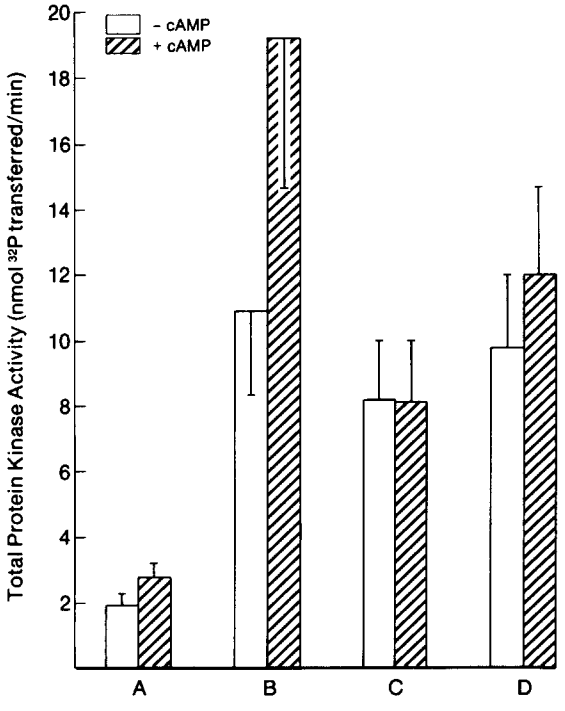

Fig. 1. The effect of treating ruptured synaptosomal membranes with cyclic AMP plus NaCl or NaCl alone on particulate or solubilized total protein kinase activity. Rat brain synaptosomal membranes were prepared and treated as described in Methods. Protein kinase activity was assayed using histone as a phosphate acceptor as described by Grant et al. [19]. (A) The supernatant following the NaCl wash. (B) The pellet following the NaCl wash. (C) The supernatant following the cyclic AMP plus NaCl wash (prior to assaying for protein kinase activity, this fraction was passed through a Sephadex G-25 column to remove the cyclic AMP added during the extraction procedure). (D) The pellet following the cyclic AMP plus NaCl wash. The data are the means ± S.E. from 11 experiments. Statistical comparison was as follows: (i) In the absence and presence of cyclic AMP; A, $P < 0.05$; B, $P < 0.05$; C, not significant; D, not significant. (ii) The cyclic AMP-dependent protein kinase activity in Group B was different from the same activity in Group D, $P < 0.05$.

There was a dramatic increase in the total protein kinase activity in the cyclic AMP plus NaCl-soluble fraction (Fig. 1, group C). The protein kinase activity in this fraction was cyclic AMP-independent (see legend to Fig. 1). As before, there was $(Na^+ + K^+)$-ATPase activity (14.2 ± 6.5 μmol P_i/mg per h) in this soluble fraction. The particulate fraction (Fig. 1, group D) displayed significant levels of protein kinase activity. This fraction, when compared to the control membrane fraction (Fig. 1, group B), had similar levels of cyclic AMP-independent protein kinase activity. However, the cyclic AMP-dependent protein kinase activity in group D (Fig. 1) was significantly lower than that in group B (Fig. 1).

It appeared that the experimental technique described by Corbin et al. [20], when extrapolated to rat brain synaptosomal membranes, could successfully remove the catalytic subunit of a cyclic AMP-dependent protein kinase.

The effects of the two treatments on $(Na^+ + K^+)$-ATPase activity are shown in Table II. The results indicate that treating the synaptosomal membranes with NaCl alone had no effect on the absolute $(Na^+ + K^+)$-ATPase activity since unwashed synaptosomal membranes had the same activity as synaptosomal membranes washed with NaCl alone (Table II). However, washing the membranes with cyclic AMP plus NaCl produced a small, but significant increase in $(Na^+ + K^+)$-ATPase activity. Washing the membranes with cyclic AMP alone did not affect the $(Na^+ + K^+)$-ATPase activity (unpublished data).

These results suggested that removal of the catalytic subunit of a membrane-bound cyclic AMP-dependent protein kinase (Fig. 1) resulted in an increase in $(Na^+ + K^+)$-ATPase specific activity (Table II). If this was indeed the case then one would expect a correlation to exist between the

TABLE II

THE EFFECT OF CYCLIC AMP PLUS NaCl or NaCl ALONE TREATMENT OF RAT BRAIN SYNAPTOSOMAL MEMBRANES ON $(Na^+ + K^+)$-ATPase SPECIFIC ACTIVITY

Rat brain synaptosomal membranes were obtained and treated as described in Methods. $(Na^+ + K^+)$-ATPase activity was assayed as described by Post and Sen [30].

Group	$(Na^+ + K^+)$-ATPase activity (μmol P_i/mg protein per h)	% of control
Untreated synaptosomal membranes	48.3 ± 4.8 [a] (9)	
NaCl-treated synaptosomal	47.5 ± 4.2 (10)	100
Cyclic AMP plus NaCl treated synaptosomal membranes	56.5 ± 4.3 [c] (10)	120.3 ± 2.8 [b,c]

[a] Values are the mean ± S.E. The values in parentheses indicate the number of experiments.

[b] The increase in $(Na^+ + K^+)$-ATPase specific activity in terms of percent stimulation ranged from a low of 7 to a high of 32.

[c] These values are significantly different from the NaCl-treated synaptosomal membranes, $P < 0.001$.

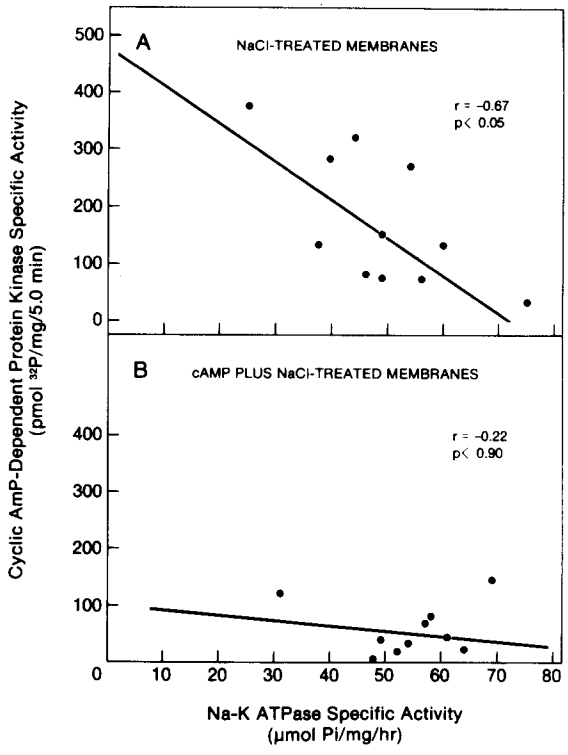

Fig. 2. The correlation between the membrane-bound cyclic AMP-dependent protein kinase activity and the membrane-bound $(Na^+ + K^+)$-ATPase specific activity following treatment of synaptosomal membranes with cyclic AMP plus NaCl or naCl alone in individual experiments. Synaptosomal membranes were obtained and treated as described in Methods. Cyclic AMP-dependent protein kinase activity was assayed as described by Grant et al. [19]. $(Na^+ + K^+)$-ATPase activity was assayed as described by Post and Sen [30]. Panel A shows the correlation that exists following treatment of the membranes with NaCl alone. Panel B shows the correlation that exists following treatment of the membranes with cyclic AMP plus NaCl. The correlation coefficient and P values in Fig. 2 were obtained by performing a linear regression analysis.

cyclic AMP-dependent protein kinase activity and the $(Na^+ + K^+)$-ATPase activity in synaptosomal membranes containing the cyclic AMP-dependent protein kinase holoenzyme. One would not expect a correlation to exist between the cyclic AMP-dependent protein kinase activity and the $(Na^+ + K^+)$-ATPase activity in synaptosomal membranes devoid of the catalytic subunit of the cyclic AMP-dependent protein kinase.

To this end the absolute cyclic AMP-dependent protein kinase activity (that is, the difference between the protein kinase activity in the presence of cyclic AMP minus that in the absence of cyclic AMP) was plotted against the $(Na^+ + K^+)$-ATPase activity from individual experiments and the results are shown in Fig. 2.

Panel A (Fig. 2) shows the negative correlation that exists between the cyclic AMP-dependent protein kinase activity and the $(Na^+ + K^+)$-ATPase in membranes containing the cyclic AMP-dependent protein kinase holoenzyme. It is apparent the individual membrane preparations which display high levels of cyclic AMP-dependent protein kinase activity also display low levels of $(Na^+ + K^+)$-ATPase activity. As expected, the converse is also true. Panel B (Fig. 2), on the other hand, shows the correlation between the cyclic AMP-dependent protein kinase activity and $(Na^+ + K^+)$-ATPase activity in membranes lacking the catalytic subunit of the cyclic AMP-dependent protein kinase. It is clear that no correlation exists between the two parameters being examined.

The interaction between the cyclic AMP-depen-

dent protein kinase and the $(Na^+ + K^+)$-ATPase enzyme was examined further by making use of a specific cyclic AMP-dependent protein kinase inhibitor. We postulated that inhibition of the cyclic AMP-dependent protein kinase should result in an increase in $(Na^+ + K^+)$-ATPase activity. To this end, a commercially available cyclic AMP-dependent protein kinase inhibitor was obtained. Prior to monitoring the effect of the inhibitor on $(Na^+ + K^+)$-ATPase enzyme activity, the specificity of the cyclic AMP-dependent protein kinase inhibitor was examined. The inhibitor caused a decrease in the level of cyclic AMP-dependent protein kinase activity in synaptosomal membranes without affecting the levels of cyclic AMP-independent protein kinase activity (results not shown). The inhibitor did not display any endogenous $(Na^+ + K^+)$-ATPase activity (results not shown).

The protein kinase inhibitor was able to increase $(Na^+ + K^+)$-ATPase activity in membranes containing the cyclic AMP-dependent protein kinase holoenzyme while it was without effect in membranes lacking the catalytic subunit of the protein kinase, except at the highest concentration used (Fig. 3).

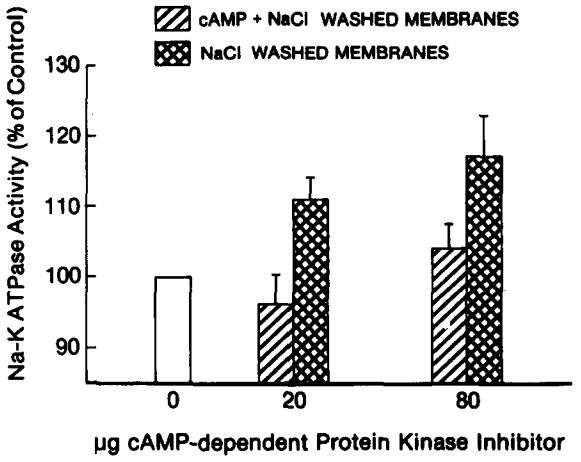

Fig. 3. The effects of a cyclic AMP-dependent protein kinase inhibitor on $(Na^+ + K^+)$-ATPase specific activity in synaptosomal membranes treated with cyclic AMP plus NaCl or NaCl alone. Rat brain synaptosomal membranes were prepared as described in Methods. $(Na^+ + K^+)$-ATPase specific activity was assayed in the presence of 20 or 80 μg cyclic AMP-dependent protein kinase inhibitor. The use of 10 μg of cyclic AMP-dependent protein kinase inhibitor did not affect the $(Na^+ + K^+)$-ATPase specific activity of either membrane preparation. Values are means ± S.E. of four separate experiments.

From the results described above we concluded that the $(Na^+ + K^+)$-ATPase presented in synaptosomal membranes could be regulated, in part, by a cyclic AMP-dependent protein kinase. The next step in this investigation was to elucidate the mechanism by which the cyclic AMP-dependent protein kinase could modulate $(Na^+ + K^+)$-ATPase enzyme activity.

To effectively demonstrate an effect of the cyclic AMP-dependent protein kinase on $(Na^+ + K^+)$-ATPase activity these two membrane components had to be purified to a certain extent. The $(Na^+ + K^+)$-ATPase enzyme and the cyclic AMP-dependent protein kinase were prepared as described in the Methods. As stated in the Introduction the particulate cyclic AMP-dependent protein kinase present in brain tissue is similar to the soluble type II isozyme. The soluble type II isozyme is capable of catalysing an intra-molecular phosphorylation of the regulatory subunit of the solubilized cyclic AMP-dependent protein kinase in the presence, but not absence, of cyclic AMP. We were able to phosphorylate the regulatory subunit of the solubilized protein kinase in the presence of cyclic AMP. The molecular weight of the phosphorylated peptide was 55000 (results not shown) which is in good agreement with the other published reports (see Ref. 21 and references therein).

We attempted to reconstitute the solubilized cyclic AMP-dependent protein kinase with either purified preparation of $(Na^+ + K^+)$-ATPase. We discovered that, despite several biochemical manipulations, we could not demonstrate an effect of the cyclic AMP-dependent protein kinase on either purified preparation of $(Na^+ + K^+)$-ATPase. These results were unexpected in that we had postulated that cyclic AMP could modulate $(Na^+ + K^+)$-ATPase activity through the initial activation of a cyclic AMP-dependent protein kinase. It appeared to us that the cyclic AMP-dependent protein kinase did not interact directly with the $(Na^+ + K^+)$-ATPase enzyme. We theorized that the cyclic AMP-dependent protein kinase activated a substrate protein which, in turn, regulated $(Na^+ + K^+)$-ATPase activity. We were of the opinion that purification of $(Na^+ + K^+)$-ATPase with SDS resulted in removal of the sub-

strate protein from the membrane and that this accounted for the lack of effect of the cyclic AMP-dependent protein kinase on the $(Na^+ + K^+)$-ATPase in the presence of cyclic AMP.

In 1977, Ueda and Greengard [29], described the presence of a neuronal membrane protein (substrate protein) which could be phosphorylated by a membrane-bound cyclic AMP-dependent protein kinase. We partially purified this substrate protein from the crude synaptosomal fraction of rat brain as described in the Methods. The substrate protein preparation could be phosphorylated by the solubilized cyclic AMP-dependent protein kinase. The molecular weight of the phosphorylated substrate protein was between 80000 and 86000 (results not shown) which is in agreement with Ueda and Greengard [29]. The substrate protein preparation did not display any endogenous cyclic AMP-dependent protein kinase or $(Na^+ + K^+)$-ATPase enzyme activities (results not shown).

We attempted to reconstitute the solubilized cyclic AMP-dependent protein kinase and the substrate protein with the purified preparations of $(Na^+ + K^+)$-ATPase. The results are shown in Table III. Using the preparation of $(Na^+ + K^+)$-ATPase that sedimented out at the 17.5% sucrose interface (specific activity = 325 μmol P_i/mg per h) we were able to demonstrate an effect of the cyclic AMP-dependent protein kinase and substrate protein on the $(Na^+ + K^+)$-ATPase activity in the presence, but not absence, of cyclic AMP. The $(Na^+ + K^+)$-ATPase enzyme activity decreased approx. 18%. The cyclic AMP-dependent protein kinase alone or the substrate protein alone, did not affect the $(Na^+ + K^+)$-ATPase activity in either the presence or absence of cyclic AMP.

The results described above led us to conclude that the overall $(Na^+ + K^+)$-ATPase activity could be inhibited by a membrane-bound cyclic AMP-dependent protein kinase. The activated protein kinase does not interact directly with the $(Na^+ + K^+)$-ATPase enzyme but appears to manifest its' effects through an intermediate protein. If the overall $(Na^+ + K^+)$-ATPase activity is indeed decreased then it should be possible to demonstrate an effect on the partial reactions of the $(Na^+ + K^+)$-ATPase enzyme. The $(Na^+ + K^+)$-ATPase enzyme reaction is known to consist of the following partial reactions which can be measured and include (1) an Na^+-dependent phosphorylation step, (ii) a K^+-dependent dephosphorylation step, and (iii) an Na^+-dependent ADP-ATP exchange step (for references, see Refs. 1 and 2). We decided, therefore, to examine the effect of the activated protein kinase and its' substrate protein on the partial reactions of the $(Na^+ + K^+)$-ATPase enzyme.

TABLE III

THE EFFECT OF CYCLIC AMP-DEPENDENT PROTEIN KINASE AND SUBSTRATE PROTEIN ON $(Na^+ + K^+)$-ATPase ACTIVITY

Reconstitution of the cyclic AMP-dependent protein kinase and the substrate protein with the $(Na^+ + K^+)$-ATPase was done as follows: 78.4 μg $(Na^+ + K^+)$-ATPase, 20 μg protein kinase and 11.7 μg substrate protein, in the volume of 0.14 ml, were incubated at 0°C. The reaction was initiated by the addition of 0.01 ml of 0.15% deoxycholate. The tubes were gently vortexed and incubated at 0°C for 60 s. The protein suspension was diluted 25-fold with 30 mM imidazole-HCl, 250 mM sucrose and 1.0 mM Na_2EDTA, pH = 7.6. Aliquots were assayed for $(Na^+ + K^+)$-ATPase activity in a mixture containing 30 mM imidazole-glycylglycine, pH = 7.6, 100 mM Na, 20 mM K, 9.0 mM $MgCl_2$ and 4.5 mM [γ-^{32}P]ATP. The released radioactive phosphate was extracted as described by Post and Sen [30]. Aliquots of the butyl acetate extract were counted in a Nuclear Chicago Mark II Liquid Scintillation counter.

	$(Na^+ + K^+)$-ATPase specific activity (μmol P_i/mg per h)	
	−cyclic AMP	+cyclic AMP (1.0 μM)
Enzyme [a]	224.0	233.3
Enzyme + protein kinase	212.3	234.2
Enzyme + substrate protein	214.3	221.4
Enzyme + protein kinase + substrate protein	233.3 (100%)	192.0 (82.2% ± 2.7) [b,c]

[a] This was the $(Na^+ + K^+)$-ATPase preparation that sedimented at the 17.5% sucrose interface.

[b] Cyclic AMP decreased the $(Na^+ + K^+)$-ATPase activity approx. 18%. These are the means ± S.E. of three separate reconstitution experiments.

[c] These values, in the presence of cyclic AMP, are significantly different from those in the absence of cyclic AMP ($P < 0.05$).

New batches of $(Na^+ + K^+)$-ATPase, cyclic AMP-dependent protein and substrate protein were prepared as outlined in the Methods. The catalytic subunit of the cyclic AMP-dependent protein kinase was dissociated from the regulatory subunit as described in the legend to Table IV. The activated protein kinase and the substrate protein were reconstituted with the $(Na^+ + K^+)$-ATPase enzyme as described in the legend to Table IV.

The results (shown in Table IV) indicate that the activated protein kinase could depress the total Na^+-dependent phosphorylation of the $(Na^+ + K^+)$-ATPase enzyme by approx. 11%. The presence of the substrate protein, along with the activated kinase resulted in a further decrease in the level of phosphorylation. The activated protein kinase and substrate protein did not appear to affect the K^+-dependent dephosphorylation step of the enzyme (results not shown).

TABLE IV

THE EFFECT OF THE CATALYTIC SUBUNIT OF THE CYCLIC AMP-DEPENDENT PROTEIN KINASE AND THE SUBSTRATE PROTEIN ON THE Na-DEPENDENT PHOSPHORYLATION OF THE $(Na^+ + K^+)$-ATPase ENZYME

The partially purified cyclic AMP-dependent protein kinase was bound to a DEAE-Sephacel column (1.6×10 cm). The column was washed with two bed volumes of 20 mM Tris-HCl, 4 mM Na_2EDTA and 0.1 mM dithiothreitol, pH=7.4. The catalytic subunit (that is, the activated protein kinase) was eluted with 10 μM cyclic AMP and 200 mM NaCl. The enzyme, the enzyme plus activated kinase, or the enzyme plus activated kinase plus substrate protein was combined as follows: 0.72 mg of $(Na^+ + K^+)$-ATPase enzyme, 101.2 pmol of protein kinase and 0.2 mg of substrate protein, in volume of 1.12 ml, were incubated at 0°C. The reaction was initiated by the addition of 0.08 ml of 0.15% deoxycholate. The tubes were gently vortexed and incubated at 0°C for 60 s. The protein suspension was diluted 5-fold with 30 mM imidazole, 1.0 mM Na_2EDTA and 0.25 M sucrose, pH=7.6. Aliquots, containing 0.054 mg protein, were phosphorylated as described by Post and Sen [30]. The values are the means of two experiments done in duplicate.

	pmol ^{32}P incorporated per mg protein	% Inhibition
Enzyme	233.1	
Enzyme + activated protein kinase	207.1	11.2
Enzyme + activated protein kinase + substrate protein	190.2	18.4

Discussion

In the last few years it has become increasingly clear that cyclic AMP and cyclic GMP can regulate the activities of several enzyme systems. With regards to the $(Na^+ + K^+)$-ATPase enzyme, cholinergic agonists have been shown to increase the $(Na^+ + K^+)$-ATPase activity in avian salt glands and rat submandibular glands. The carbachol-mediated increase in enzyme activity appears to be due to cyclic GMP (Ref. 8, and references therein). Cyclic AMP, on the other hand, has been shown to inhibit $(Na^+ + K^+)$-ATPase activity in membrane preparations from kidney, [32], liver [33] and heart [34]. Several classes of cyclic AMP-dependent protein kinases exist in mammalian brain including both soluble and membrane-bound protein kinases. The membrane-bound kinases are present in nerve endings [18] and appear to be located both pre- [35] and post-synaptically [18]. The post-synaptic cyclic AMP-dependent protein kinases have been postulated to be involved in regulating the permeability of the post-synaptic membrane to ions [24].

We would like to suggest that the changes in intracellular ion concentrations that occur within both pre- [35] and post-synaptic [18] structures may be partially due to the inhibition of the $(Na^+ + K^+)$-ATPase enzyme system as well as to changes in the passive permeability of the membrane to ions. In this investigation evidence has been presented that suggests that cyclic AMP inhibits the $(Na^+ + K^+)$-ATPase enzyme in rat brain. The inhibition is not direct but involves the initial activation of a membrane-bound cyclic AMP-dependent protein kinase. Removal of the catalytic subunit of the membrane-bound cyclic AMP-dependent protein kinase leads to a decrease in particulate cyclic AMP-dependent protein kinase activity (Fig. 1) and a concomitant increase in $(Na^+ + K^+)$-ATPase activity (Table II).

Further evidence pointing to a relationship between the protein kinase and the $(Na^+ + K^+)$-ATPase enzyme comes from the finding that a

protein kinase inhibitor was able to activate the $(Na^+ + K^+)$-ATPase enzyme only when the protein kinase holoenzyme was present in the synaptosomal membranes but not in its absence (Fig. 3). The protein kinase inhibitor is a heat stable protein that specifically inhibits cyclic AMP-dependent protein kinases without affecting other protein kinases [36]. The inhibitor decreases the cyclic AMP-dependent protein kinase activity by interacting with the dissociated catalytic subunit but not with the cyclic AMP-dependent protein kinase holoenzyme [36]. It has recently been shown that the cyclic AMP-dependent protein kinase inhibitor present in brain and skeletal muscle have many properties in common [37]. It is possible that the inhibitor used in these experiments shares these properties and that this accounts for the fact that it is capable of inhibiting the cyclic AMP-dependent protein kinase in synaptosomal membranes.

Although we have not attempted to characterize the membrane-bound cyclic AMP-dependent protein kinase, a recent report by Rubin et al. [22] has indicated that the cyclic AMP-dependent protein kinase present in bovine brain is similar to the soluble type II protein kinase. The kinases exhibit nearly identical immunological, physio-chemical, and structural properties.

Reconstitution of the protein kinase and the substrate protein with the $(Na^+ + K^+)$-ATPase enzyme resulted in a small, but significant decrease in $(Na^+ + K^+)$-ATPase activity in the presence of cyclic AMP (Table III). We also attempted to reconstitute the protein kinase and the substrate protein with the $(Na^+ + K^+)$-ATPase preparation of higher specific activity (575 μmol P_i/mg per h) but could not demonstrate any effect on the $(Na^+ + K^+)$-ATPase activity in the presence of cyclic AMP. One possible reason for this may be that the membrane matrix of this $(Na^+ + K^+)$-ATPase preparation was sufficiently disrupted by the detergent treatment such that the protein kinase and substrate protein could not insert into the membrane. Preliminary experiments indicate that the inhibition of the $(Na^+ + K^+)$-ATPaes enzyme appears to be due to the inhibition of the Na^+-dependent phosphorylation step of the reaction mechanism (Table IV).

From the above discussion we feel than an interaction between cyclic AMP and $(Na^+ + K^+)$-ATPase does exist. Cyclic AMP does not interact directly with the $(Na^+ + K^+)$-ATPase. The effects of cyclic AMP are expressed through soluble (unpublished results) and membrane-bound cyclic AMP-dependent protein kinases. The cyclic AMP-dependent protein kinases do not interact directly with the $(Na^+ + K^+)$-ATPase enzyme system. It appears that phosphorylation of a substrate protein by the cyclic AMP-dependent protein kinases is a necessary step to modulate the activity of $(Na^+ + K^+)$-ATPase. A scheme showing the interactions of the protein kinase and the substrate protein with the $(Na^+ + K^+)$-ATPase enzyme is outlined in Fig. 4.

$(Na^+ + K^+)$-ATPase is an enzyme that is intimately involved in several physiological processes. Since changes in the $(Na^+ + K^+)$-ATPase activity will affect the cellular processes subserved by the enzyme system, it is imperative that, for normal cell function, dramatic changes in enzyme activity and ions fluxes should be avoided. We would like to suggest that the modulation of $(Na^+ + K^+)$-ATPase activity by cyclic AMP to the extent of

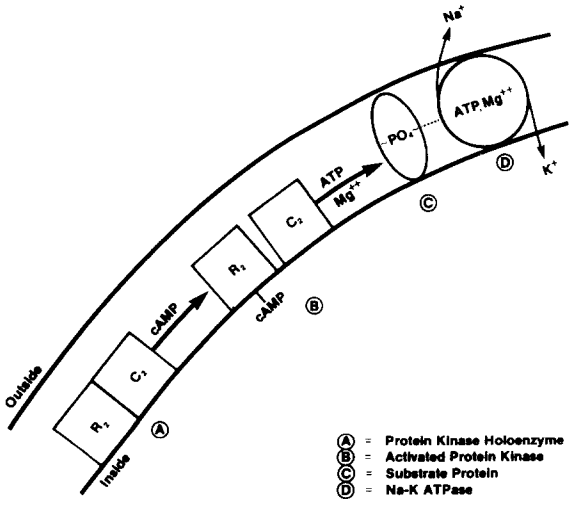

Fig. 4. Postulated interaction of the cyclic AMP-dependent protein kinase and it's substrate protein with the $(Na^+ + K^+)$-ATPase enzyme. It is believed that cyclic AMP inhibits $(Na^+ + K^+)$-ATPase (D) by initially activating a membrane-bound cyclic AMP-dependent protein kinase (A). The activated kinase transfers the terminal phosphate of ATP in the presence of Mg^{2+} to a substrate protein (D). Phosphorylation of the substrate protein (C) inhibits the $(Na^+ + K^+)$-ATPase enzyme.

20–30% reported in this and a previous communication [25] is physiologically significant.

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References

1 Schwartz, A., Lindenmeyer, G.E. and Allen, J.C. (1975) Pharmacol. Rev. 27, 3–134
2 Jørgensen, P.L. (1980) Physiol. Rev. 60, 864–917
3 Meyer, E.M. and Cooper, J.R. (1981) J. Neurochem. 36, 467–475
4 Vizi, E. (1978) Neuroscience 3, 367–384
5 Lang, S. and Blaustein, M.P. (1980) Circ. Res. 46, 463–470
6 Schied, C.R., Honeyman, T.N. and Fay, F.S. (1979) Nature 277, 32–36
7 Bihler, I. and Sawh, P.C. (1979) J. Mol. Cell. Cardiol. 2, 404–414
8 Stewart, D.J. and Sen, A.K. (1981) Am. J. Physiol. 240, C207–C214
9 Rangaraj, N. and Kalant, H. (1978) Biochem. Pharmacol. 27, 1139–1144
10 Moore, R.D. (1973) J. Physiol. London 232, 23–45
11 Edelman, I.S. and Ismail-Beigi, F. (1973) Rec. Prog. Horm. Res. 30, 235–257
12 Stewart, D.J., Semple, E., Swart, C. and Sen, A.K. (1976) Biochim. Biophys. Acta 419, 150–163
13 Lingham, R.B., Stewart, D.J. and Sen, A.K. (1980) Biochim. Biophys. Acta 601, 229–234
14 Lo, C.S. and Edelman, I.S. (1976) J. Biol. Chem. 251, 7834–7840
15 Lin, M.H. and Akera, T. (1978) J. Biol. Chem. 253, 723–726
16 Nimmo, H. and Cohen, P. (1977) Adv. Cyclic Nucleotide Res. 8, 145–266
17 Rangel-Aldoa, R., Kupiec, J. and Rosen, O. (1979) J. Biol. Chem. 254, 2499–2508
18 Uno, I., Ueda, T. and Greengard, P. (1977) J. Biol. Chem. 252, 5164–5174
19 Grant, W., Breithaupt, T. and Cunningham, E. (1979) J. Biol. Chem. 254, 5726–5733
20 Corbin, J., Sugden, P., Lincoln, T. and Keely, S. (1977) J. Biol. Chem. 252, 3854–3861
21 Rubin, C., Rangel-Aldoa, R., Sarkar, D., Erlichman, J. and Fleischer, N. (1979) J. Biol. Chem. 254, 3797–3805
22 Rubin, C. and Rosen, O. (1975) Annu. Rev. Biochem. 4, 831–887
23 Beam, K.G., Alper, S.L., Palade, G.E. and Greengard, P. (1979) J. Cell. Biol. 83, 1–15
24 Greengard, P. (1976) Nature 260, 101–108
25 Sen, A.K., Murthy, R., Stancer, M., Awad, I., Godse, D. and Grof, P. (1976) in Membranes and Disease (Bolis, L., Hoffman, J. and Leaf, A., eds.), pp. 109–122, Raven Press, New York
26 Whittaker, P. and Barker, L. (1972) in Methods in Neurochemistry (Fried, R., ed.), Vol. 12, pp. 1–52, Marcell Dekker, New York
27 Sweadner, K. (1978) Biochim. Biophys. Acta 508, 486–499
28 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52
29 Ueda, T. and Greengard, P. (1977) J. Biol. Chem. 252, 5155–5163
30 Post, R.L. and Sen, A.K. (1967) in Methods in Enzymology (Estabrook, R. and Pullman, eds.), Vol. 10, pp. 762–768, Academic Press, New York
31 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
32 Braughler, J. and Corder, C. (1978) Biochim. Biophys. Acta 424, 455–465
33 Barnabei, O., Luly, P., Tomasi, V., Trevisani, A. and Tria, E. (1973) in Advances in Enzyme Regulation (Weber, G., ed.), Vol. 2, pp. 274–290, Permagon Press, New York
34 Limas, C., Notargiacomo, V. and Cohn, J. (1973) Cardiovasc. Res. 7, 477–481
35 Therien, M. and Mushynski, W. (1979) Biochim. Biophys. Acta 585, 188–200
36 Walsh, D.A., Ashby, D.C. (1973) Rec. Prog. Horm. Res. 29, 329–359
37 DeMaille, J., Peters, K., Strandjord, P. and Fischer, E. (1978) FEBS Lett. 86, 113–116