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SYNAPTOSOMAL (Ca^{2+}–Mg^{2+})-ATPASE ACTIVITY MODULATION BY CYCLIC AMP

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Abstract—Dibutyryl cyclic AMP, in a concentration-dependent manner, increased synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity, but in synaptic plasma membranes lacked any effect. The maximal enzyme activity in synaptosomes was increased by 38%, leaving unaltered the extrasynaptosomal Ca^{2+} concentration necessary to reach it. In the presence of 5 μM cyclic AMP, cyclic AMP-dependent protein kinase increased (30%) maximal (Ca^{2+}–Mg^{2+})-ATPase activity in synaptic plasma membranes, but the apparent affinity for Ca^{2+} was not modified. This effect was partially inhibited (60%) by a cyclic AMP-dependent protein kinase inhibitor. The data suggest that synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity is modulated by a cyclic AMP-dependent phosphorylation reaction.

Several mechanisms have been proposed to act in concert to maintain the resting intrasynaptosomal free Ca^{2+} concentrations within a narrow range. Thus, Ca^{2+} can be bound by a variety of proteins [1, 2] and can be sequestered by intrasynaptosomal organelles such as the mitochondria [3] and the endoplasmic reticulum [4]. Furthermore Ca^{2+} extrusion through synaptic plasma membrane (SPM)† by a high affinity ATP-driven Ca^{2+} pump, (Ca^{2+}–Mg^{2+})-ATPase [5] or by a Na^+/Ca^{2+} exchange system [6] have been described.

The high-affinity Ca^{2+} pump has been shown to be stimulated by calmodulin [5, 7], but the modulation by intracellular second messengers is not yet well known. In several preparations of cardiac tissue the (Ca^{2+}–Mg^{2+})-ATPase activities are regulated by a cAMP-dependent phosphorylation reaction [8, 9]. However in brain the effect of cAMP on (Ca^{2+}–Mg^{2+})-ATPase activity is still unknown.

Synaptosomes isolated from rat brain retain many of the functional properties of intact never terminals [10]. A substantial concentration of cAMP-dependent protein kinase is present in synaptosomal fractions [11–13]. This enzyme is able to phosphorylate different intrasynaptosomal proteins and possibly the synaptosomal (Ca^{2+}–Mg^{2+})-ATPase.

In this work we studied the effects of dibutyryl cAMP (dcAMP) or cAMP-dependent protein kinase (cAMP–PK) on (Ca^{2+}–Mg^{2+})-ATPase activity in syaptosomes or SPM, respectively.

MATERIALS AND METHODS

Chemicals. Dibutyryl cAMP, cAMP, bovine heart cAMP-dependent protein kinase and bovine heart cAMP-dependent protein kinase inhibitor (Type II) were purchased from Sigma Chemical Co. (St Louis, MO). ATP disodium salt was from Merck (Darmstadt, F.R.G.). All other reagents were of analytical grade.

Isolation of synaptosomes and SPM. Synaptosomes and SPM were prepared from whole brains of female Sprague–Dawley rats, weighing 200–250 g, using the method described by Dodd *et al.* [14] and Jones and Matus [15], respectively.

The synaptosomal pellet was suspended in 0.32 M sucrose, 20 mM Tris–HCl, pH 7.4, at a concentration of 2.0–2.5 mg protein/ml. The SPM pellet was suspended in 20 mM Tris–HCl, pH 7.4 at concentration of 0.5–1.0 mg protein/ml.

The protein content was determined by the method of Lowry *et al.* [16] with bovine serum albumin as a standard.

ATPase assays. ATPase activity was measured by the colorimetric determination of P_i hydrolised from ATP by the method of Fiske and Subbarrow as modified by Lebel *et al.* [17].

Synaptosomes (200–250 μg protein) or SPM (50–100 μg protein) were suspended in incubation media (final volume 1 ml) containing 20 mM Tris–HCl, pH 7.4; 0.75 mM $MgCl_2$; 0.4 mM EGTA; 0.1 mM ouabain; 0.5 mM sodium azide; with (for total ATPase activity) or without (for basal Mg^{2+}-ATPase activity) addition of varying concentrations of $CaCl_2$ (0.150–0.375 mM), which correspond to (0.1–3.0 μM) free Ca^{2+} concentrations as calculated by the method described by Bartfai [18]. When enzymatic activity was investigated in intact synaptosomes, the isotonicity of the reaction media was maintained by the addition of sucrose. Reaction mixtures were preincubated at 37° for 10 min. The reaction was started by the addition of 1.5 mM ATP (final concentration). After 20 min incubation the reaction was stopped with 1 ml ice-cold 10% (wt/vol) trichloroacetic acid. The drugs were added just before incubation.

Reagents blanks, consisting of the complete assay mixture with added trichloroacetic acid, were not

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† Abbreviations used: SPM, synaptic plasma membrane; dcAMP, dibutyryl cyclic AMP; cAMP-PK, cyclic AMP-dependent protein kinase.

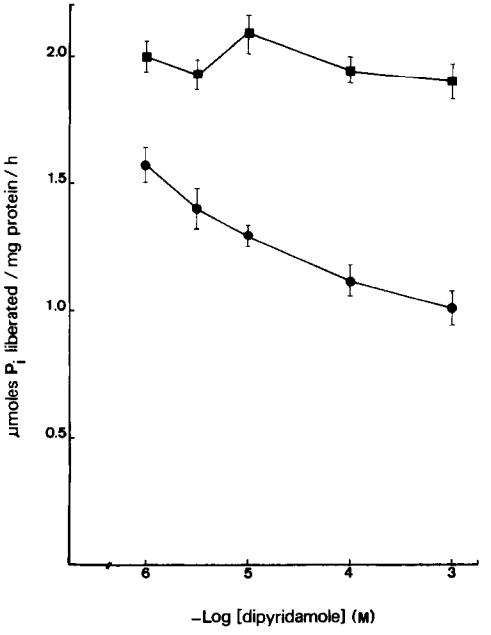

Fig. 1. Effect of dipyridamole on SPM or synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity. Synaptosomes or SPM were incubated in reaction mixtures containing 0.34 mM $CaCl_2$ (1.2 µM free Ca^{2+}) for synaptosomes or 0.275 mM $CaCl_2$ (0.5 µM free Ca^{2+}) for SPM, in the presence of varying dipyridamole concentrations (10^{-6}–2×10^{-3} M). (●) (Ca^{2+}–Mg^{2+})-ATPase activity in synaptosomes, (■) (Ca^{2+}–Mg^{2+})-ATPase activity in SPM. The results are means ± SE (N = 3). Control (Ca^{2+}–Mg^{2+})-ATPase activity in synaptosomes was 1.514 ± 0.057 and control (Ca^{2+}–Mg^{2+})-ATPase activity in SPM was 1.926 ± 0.039. Statistically significant differences became apparent at 5×10^{-6} M dipyridamole, with a $P < 0.001$. (Scheffé's Test.)

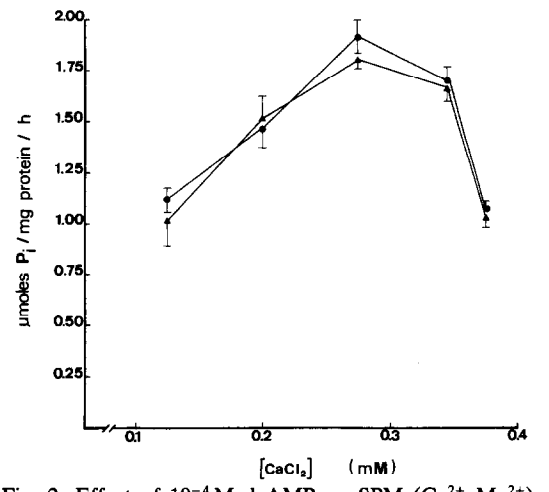

Fig. 2. Effect of 10^{-4} M dcAMP on SPM (Ca^{2+}–Mg^{2+})-ATPase activity. The effect of dcAMP (10^{-4} M) on Ca^{2+} concentration response curves (over the range of 0.150–0.375 mM $CaCl_2$ which corresponds to 0.1–3.0 µM free Ca^{2+}) was assayed in SPM as described in Materials and Methods. Control (●); dcAMP (▲). Data are presented as mean ± SE (N = 5).

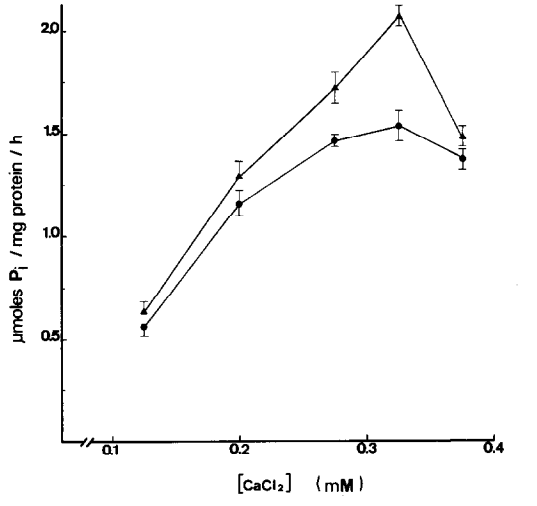

Fig. 3. Effect of 10^{-4} dcAMP on synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity. Synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity was assayed as described in Materials and Methods, as a function of Ca^{2+} concentrations (0.150–0.375 mM $CaCl_2$ or 0.1–3.0 µM free Ca^{2+}), in presence or absence of 10^{-4} M dcAMP. Control (●); dcAMP (▲). Each point represents mean ± SE (N = 4). Statistically significant differences were found in dcAMP vs control ($P < 0.001$). (Two-way ANOVA.)

incubated.

(Ca^{2+}–Mg^{2+})-ATPase activity represents the difference between total ATPase and basal Mg^{2+}-ATPase activities and was expressed as µmoles P_i/mg protein/hr.

The assay was linear with the amount of protein over the range between 100–400 µg protein for synaptosomes and 25–150 µg for SPM, and was also linear with time, between 5 and 30 min.

Statistics. The data are presented as means ± SE of separate determinations each performed in triplicate. The mean values were compared by variance analysis. In some cases, when *F* was significant, the difference between means was determined using Scheffé's test.

RESULTS

It is generally assumed that ATP does not cross plasma membrane. Evidence has been provided however of the existence of specific ATP uptake system in membranes [19]. We have assessed that ATP added extrasynaptosomally can be used as substrate for synaptosomal (Ca^{2+}–Mg^{2+})-ATPase. In this context, at 0.325 mM extrasynaptosomal $CaCl_2$ (which corresponds to 1 µM free Ca^{2+}) (Ca^{2+}–Mg^{2+})-ATPase activity in synaptosomes preloaded with ATP, washed and incubated in its absence was not significantly different (1.321 ± 0.098, N = 4) from enzyme activity measured in synaptosomes directly incubated with ATP (1.373 ± 0.027, N = 4). Moreover, dipyridamole, an inhibitor of adenosine and ATP uptake [19], decreased in a concentration-dependent manner (Ca^{2+}–Mg^{2+})-ATPase activity in synaptosomes, but in SPM lacked any effect. Maxi-

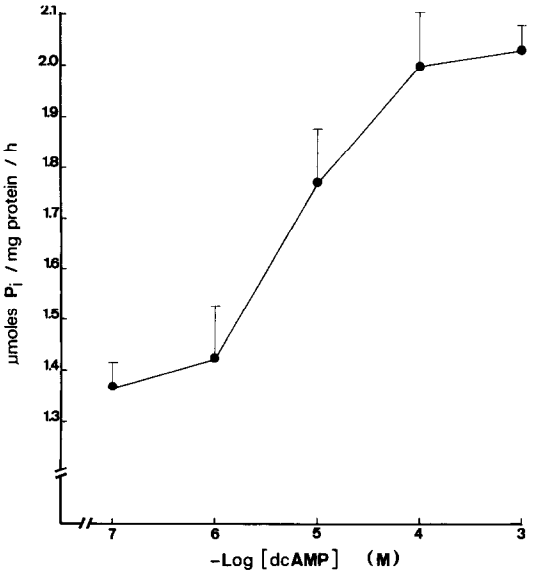

Fig. 4. Synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity as a function of dcAMP concentrations. Synaptosomes were incubated in the presence of varying concentrations of dcAMP (10^{-7}–10^{-3} M) at optimal concentrations of $CaCl_2$, $MgCl_2$ and ATP (0.34 mM or 1.2 μM free Ca^{2+}, 0.75 mM and 1.5 mM, respectively). The control value for (Ca^{2+}–Mg^{2+})-ATPase activity was 1.421 ± 0.098 μmoles P_i/mg protein/hr (N = 4). Data are presented as mean ± SE (N = 4). Statistically significant differences became apparent at 5×10^{-6} M dcAMP, with a $P < 0.001$. (Scheffé's test.)

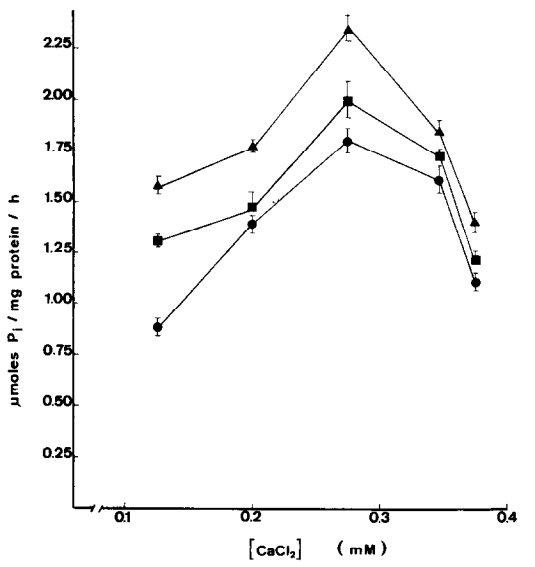

Fig. 5. Effects of cAMP-PK on (Ca^{2+}–Mg^{2+})-ATPase activity in SPM. SPM were incubated in reaction mixtures containing varying Ca^{2+} concentrations (0.150–0.375 mM $CaCl_2$ or 0.1–3.0 μM free Ca^{2+}), in the presence or absence of 20 μg cAMP-PK + 5 μM cAMP or 20 μg cAMP-PK + 5 μM cAMP + 10 μg cAMP-PK inhibitor. (●) Control, (▲) cAMP-PK + cAMP, (■) cAMP-PK + cAMP + cAMP-PK inhibitor. Each point represents mean ± SE (N = 3). Two-way analysis of variance of data indicate that statistically significant differences exist between cAMP-PK + cAMP vs control ($P < 0.001$) and cAMP-PK + cAMP vs cAMP-PK + cAMP + cAMP-PK inhibitor ($P < 0.01$).

mal ATPase inhibition (38%) was reached at 1 mM dipyridamole (Fig. 1). Furthermore, synaptosomes incubated in the presence of adenosine alone did not show ATPase activity.

SPM were incubated in reaction media containing varying concentrations of $CaCl_2$ (0.150–0.375 mM), as described in Materials and Methods. As shown in Fig. 2 control (Ca^{2+}–Mg^{2+})-ATPase activity increased from 0.150 to 0.275 mM Ca^{2+}. Maximal activity (1.922 ± 0.082, N = 5) was achieved at 0.275 mM Ca^{2+}. Higher Ca^{2+} concentrations inhibited ATPase activity. The presence of dcAMP (10^{-4} M) lacked effect on the SPM (Ca^{2+}–Mg^{2+})-ATPase activity at all Ca^{2+} concentrations assayed.

Ca^{2+} concentration–response curves (0.150–0.375 mM) in synaptosomes in the presence or the absence of 10^{-4} M dcAMP are represented in Fig. 3. Increasing the calcium concentration up to 0.34 mM enhanced the control synaptosomal enzyme activity. Maximal (Ca^{2+}–Mg^{2+})-ATPase activity (1.504 ± 0.075, N = 4) was achieved at 0.34 mM Ca^{2+}. Higher Ca^{2+} concentrations decreased the control synaptosomal ATPase activity. An amount of 10^{-4} M dcAMP, added to the incubation media, increased the synaptosomal (Ca^{2+}-Mg^{2+})-ATPase activity at all Ca^{2+} concentrations assayed. The maximal activity was elevated (38%) over the control value, but the extrasynaptosomal Ca^{2+} concentration to reach it was unaltered. Two-way analysis of variance of data in Fig. 3 indicate that the synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity was both dependent on Ca^{2+} concentration and on dcAMP.

dcAMP Induced a concentration-dependent increase on synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity. Maximal activity was reached at 10^{-4} M dcAMP and was 1.995 ± 0.096 μmoles P_i/mg protein/hr (about 35% over the control value). ($EC_{50} = 5 \times 10^{-6}$ M, Fig. 4).

SPM and synaptosomal basal, Mg^{2+}-ATPase activities were not altered by dcAMP.

The effect of cAMP-PK on (Ca^{2+}–Mg^{2+})-ATPase activity in SPM was investigated (Fig. 5). SPM were incubated in reaction mixtures containing varying Ca^{2+} concentrations (0.150–0.375 mM) in the presence or absence of 20 μg cAMP–PK and 5 μM cAMP. Maximal control enzyme activity (1.815 ± 0.052, N = 3) was achieved at 0.275 mM Ca^{2+}. CAMP–PK increased SPM (Ca^{2+}–Mg^{2+})-ATPase activity at all Ca^{2+} concentrations assayed. The maximal activity was enhanced (30%) over the control value, leaving unaltered the Ca^{2+} concentration necessary to reach it. cAMP-PK effect on SPM (Ca^{2+}–Mg^{2+})-ATPase activity was partially reversed (60%) by 10 μg cAMP–PK inhibitor.

Statistically significant differences were found between cAMP–PK vs control ($P < 0.001$) and cAMP–PK vs cAMP–PK inhibitor ($P < 0.01$). cAMP–PK, cAMP–PK inhibitor and cAMP alone did not modify either the Mg^{2+}–ATPase or the (Ca^{2+}–Mg^{2+})-ATPase activities in SPM (data not shown).

DISCUSSION

The indirect data presented above suggest that

ATP added extrasynaptosomally can be used as substrate for synaptosomal (Ca^{2+}–Mg^{2+})-ATPase activity, probably due to its ability to cross synaptic plasma membrane.

(Ca^{2+}–Mg^{2+})-ATPase activity stimulated by calmodulin [5, 7] has been amply described, but the possibility that intracellular cAMP modulates the ATPase activity is as yet a matter of controversy. In the heart and sarcoplasmic reticulum it has been shown that (Ca^{2+}–Mg^{2+})-ATPase activity is modulated by a cAMP-dependent phosphorylation reaction [8, 9]. In contrast, a study in SPM by Stauderman *et al.* found that cAMP was not able to modify enzyme activity [20]. It has to be pointed out however that only a direct action of cAMP was analysed. In agreement with the finding of Stauderman *et al.* dcAMP did not modify SPM (Ca^{2+}–Mg^{2+})-ATPase activity which makes a direct action of cAMP with the enzyme highly unlikely.

Intact synaptosomes contain a cAMP-dependent protein kinase [11–13]. It is therefore possible that brain (Ca^{2+}–Mg^{2+})-ATPase is also a substrate for cAMP-dependent protein kinase. This view is supported by the fact that dcAMP affected (Ca^{2+}–Mg^{2+})-ATPase only in synaptosomes and in a concentration-dependent manner by increasing maximal enzyme activity without modifying the extrasynaptosomal Ca^{2+} concentration necessary to reach it. This hypothesis is further supported by the fact that cAMP–PK increased maximal (Ca^{2+}–Mg^{2+})-ATPase activity in SPM, but did not modify the apparent affinity for Ca^{2+}. This effect was partially inhibited by cAMP–PK inhibitor.

Our results indicate that cAMP exerts its modulatory action on synaptosomal (Ca^{2+}–Mg^{2+})-ATPase, possibly by a cAMP-dependent phosphorylation reaction.

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