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Inactivation of ecto-ATPase activity of rat brain synaptosomes [1]

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Abstract

The ecto-ATPase activity of synaptosomes plasma membrane decays exponentially as a function of time from 0.35 ± 0.05 to 0.08 ± 0.02 μmol ATP hydrolyzed per min per mg synaptosome protein. The first-order rate constant of inactivation is dependent on the Mg-ATP concentration varying from 0.042 ± 0.001 min^{-1} with 30 μM ATP up to 0.216 ± 0.003 min^{-1} with 2 mM ATP. The non-hydrolyzable ATP analogue, β-γ-methyleneadenosine 5′-triphosphate, did not produce inactivation of the ecto-ATPase activity. Thus, the inactivation of the ecto-ATPase activity requires hydrolysis of ATP. Product inhibition can be excluded because ADP, AMP, adenosine and inorganic phosphate up to 1 mM had no effect on the inactivation of the ecto-ATPase. Concanavalin A partially protected against the ATP-dependent inactivation. The ecto-ATPase inactivation produced by Mg-ATP is partially reverted by centrifugation, removal of the supernatant and resuspension of synaptosomes in a fresh medium. This partial reversion occurs in parallel to the release to the supernatant of phosphorylated protein(s) of 90–95 kDa. Alkaline phosphatase treatment fully reverts the ecto-ATPase inactivation. We conclude that the ATP-induced inactivation is mediated, at least partially, by phosphorylation of membrane proteins.

Keywords: Synaptosome; Plasma membrane; Ca^{2+}/Mg^{2+} ecto-ATPase; Extracellular ATP; Concanavalin A

1. Introduction

The role of ATP on cell signalling seems to be mediated through ATP receptors on the extracellular surface of the cell membrane [1]. In addition, ATP can serve as a substrate for ecto-protein kinases, being involved in the extracellular phosphorylation of proteins [1–3]. The local extracellular ATP concentration is modulated by the activity of ectonucleotidases [1,4], which are responsible for hydrolysis of extracellular ATP. In synaptosomes, extracellular ATP is sequentially dephosphorylated to ADP, AMP and adenosine [5]. The first step, the hydrolysis of ATP, is mediated by an ecto-ATPase activity. It is not clear whether the hydrolysis from ADP to AMP is carried out by the same enzyme or by an additional ecto-enzyme [4,6]. These ecto-enzymes are likely play a key role in the modulation of extracellular ATP-induced effects.

The physiological roles of these ecto-ATPases are still unclear [4]. Several hypothesis have been raised. Alterations in ecto-ATPase activity have been implied in the development and maintenance of human temporal lobe epilepsy [7]. Moreover, an ecto-ATPase of hepatocytes has been identified as a substrate for the insulin receptor tyrosine kinase [8]. Recently [9], several isoforms of the hepatocyte cell-adhesion molecule (cell-CAM 105) have been shown to be identical with the liver plasma membrane ecto-ATPase and to be phosphorylated in vitro. In addition, an ecto-enzyme that binds ATP is either a potential candidate for being an ATP receptor itself, or may be located near the receptor to modulate the ATP availability. Extracellular ATP modulates the cellular excitability by changing the membrane permeability and the cytoplasmic Ca^{2+} level in several cell types [10,11]. Recently, we have shown that extrasynaptosomal ATP increases the intrasynaptosomal free Mg^{2+} concentration and that ATP has to be hydrolyzed to exert this effect [12].

Ecto-ATPases from plasma membrane have been isolated from several tissues including rabbit skeletal muscle [13,14], sheep kidney medulla [15], rat heart [16], and

Abbreviations: AMPPCP, β-γ-methyleneadenosine 5′-triphosphate; Con-A, concanavalin A; U, μmol product per min per mg protein

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[1] We will refer to the activities studied here as Mg^{2+}-ATPase, Ca^{2+}-ATPase or ADPase, depending on the actual substrate: Mg-ATP, Ca-ATP, ADP, respectively. We do not imply the existence of separate enzymes.

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bovine brain synaptic membranes [17]. The ecto-ATPases seem to differ from each other concerning their molecular weights and subunit structure but share certain properties that distinguish them from the Ca^{2+} pump: namely, they are activated by Ca^{2+} and Mg^{2+} with low affinity and hydrolyze other nucleoside triphosphates besides ATP [4,18]. In contrast, the Ca^{2+} pump is activated by micromolar concentrations of Ca^{2+} and requires Mg-ATP as a substrate.

In several rat tissues, including skeletal muscle [19], heart [16,20], myometrium [21] and isolated intact mesenteric small arteries [22], the Mg^{2+}/Ca^{2+} ATPase activity is not linear as a function of time. However, the molecular mechanisms underlying this inhibition remain to be elucidated. We have noticed earlier that the ATPase activity of rat brain synaptosomes decays as a function of time during its assay in vitro [23]. In this study, we report that the inhibition process of the Mg^{2+}-ATPase(1) activity of synaptosomes is modulated by Mg-ATP, probably through phosphorylation of proteins associated with the plasma membrane.

2. Materials and methods

Synaptosomes have been prepared from Wistar rat brains as indicated by García-Martín and Gutiérrez-Merino [24]. Plasma membrane vesicles were prepared from synaptosomes by hypotonic lysis as described elsewhere [24]; these preparations will subsequently be referred to as synaptic plasma membrane vesicles. The percentage of inverted vesicles was estimated as in Ref. [24] and it was found to be approx. 40% of total vesicles. Protein concentration was measured by the method of Bradford [25]. The ATPase activity was measured at 25°C using the coupled enzyme pyruvate kinase-lactate dehydrogenase [24] and by following the release of inorganic phosphate using a modified Fiske and Subbarow method [26]. The composition of the standard assay medium was the following: 50 mM Tes (pH 7.4), 0.1 M KCl, 2 mM $MgCl_2$, 2 mM ATP, 2 mM β-mercaptoethanol, 5 mM sodium azide and 125 μg of protein per ml. For the kinetic assays with the coupled enzyme system, 0.42 mM phosphoenolpyruvate, 0.22 mM NADH, 35 μg/ml pyruvate kinase and 30 μg/ml lactate dehydrogenase were added to the assay medium. The protein concentration in these assays was 25 μg of protein per ml. Since the coupled enzyme system requires Mg^{2+}, this assay was only used for Mg^{2+}-ATPase activity measurements. Whenever the Mg^{2+}-ATPase activity of broken synaptosomes or vesicles was measured, 1 mM EGTA was included in the assay to eliminate the contribution of $(Ca^{2+} + Mg^{2+})$-ATPase activity (Ca^{2+} pump). Other modifications of the assay medium are indicated in the text or legends to the figures where appropriate. When the coupled enzyme assay was used, the ATPase activity was calculated as a function of time from the slope of the absorbance change at 340 nm at different times (t_i) after starting the reaction, averaging from ($t_i - 0.5$) to ($t_i + 0.5$) min. The results obtained with this operational protocol were identical (±5%) to those produced at t_i by derivatization of the non-linear regression function fit to selected series of absorbance versus time datasets. This is a difference lower than the average error of the data reported in this study, obtained from, at least, three different synaptosome preparations, in each case by duplicate measurements. To get a faster response of the coupled enzyme system, the concentrations of pyruvate kinase and of lactate dehydrogenase were twice those currently used in previous studies from this laboratory [23,24,27]. Routinely, we confirmed that in less than 15 s after starting the reaction the coupled enzyme system was operating under steady-state conditions, by means of addition of 10–20 nmol of ADP at different times after the reaction was started with sarcoplasmic reticulum membranes or purified Ca^{2+},Mg^{2+}-ATPase prepared as in Ref. [27]. The Na^+,K^+-ATPase contribution to the total ATPase activity was assessed by measuring the effect of 1 mM ouabain. Under our experimental conditions this contribution was less than 5% of total Mg^{2+}-ATPase activity, and, therefore, was neglected.

ADPase activity was determined by following the release of inorganic phosphate as indicated above for the Ca^{2+}/Mg^{2+}-ATPase, using 2 mM ADP as substrate.

The pretreatment of synaptosomes and vesicles with alkaline phosphatase was done as in Ref. [28]. Briefly, synaptosomes or vesicles in 50 mM Tes (pH 7.7) and 0.1 M KCl were incubated at 37°C for 20 min with 30–40 μg/ml alkaline phosphatase. To eliminate the alkaline phosphatase, vesicles or synaptosomes were spun in a microcentrifuge for 20 min at $20\,000 \times g$. Pellets were washed three times in 10 volumes of ice-cold 50 mM Tes (pH 7.4) and 0.1 M KCl and then resuspended in the same buffer. The removal of alkaline phosphatase was checked measuring the rate of hydrolysis of 2 mM p-nitrophenyl phosphate.

2.1. Phosphorylation assays

Synaptosomes were diluted at a final concentration of 1 mg protein per ml in 50 mM Tes (pH 7.4), 0.1 M KCl and 2 mM $MgCl_2$. Reactions were started by addition of 1 mM [γ-^{32}P]ATP (2.8×10^5 dpm/nmol) and stopped after 30 min at room temperature by addition of 50 μl to 100 μl of a solution containing: 2% (w/vol) sodium dodecyl sulfate, 1.25% (vol/vol) β-mercaptoethanol, 20% (vol/vol) glycerol, 0.025% (w/vol) bromophenol blue and 0.312 mM Tris-HCl (pH 6.8) (stop solution), and heating at 100°C for 3 min. SDS-PAGE of proteins was done on 10% polyacrylamide gels using the discontinuous buffer system of Laemmli [29]. Gels were stained with Coomassie Brilliant Blue (R-250) and dried for autoradiography.

2.1.1. HPLC

Separation of the nucleotides was performed using an ion-pair reverse-phase system on a Nucleosil 120 C18 (5 μm; 15 × 0.46 cm) column at 25 °C. The solvents used were: (A) 100 mM KH_2PO_4 (pH 6), containing 8 mM tetrabutylammonium phosphate; (B) solvent A and methanol (70/30 vol/vol) (pH 5.5). The following conditions were used:

Time (min)	% of solvent A
0–2.5	100
5	80
10	60
13–19	0
19.1–22	100

At a flow rate of 1.5 ml/min, the elution of nucleotides was monitored at a wavelength of 260 nm. The retention times typically obtained were: adenosine 8.48 min, AMP 8.92 min, ADP 13.81 min and ATP 16.98 min.

2.2. Other methods

The densitogram of the autoradiographs was obtained by scanning the film on a Shimadzu CS-9000 densitometer.

Free Ca^{2+} concentrations were fixed using EGTA, with an apparent dissociation constant of the Ca^{2+}-EGTA complex of $10^{-7.2}$ at pH 7.4 [30]. When needed the concentrations of free cations, Ca-ATP and Mg-ATP in the assay medium were calculated by using a program developed for multiple equilibrium analysis by Perrin and Sayce [31]. The following dissociation constants (K_d) were used: K_d (Ca-ATP) = 1.17×10^{-4} M and K_d (Mg-ATP) = 2.46×10^{-5} M [32].

2.3. Expression of results

Results are given as mean ± standard deviation for the number of preparations (n) given in parentheses. Unless stated otherwise, the data shown in the figures are the average of the results obtained with three different preparations of synaptosomes, in each case by duplicate ($n = 6$).

The ATPase activity is expressed as U, i.e., μmol ATP hydrolyzed/min/mg of protein.

2.4. Materials

[γ-^{32}P]ATP was obtained from DuPont (Belgium). Alkaline phosphatase (2000 U), pyruvate kinase (200 U) and lactate dehydrogenase (550 U) were from Boehringer Mannheim. Lectins, nucleotides, buffers and salts were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

3. Results

3.1. Ca^{2+}/Mg^{2+} activated ATPase in synaptosomes and in synaptic plasma membrane vesicles

Fig. 1 shows the time-dependence of the Mg^{2+}-ATPase activity measured in synaptosomes and in synaptic plasma membrane vesicles. The Mg^{2+}-ATPase activity decayed exponentially as a function of time up to 20% of the initial activity. Identical results were obtained when the ATPase activity was measured as a function of time using the modified Fiske-Subbarow and the coupled enzyme methods indicated in Section 2 (data not shown). This time-dependent inhibition of the ATPase activity is similar in synaptosomes and in synaptic plasma membrane vesicles, although those synaptic plasma membrane vesicles are approx. 40% inverted plasma membrane vesicles [24,33]. The data shown in Fig. 1 suggest that this ATPase activity is mostly due to ecto-enzyme(s), because a similar inhibition is observed in broken and intact synaptosomes, and the Mg^{2+}-ATPase activity of synaptic plasma membrane vesicles is only slightly higher than that of intact synaptosomes. These data unravelled the presence of an inactivation process of the ecto-ATPase activity of the plasma membrane of rat synaptosomes. The decay of the Mg^{2+}-ATPase activity can be fitted to a single exponential process. The first-order half-time of inactivation obtained from data shown in the Fig. 1 were 1.41 ± 0.12 ($n = 9$), 2.66 ± 0.25 ($n = 9$) and 2.47 ± 0.23 ($n = 6$) min for vesicles, intact or broken synaptosomes, respectively.

This inhibition is dependent on ATP in the assay medium (Fig. 2), because without ATP there was no measurable inactivation. Additionally no significant inactivation was obtained with the non-hydrolyzable ATP-analog, AMPPCP, in the preincubation medium, showing that ATP has to be hydrolyzed to produce the observed inhibi-

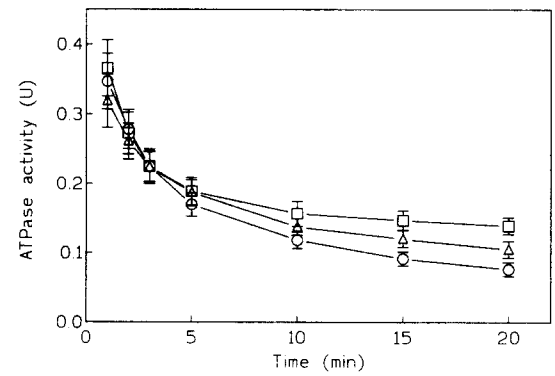

Fig. 1. Mg^{2+}-ATPase activity of intact (open triangles) and broken (open circles) synaptosomes and of synaptic plasma membrane vesicles (open squares). The activity was measured as a function of time in the standard assay medium using the coupled enzyme system at 25°C as indicated in Section 2. The error bars represent the standard deviation of six independent experiments.

tion of the Mg^{2+}-ATPase activity. The possibility of product inhibition was investigated, and the effects of all the possible products were explored. The concentrations of the products accumulated in the reaction medium during the assay (ADP, AMP and adenosine) were measured by HPLC. The reaction was started with 1 mM ATP and stopped with 5% perchloric acid after 10 min, at time that produced a large inactivation of the ecto-ATPase activity (see below, Fig. 5). The samples were neutralized and analyzed by HPLC as indicated in Section 2. The final concentration of ADP, AMP and adenosine produced were 0.38 mM, 0.227 mM and < 14 μM, respectively. The addition to the assay medium of ADP, AMP, adenosine and phosphate up to 1 mM had no effect on the inactivation process of the ATPase activity. This indicated that the inactivation of the Mg^{2+}-ATPase activity is not due to the accumulation of the products of ATP hydrolysis.

It has been reported that Mg^{2+} or Ca^{2+} produce a similar stimulation of ecto-ATPase activities [4,18]. Thus, we measured the effect of replacing 2 mM Mg^{2+} by 2 mM Ca^{2+} in the assay medium. As shown in Fig. 3, in the presence of Ca^{2+} the ATPase activity also decayed exponentially. The effects of Ca^{2+} and Mg^{2+} were not additive, thereby indicating that both Ca^{2+} and Mg^{2+}-ATPase activities are probably carried out by the same enzyme(s). It is noteworthy that the high affinity Ca^{2}-stimulated and Mg^{2+}-dependent-ATPase activity, determined from the difference of the rate of P_i release in the presence of 2 mM Mg^{2+} and 50 μM Ca^{2+} and the rate of P_i release after addition of 2 mM EGTA remained constant during incubation times up to 30 min (data not shown).

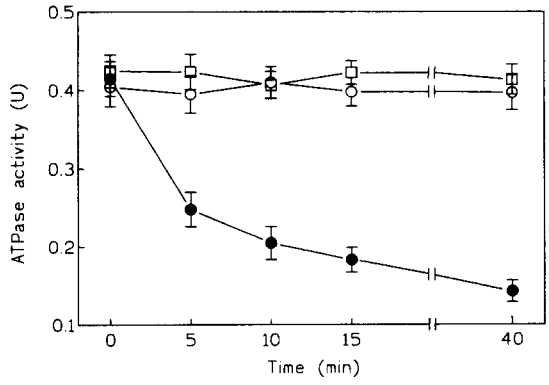

Fig. 2. Ecto-ATPase activity of synaptosomes as a function of the incubation time in the presence and absence of ATP and in the presence of AMPPCP. Synaptosomes were incubated in 50 mM Tes (pH 7.4), 0.1 M KCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol and without ATP (open squares), or with 2 mM ATP (filled circles), or with 2 mM AMPPCP (open circles) for different times. At the times indicated in the abscissa, 0.1 ml were pooled to measure initial rate of the ATPase activity in the standard assay medium. The initial rate activity data shown in the figure were obtained from the initial slope of the traces of absorbance versus time, averaged during the first minute. The ecto-ATPase activity was measured using the coupled enzyme system at 25°C as indicated in Section 2

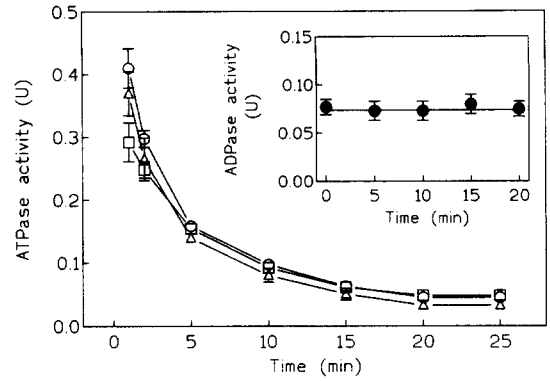

Fig. 3. Ca^{2+} or Mg^{2+} ATPase activity of synaptosomes. The ATPase activity was measured at 25°C in the presence of 2 mM Mg^{2+} (open circles) or 2 mM Ca^{2+} (open squares) or 2 mM Mg^{2+} and 2 mM Ca^{2+} (open triangles), by following the release of inorganic phosphate as a function of time as indicated in Section 2. Open circles, Mg^{2+}-ATPase activity; open triangles, Ca^{2+}-ATPase activity and open squares, Ca^{2+}/Mg^{2+}-ATPase activity. Inset: ADPase activity obtained under identical conditions except that 2 mM ATP was substituted by 2 mM ADP.

Previous studies have shown that both ATP and ADP are substrates of the ecto-ATPase [4,18]. We have determined the ADPase activity in the experimental conditions used for ATPase measurements by replacing ATP by ADP. As shown in the inset of Fig. 3, the added ADP was hydrolyzed by the synaptosomes with a specific activity that is independent of the incubation time. This is consistent with the non-involvement of ADP and of products of ADP hydrolysis in the inactivation of the ecto-ATPase as discussed above.

The involvement of non-specific phosphatases in the hydrolysis of ATP can be neglected, as 2 mM p-nitrophenyl phosphate was hydrolyzed with a rate 0.006 ± 0.002 μmol/min per mg protein.

3.2. Modulation of the inactivation of the ecto-ATPase

It has been shown that con-A activates some non-specific Mg^{2+}-ATPases [4]. Therefore, we measured the effect of preincubation with con-A and succinyl Con-A on the Mg^{2+}-ATPase activity of synaptosomes. Fig. 4 shows the effects of preincubation with ATP and con-A on the ATPase activity. We have found that con-A can partially prevent the time-dependent inactivation produced by ATP. However, when the membranes were preincubated with ATP and con-A, con-A was unable to exert a significant protection against the inactivation of Mg^{2+}-ATPase produced by ATP. Succinyl Con-A, a dimeric derivative of the lectin, which blocks its ability to produce cross-linking of membrane proteins [34], had no effect on the inactivation process at concentrations up to 150 μg/ml. This suggests that the cross-linking effect of con A is involved in the protection afforded by this lectin against inactivation of the Mg^{2+}-ATPase activity by ATP.

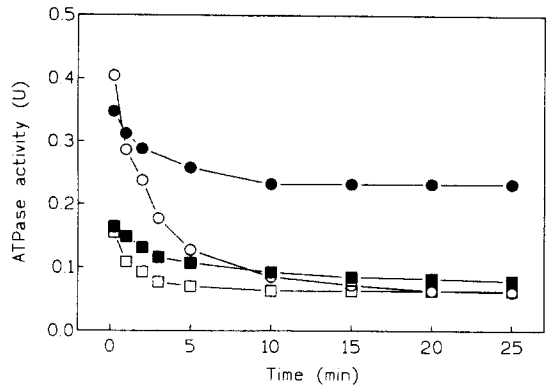

Fig. 4. Effect of preincubation with con-A and ATP on the time dependence of the Mg^{2+}-ATPase activity of synaptosomes. Synaptosomes were preincubated in the standard assay medium at 25°C for 15 min without ATP or con-A (open circles), or supplemented with: 2 mM ATP and 50 μg/ml con-A (open squares), 2 mM ATP only (filled squares), or 50 μg/ml con-A only (filled circles). The Mg^{2+}-ATPase activity was measured as a function of time in the standard assay medium using the coupled enzyme assay. The data shown are those obtained in a typical experimental series, and are representative of the results obtained with three different preparations of synaptosomes, in each case by duplicate ($n = 6$).

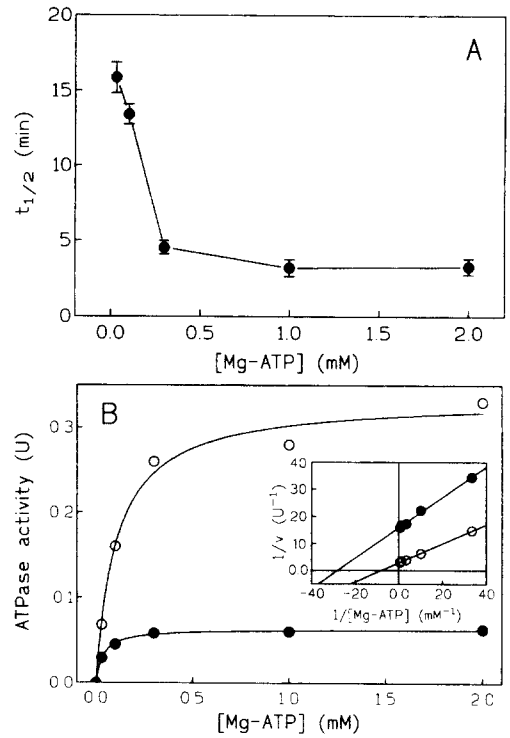

Fig. 5. Dependence of the Mg^{2+}-ATPase activity of synaptosomes on the concentration of Mg-ATP. The ATPase activity was measured as a function of time with the coupled enzyme system at 25°C as indicated in Section 2. The Mg-ATP concentration was varied from 0.03 to 2 mM in the presence of 2 mM $MgCl_2$ in the assay medium. (A) Dependence of the half-time of the inactivation process ($t_{1/2}$) upon Mg-ATP concentration. (B) Dependence of the Mg^{2+}-ATPase activity upon Mg-ATP concentration: filled circles, final or stable ATPase activity and open circles, labile or inactivated ATPase activity. Inset: Linewaver-Burk presentation of the data.

As the inactivation of the ecto-ATPase is an ATP-dependent process, we have studied the dependence of the inactivation with ATP concentration. The results obtained are shown in the Fig. 5. The increase of ATP concentration from 0.03 to 2 mM accelerated the inhibition process (panel A) and increased both: the labile and the final ATPase activities (panel B). The values of the apparent K_m for Mg-ATP obtained from reciprocal plots (insert of panel B) were 0.122 mM and 0.035 mM for the labile and for the final activities, respectively. These values are similar to the K_m values of ATP reported for another ecto-ATPases [5,35].

The possibility that the inactivation of the Mg^{2+}-ATPase activity could be related with aggregation of synaptosomes was considered. However, an increase of concentration of the synaptosomes in the assay medium between 0.07 and 1 mg of protein/ml did not produce a significant change on the kinetic parameters of the inactivation of Mg^{2+}-ATPase (half-time, $t_{1/2}$, and final Mg^{2+}-ATPase activity). Therefore, the simplest hypothesis of aggregation as the cause of the inactivation of the Mg^{2+}-ATPase activity can be excluded.

The effects of preincubation with Mg^{2+} and Ca^{2+} on the inactivation of the Ca^{2+}/Mg^{2+}- ATPase were investigated. In a separate series of experiments, synaptosomes were incubated in the presence of several concentrations of these cations, from 0 to 5 mM, and then the ATPase activity was measured in the standard assay medium. The results obtained are presented in the Figs. 6 and 7, respectively. The increase of Mg^{2+} and Ca^{2+} concentration in the preincubation medium resulted in the acceleration of inactivation process. The addition of the Ca^{2+} ionophore calcimycin up to 40 μg/mg synaptosomal protein had no

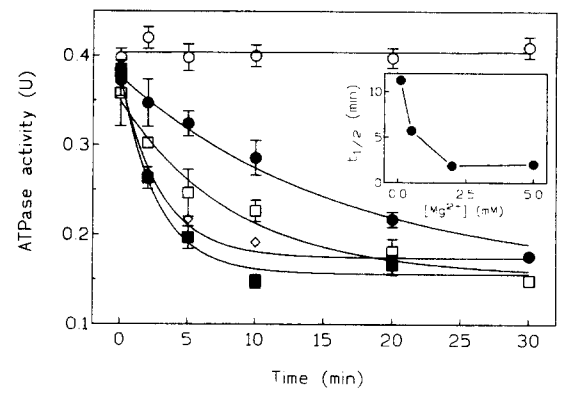

Fig. 6. Effect of Mg^{2+} on the time dependent inactivation of the ecto-ATPase activity. The ATPase activity was measured with the coupled enzyme system at 25°C. Synaptosomes were incubated during the times indicated in the abscissa in the presence of several Mg^{2+} concentrations: 0.1 mM (filled circles), 0.5 mM (open squares), 2 mM (filled squares) and 5 mM (open diamonds). Open circles, results obtained for incubation of synaptosomes without added Mg^{2+} and with 1 mM EDTA. At the times indicated in the figure the initial ATPase activity was determined in the standard assay medium as indicated in the legend to the Fig. 2. Inset: dependence of the half-time of the inactivation process ($t_{1/2}$) upon Mg^{2+} concentration.

effect on the time dependence of the inactivation of the ATPase activity (results not shown).

Increasing the temperature from 25 to 37°C increased the final ATPase activity, from 0.08 ± 0.01 U to 0.13 ± 0.006 U ($n = 6$), and decreased the half-time of the inactivation process, from 3.15 ± 0.14 min to 2.23 ± 0.1 min ($n = 6$). These results showed that the final Mg^{2+}-ATPase activity and the inactivation process are temperature-sensitive.

3.3. The inactivation of the ecto-ATPase activity is a reversible process

It has been reported that the inactivation of Ca^{2+}/Mg^{2+} nucleotide phosphohydrolases in myometrium can be fully reverted by washing the cells after preincubation with ATP [21]. This raised the question of whether the ATP-inactivation of Ca^{2+}/Mg^{2+}-ATPase of synaptosomes is reversible. Fig. 8A shows that the labile ATPase activity could be partially restored by centrifugation of synaptosomes after preincubation with ATP, removal of the supernatant and resuspension in fresh medium. The partial reversal of the ATPase activity is not observed when the pellet is resuspended in the incubation medium. Because the blockade of the recovery of the ecto-ATPase activity when synaptosomes are resuspended in the supernatant cannot be explained by the accumulation of the products of ATP hydrolysis (see above), we investigated next whether protein phosphorylation could be underlying the ATP-inactivation of the ecto-ATPase activity.

If the inactivation of the ecto-ATPase produced by ATP is due to protein phosphorylation, treatment of synaptosomes with alkaline phosphatase should revert the observed inactivation. To determine whether the observed

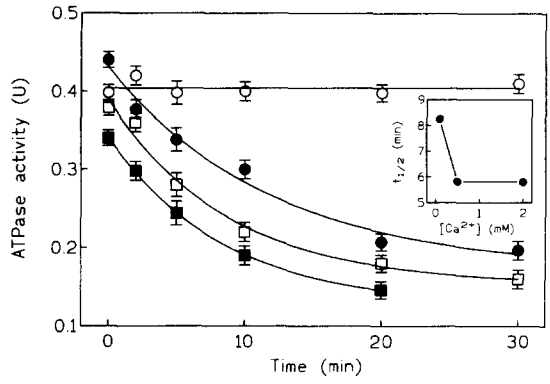

Fig. 7. Dependence on the Ca^{2+} concentration of the time-dependent inactivation of the ecto-ATPase activity. The ATPase activity was measured with the coupled enzyme system at 25°C. Synaptosomes were incubated during the times indicated in the abscissa in the presence of several Ca^{2+} concentrations: 0.1 mM (filled circles), 0.5 mM (open squares), and 2 mM (filled squares). Open circles, results obtained on incubation of synaptosomes without added Ca^{2+} and with 1 mM EDTA. At the times indicated in the figure the initial ATPase activity was determined in the standard assay medium. Inset: dependence of the half-time of the inactivation process ($t_{1/2}$) upon Ca^{2+} concentration.

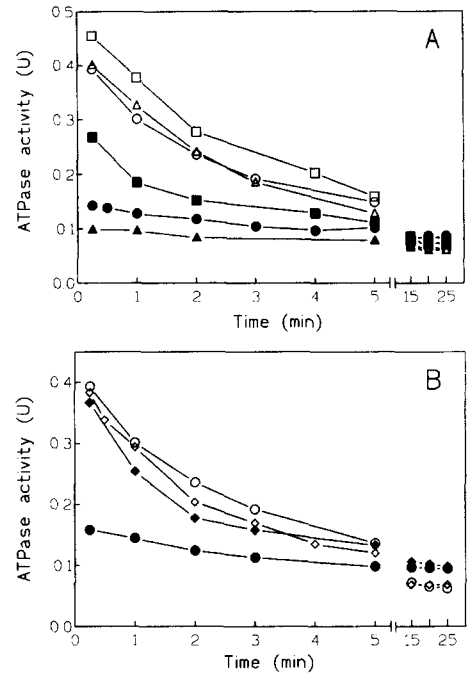

Fig. 8. Reversal of the ATP-induced inactivation of the ecto-ATPase activity of synaptosomes. (A) ATPase activity as a function of time of synaptosomes preincubated with (filled symbols) and without (open symbols) ATP for 30 min at 25°C in the standard assay medium containing 2 mM $MgCl_2$. Circles, Mg^{2+}-ATPase activity of control, not washed, synaptosomes. Squares, Mg^{2+}-ATPase activity of the synaptosome pellet resuspended in fresh reaction medium. Triangles, Mg^{2+}-ATPase activity of synaptosome pellet resuspended in the supernatant. (B)ATPase activity as a function of time of synaptosomes after preincubation with (filled symbols) and without (open symbols) ATP for 30 min at 25°C in the standard assay medium containing 2 mM $MgCl_2$. After the preincubation synaptosomes were treated with alkaline phosphatase (diamonds) as indicated in Section 2. Also included are the results obtained for controls (circles) carried out without alkaline phosphatase treatment. The data shown are those obtained in a typical experimental series, and are representative of the results obtained with three different preparations of synaptosomes, in each case by triplicate ($n = 9$).

inhibition of the Mg^{2+}-ATPase activity could be reverted by alkaline phosphatase treatment, synaptosomes were preincubated at 25°C for 30 min with 2 mM Mg^{2+} and 2 mM ATP. Then the synaptosomes were treated with alkaline phosphatase as indicated in Section 2. The ecto-ATPase activity of synaptosomes pretreated with alkaline phosphatase is shown in the Fig. 8B. Two controls were run in these series of experiments: (1) synaptosomes preincubated in the absence of ATP were also treated with alkaline phosphatase, and (2) synaptosomes preincubated with ATP were centrifuged and resuspended in the standard assay medium but not treated with alkaline phosphatase (see Fig. 8A). In contrast to the partial reversion of the Mg^{2+}-ATPase activity obtained by centrifugation and resuspension in a fresh medium (washing step), the treatment with alkaline phosphatase fully reverted the inhibition induced by ATP. Synaptosomes preincubated without ATP were unaffected by the washing step. These results indicated that the ATP-induced inhibition is modulated by protein

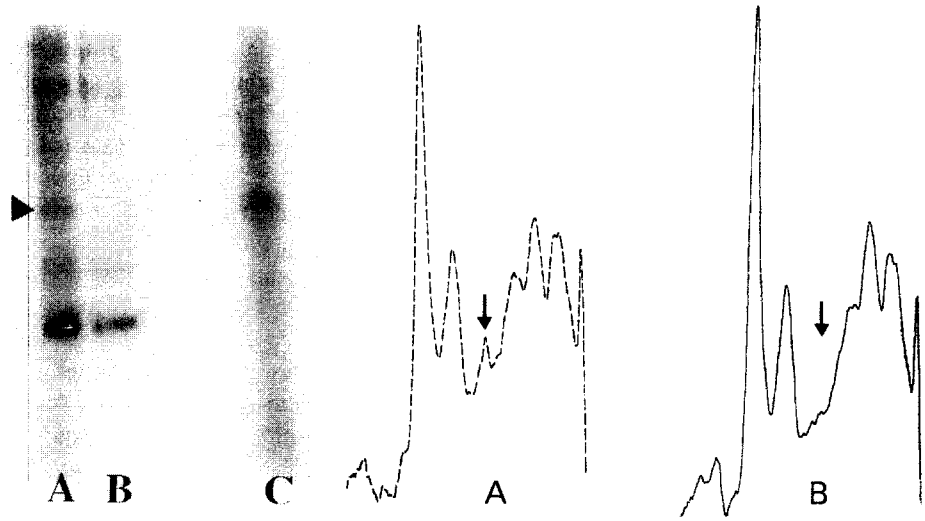

Fig. 9. Effect of the washing step, which produced reversion of the inactivation of the ecto-ATPase activity, on the phosphorylation pattern of rat brain synaptosome proteins, revealed by autoradiography. Synaptosomes were incubated in an assay medium containing 1 mM [γ-^{32}P]ATP (2.8×10^5 dpm/nmol) for 30 min at 25°C, to allow for completion of the inactivation process of the ecto-ATPase (see Section 2 for further experimental details). Then, an aliquot of this suspension was added to the stop solution and subjected to SDS-PAGE (33 μg of protein), as indicated in Section 2 (lane A of the autoradiography). The rest of the suspension was spun at 4°C for 20 min at 20000 × g, and the pellet was resuspended in a fresh medium containing 100 mM KCl and 50 mM Tes (pH 7.4). Samples of the resuspended pellet and of the supernatant were treated for SDS-PAGE as indicated in Section 2, then loaded on lanes B (33 μg of protein) and C (3 μg of protein), respectively. The autoradiography of the gel was obtained with 100 h exposure at −80°C, using an amplifying screen. The densitograms of lanes A and B of the autoradiography are shown in the figure. The arrows in the densitograms indicate the position of the protein band centered at 93 kDa band. The results shown are those obtained in a typical experimental series, and are representative of the results obtained with three different preparations of synaptosomes, in each case by triplicate ($n = 9$).

phosphorylation. In an attempt to clarify this point, synaptosomes were incubated with 1 mM [γ-^{32}P]ATP and 2 mM Mg^{2+} at 25°C. After 30 min, the synaptosomes were spun and resuspended in: (1) the same reaction mixture and (2) in a fresh reaction mixture. The samples were analyzed by polyacrylamide gel electrophoresis and autoradiography. The results obtained are shown in Fig. 9. The results revealed that the extent of phosphorylation of proteins of synaptosomes with molecular weights between 90–95 kDa was significantly decreased by a simple washing step of synaptosomes. Partial phosphorylation was already observed after 1 min incubation with 1 mM [γ-^{32}P]ATP of synaptosomes at a protein concentration of 1 mg per ml (data not shown). After centrifugation of the synaptosomes, a phosphorylated band of 90–95 kDa appeared in the supernatant (in parallel to a large decrease of this band in the pellet). These results indicated that the binding of protein(s) of 90–95 kDa to the plasma membrane is modulated by phosphorylation/dephosphorylation.

4. Discussion

The Mg^{2+}/Ca^{2+} ecto-ATPase activity of synaptosomes decays as a function of time up to approx. 20% of its initial value. The ecto-ATPase activity studied here is different to the Ca^{2+} pump, which does not show a time-dependent inactivation and is stimulated by much lower Ca^{2+} concentrations. It also shows that ecto-ADPase activity of synaptosomes remains constant as a function of time. The decrease of the rate of ATP hydrolysis with the incubation time was not due to a decrease of the substrate concentration because the ATP concentration in the assay medium is kept constant when the coupled enzyme assay is used. In intact myometrial cells and in isolated plasma membranes derived from them has been shown that the inactivation of the labile ATPase is probably due to product inhibition caused by the accumulation of inorganic phosphate in the assay medium, being the IC50 about 0.3 mM. Nevertheless, in our system inhibition by product can be excluded since preincubation with adenosine, Pi, AMP and ADP up to 1 mM had no effect on the inactivation process. These results are similar to those reported for rat heart purified Ca^{2+}/Mg^{2+} ATPase [16], skeletal muscle low-density vesicles [19] and for intact rat mesenteric small arteries [22]. However, ATP hydrolysis is required to observe loss of activity of the ecto-ATPase, because incubation with AMPPCP does not produce a significant inactivation of this enzymatic activity (Fig. 2).

The inhibition of the ecto-ATPase activity can be fit by an apparent first order process. The overall pattern of the rate of ATP hydrolysis shown in the Fig. 1 is remarkably similar to that seen when ATP is hydrolyzed by the ecto-ATPase activity of myometrial cells [21]. A similar pattern was also described with the ecto-ATPase purified from the plasma membrane of rat heart [16]. With these systems the hydrolysis of ATP was not linear as a function of time.

We investigated the pattern of ATP hydrolysis over a

range of ATP concentrations between 30 μM and 1 mM, because it has been estimated that the ATP concentration at cholinergic synapse [36] and other synapses [37] could reach between 70 μM and 1 mM. The apparent K_m value for ATP of the ecto-ATPase was similar to that reported elsewhere for the ecto-ATPase activity of synaptosomes [35] and of striatal cholinergic synapse [5], but significantly lower than that reported for myometrium and rat heart sarcolemma [21,38]. In intact rat mesenteric small arteries, Juul et al. [22] showed that ATP modulated the ecto-ATPase activity, which was reported to be ATP concentration-dependent and that led to a decreased affinity for the substrate. Although we have also found a change in substrate affinity, in this case we found a moderate increase of affinity, i.e., from 122 to 35 μM.

The effect of Mg^{2+} and Ca^{2+} on the inactivation process of the ecto-ATPase demonstrated that both Mg-ATP and Ca-ATP produced inhibition of the ecto-ATPase. It is to be recalled that the ecto-ATPase can use both Mg-ATP and Ca-ATP as substrate. The rate constant of the inactivation process of ATPase is similar in the presence of Mg-ATP or Ca-ATP in the assay medium. However, as shown in Figs. 6 and 7, the preincubation with Ca^{2+} slows down the inactivation of the ecto-ATPase, pointing out an additional role of Ca^{2+} in the modulation of the inactivation process of the ecto-ATPase induced by Mg-ATP.

Other ecto-ATPases, such as the skeletal muscle transverse tubule Mg^{2+}-ATPase and heart plasma membrane Mg^{2+}/Ca^{2+} ATPase, have been suggested to be glycoproteins because of its interaction with con-A [38,39]. Preincubation of synaptosomes with con-A blocked the inactivation of the ecto-ATPase produced by ATP. Con-A is unable to prevent the ATP-induced inactivation of the ecto-ATPase when ATP is present before con-A addition, therefore suggesting that con-A may prevent ATP binding to a modifier site. Once ATP is bound to this putative site, con-A cannot induce their antagonizing effects. Inactivation by ATP binding to a modulator, non-catalytic, site should be a reversible process. The reversibility of the ATP-induced inactivation of the ecto-ATPase was confirmed experimentally.

The ATP-induced inhibition is fully reverted by treatment with alkaline phosphatase. This indicated that phosphorylation of some components of the membrane is involved in the mechanism underlying this inhibition. Ecto-protein phosphorylation would constitute a reversible process that may modulate the external concentration of ATP, modulating thereby ATP-dependent cell signalling pathways. This hypothesis is also supported by the correlation between partial reversion of the ATPase activity and the release to the external medium of phosphorylated protein(s) of synaptosomes of 90–95 kDa (Fig. 9). In this regard, it may be pointed out that an ecto-ATPase of M_r 120 000 has been identified [8] in liver cells that is phosphorylated in tyrosine residues by the kinase activity of the insulin receptor. It has been suggested that this phosphorylation could affect the enzymatic activity of the ecto-ATPase. Partial reversion of the ATP-induced inactivation of the ecto-ATPase is attained by centrifugation of synaptosomes and resuspension in a fresh medium. As the phosphorylated protein(s) of 90–95 kDa is (are) largely released to the supernatant, the simplest hypothesis is that it corresponds to protein(s) associated with the external surface of the plasma membrane of synaptosomes, whose association with the plasma membrane is modulated by phosphorylation. A 90–95 kDa protein is likely to play a major role of the ecto-ATPase activity of synaptosomes because resuspension of the synaptosome pellet in the supernatant maintains the ecto-ATPase activity largely inactivated, and treatment with alkaline phosphatase reverts the inhibition of the ATPase. It is worth noting here that Beeler et al. [19] proposed that the regulation by ATP of the ecto-ATPase of low-density vesicles from rat skeletal muscle requires the mobility of proteins within the membrane.

In summary, the results reported in this paper show that the ecto-ATPase activity of rat brain synaptosomes is largely inactivated as a function of the time of incubation with ATP following a first-order kinetic process. This inactivation is ATP-dependent and requires ATP hydrolysis, and is likely mediated by phosphorylation of 90–95 kDa protein(s) associated with the external surface of the plasma membrane of synaptosomes.

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