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Isolation of a Ca^{2+} or Mg^{2+}-activated ATPase (ecto-ATPase) from bovine brain synaptic membranes

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An ATPase was isolated from synaptosomal plasma membranes derived from bovine cerebral cortex. The protein has an apparent molecular mass of 50 kDa and a p*I* of 5.3 to 5.9. It can be labelled by incubation of intact synaptosomes with azido-GTP or azido-ATP. The isolated ATPase can be activated to a similar extent in the presence of millimolar concentrations of Mg^{2+} or Ca^{2+}. It does not hydrolyze ADP. Maximal activity is obtained between pH 7.5 and 8.5. Typical inhibitors of cytoplasmic ATPases do not affect enzyme activity. The enzyme is specifically inhibited after previous incubation of intact synaptosomes in the presence of the slowly membrane-permeable enzyme inhibitor diazotized sulfanilic acid. Incubation of intact synaptosomes with diazotized sulfanilic acid results in a small increase in the apparent molecular mass of the enzyme. Our results suggest that the active site of the membrane bound enzyme faces the extracellular medium. It thus would represent an ecto-ATPase.

Introduction

ATP is an important intercellular messenger substance that can act via specific membrane-bound receptors. Amongst these are ligand-gated ion channels, receptors functionally coupled to trimeric G-proteins as well as presumably receptors forming transmembrane aqueous pores [1]. Furthermore, ATP can serve as a co-substrate for surface-located ecto-protein kinases and thus play a role in the extracellular phosphorylation of proteins [2,3]. Regulated and Ca^{2+}-dependent release of ATP has been described for systems that contain concentrated packages of ATP in their secretory vesicles. These include cholinergic and adrenergic synaptic vesicles, chromaffin granules and also granules of blood platelets [4]. ATP can act as a neurotransmitter both, in the peripheral and central nervous system [5–7]. But ATP can also be released from the activated underlying postsynaptic cell [8,9]. Until recently source and cellular mechanism of this type of release were enigmatic. But the recent discovery that the product of the well-characterized multidrug resistance gene may function as a plasma membrane ATP channel [10] points to a possible ubiquitous mechanism for cellular ATP release.

The physiological activity of extracellularly released ATP demands mechanisms of inactivation. In many cellular systems a complete cascade of surface located ecto-enzymes that hydrolyze ATP to adenosine has been described. These include fractions of intact synaptosomes isolated from nerve terminals in mammalian and non-mammalian systems [11,12]. The first step, the hydrolysis of ATP, is mediated by an enzyme (ecto-ATPase) with characteristics different from well-characterized ATPases with intracellular active sites. The last step in the hydrolysis cascade is the formation by 5′-nucleotidase of adenosine from AMP [13]. Extracellularly applied ADP is also hydrolysed by intact cells but it is not yet clear whether this is due to the presence of an additional enzyme. It has been suggested that the ecto-ATPase is in fact a diphosphohydrolase (apyrase, EC 3.6.1.5) and capable of hydrolyzing both, ADP and ATP [14–20]. Alternatively, activity of ATP:AMP phosphotransferase (adenylate kinase, EC 2.7.4.3) may account for the removal of ADP [21]. A third possibility is the simultaneous presence at the cell surface of both, an ecto-apyrase and a non-ADP-hydrolyzing ecto-ATPase [22].

ATP hydrolyzing ecto-enzyme activity has been characterized in detail in intact cellular systems. In brain, the ecto-ATPase is about equally activated by millimolar concentrations of Ca^{2+} or Mg^{2+} alone. It has a very broad substrate specificity hydrolyzing also a variety of other purine and pyrimidine 5′-trinucleotides and it cannot be effectively inhibited by known ATPase

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inhibitors. In the central nervous system, activity of ecto-ATPase could be involved in the degradation of presynaptically and possibly also postsynaptically released ATP, as well as in the hydrolysis of ATP (or other nucleoside triphosphates) released under conditions of hypoxia. Thus, the enzyme is expected to have a major role in the extracellular metabolism of brain nucleotides. Here we report the purification and characterization of a 50-kDa enzyme that is contained in synaptosomal plasma membranes and shares properties of the ecto-ATPase contained in fractions of intact synaptosomes.

Materials and Methods

Isolation of synaptosomes

Bovine brain obtained from the slaughterhouse was transported to the laboratory on ice. Synaptosomes were isolated similar to the method previously described [23]. 4–5 g of cerebral cortex were cleared of the white matter and homogenized in 0.32 M sucrose (20 mM Hepes-NaOH (pH 7.4). After two differential centrifugation steps ($1000 \times g$ for 10 min; $12000 \times g$ for 20 min) the pellet was resuspended in isotonic sucrose (3 ml/g wet weight) and layered onto a density gradient containing Hepes-buffered solutions of 0.8 M and 1.1 M sucrose. After centrifugation ($75000 \times g$ for 150 min) synaptosomes were harvested at the 0.8/1.1 M sucrose interface. For incubation experiments, synaptosomes harvested from the hypertonic sucrose solution were readjusted to isotonic conditions by slowly adding Hepes-buffered 0.2 M sucrose solution until the sucrose concentration was 0.32 M.

Isolation of presynaptic plasma membranes from synaptosomes

Synaptosomes harvested from the 0.8/1.1 M sucrose interface were centrifuged ($40000 \times g$ for 30 min) and resuspended in 20 mM Hepes buffer (3 ml/g wet weight (pH 7.4)) to release cytoplasmic contents by hypotonic shock. The suspension was layered onto a sucrose density gradient containing Hepes buffered solutions of 0.3 M, 0.8 M and 1.15 M sucrose and centrifuged at $75000 \times g$ for 150 min. Presynaptic plasma membranes (SPM) were harvested at the 0.8/1.15 M sucrose interface and stored at −80°C for further experimentation.

Isolation of plasma membranes from a fraction enriched in synaptosomes

This procedure was used in experiments to probe for different isolation procedures of the ATPase demanding a high yield of plasma membranes. 60 g of cerebral cortex were homogenized in isotonic sucrose solution (0.32 M, 20 mM Hepes-NaOH (pH 7.4)) and subjected to a number of centrifugation steps. The supernatant of the first centrifugation ($3000 \times g$ for 10 min) was recentrifuged at $12000 \times g$ for 20 min and the resulting pellet was subjected to osmotic lysis by addition of 20 mM Hepes buffer (5 vols./g wet weight). The pellet obtained after renewed centrifugation (28000 for 45 min) was resuspended in hypotonic Hepes buffer (1 vol./g wet weight) and layered onto a cushion of Hepes-buffered 0.8 M sucrose. After centrifugation ($28000 \times g$ for 60 min) the resulting pellet was resuspended in a solution of Hepes buffered 2 M KCl (4 vols./g wet weight) to remove an excess of peripheral membrane proteins. The pellet containing the plasma membranes (PM) obtained after recentrifugation ($28000 \times g$ for 45 min) was resuspended in 20 mM Hepes-buffer (2 vols./g wet weight) and stored at −80°C.

Isolation of the Mg^{2+} or Ca^{2+} ATPase

After addition of polydocanol (polyoxyethylen-9-laurylether, Sigma) (2% (v/v)) the plasma membrane fraction was agitated for 30 min. After further 10 min insoluble constituents were removed by centrifugation ($200000 \times g$ for 30 min) and the supernatant was dialyzed (Visking dialysis tubing, type 20/32, Serva) against 20 mM Hepes buffer to reduce the detergent concentration. The dialysate was concentrated using an Amicon ultrafiltration unit (YM 30 membranes, 30-kDa exclusion). The concentrated extract could be stored at −80°C for several months. The detergent extract was subjected to chromatography on Sephacryl S-300 (Pharmacia, length 165 cm, diameter 2.5 cm) and eluted with 20 mM Hepes buffer in the presence of 0.1% polydocanol and 120 mM NaCl. Fractions with the highest activity in Ca^{2+}-ATPase were concentrated using the Amicon ultrafiltration unit (YM 10 membranes, 10-kDa exclusion) and diluted with 20 mM Hepes buffer and 0.1% polydocanol to a final concentration of 40 mM NaCl. They were subsequently subjected to ion-exchange chromatography on DEAE-MemSep 1000 (Millipore). Bound protein was eluted with polydocanol (0.1%) and Hepes buffer containing solutions of 100 mM and 400 mM NaCl. The Ca^{2+}-ATPase containing fractions of the column eluate were further separated by glycerol density gradient centrifugation. The continuous glyerol gradient (7.5%–30% (w/v)) contained 20 mM Hepes buffer, 0.1% polydocanol and 100 mM NaCl. After centrifugation ($75000 \times g$ for 20 h) fractions were collected from the tube by suction starting from the bottom of the gradient.

Labelling with azido-GTP and azido-ATP

For labelling with [α-^{32}P]8-azido-GTP (spec. act. 5.2 Ci/mmol, ICN) isolated synaptosomes were incubated in isotonic sucrose solution (0.5 ml final volume) containing azido-GTP (0.34 nmol, 1.78 μCi) in the presence of (a) 1 mM $CaCl_2$, 5 mM ATP; (b), 1 mM

$MgCl_2$, 5 mM ATP and (c), 1 mM $MgCl_2$, 5 mM ATP, 1% Triton X-100. After illumination (254 nm) for 5 min samples were immediately precipitated using TCA (5% (v/v) final volume) and prepared for SDS-PAGE and autoradiography.

For labelling with [α-^{32}P]8-azido-ATP (spec. act. 10 Ci/mmol) Ca^{2+}-ATPase containing protein fractions as specified in Results were used. They were incubated with 20 mM Hepes buffer and 0.1% polydocanol containing azido-ATP (153 nM, 9.32 μCi) in the presence of ATP (10 μM) and $CaCl_2$ (2 mM). After illumination for 3 min they were precipitated with TCA (5% final volume) and prepared for SDS-PAGE and autoradiography.

Production of an anti-ecto-ATPase antibody

The protein of 50 kDa and p*I* 5.3–5.9 obtained after chromatography on DEAE-MemSep 1000 and two-dimensional PAGE was cut out, electroeluted from the gel and lyophylized. Polyclonal antisera were raised in rabbits. The IgG fractions derived from the antisera (DEAE Affi-Blue, Bio-Rad) were purified by immunoadsorption on strips of nitrocellulose containing the 50-kDa protein band obtained after DEAE-MemSep chromatography, SDS-PAGE and transfer to nitrocellulose.

Electrophoretical procedures and immunodetection

SDS-PAGE [24] was carried out on minigels (1-mm thick) with acrylamide concentrations of 5% (w/v) for the stacking gel and 10% (w/v) for the running gel. Molecular masses were determined by standard proteins (Merck). Two-dimensional PAGE was performed according to O'Farrell [25] in the presence of carbamylated creatine phosphokinase standard (Pharmacia). Proteins were identified by staining with Coomassie brilliant blue or by silver staining [26]. After transfer to nitrocellulose [27] immunodetection was performed using horseradish-conjugated (ECL-system, Amersham) or ^{125}I-labelled second antibodies.

Analytical procedures

Activity of hydrolysis of ATP, ADP or AMP (5'-nucleotidase) and also of GTP, GDP, UTP, ITP and CTP was determined by analysis of inorganic phosphate [28]. For determination of non-specific phosphatase activity *p*-nitrophenyl phosphate, phospho-*enol*pyruvate, and glucose 6-phosphate were used instead of nucleoside triphosphates. Substrate concentrations were 1 mM with either $MgCl_2$ (1 mM) or $CaCl_2$ (4 mM) added. Incubation was performed for 10 min at 37°C. Dependence of enzyme activity on pH was determined within the pH range 3–11 using the following buffer systems: pH 3–6, sodium citrate (100 mM); pH 6.5–7.5, Hepes-NaOH (100 mM); pH 8–8.5, Tris-HCl (100 mM); pH 9–11, sodium borate (50 mM). To study the effect of other cations on ATPase activity the following compounds (1 mM) were applied: $ZnCl_2$, $SrCl_2$, $LaCl_3$, $SnCl_2$, $CoCl_2$, $FeCl_3$, LiCl, $CeCl_3$, $MnCl_2$, $CuSO_4$ and $NiCl_2$. Protein was determined according to Peterson [29] with BSA as a standard.

Inhibition of ecto-enzyme activity by diazotized sulfanilic acid

To determine the cellular localization of the active site of the Ca^{2+}-ATPase, fractions of intact synaptosomes were incubated in isotonic sucrose solution for 10 min in diazotized sulfanilic acid (DSA, 6 mM) similar to the procedure previously described [11]. The reaction was stopped by addition of bovine serum albumine to a final concentration of 1.5 mg/ml and fractions were kept on ice for analysis and for further subcellular fractionation and isolation of Ca^{2+}-ATPase. In control experiments synaptosomes were lyzed either by freezing and thawing or by addition of Triton X-100 to a final concentration of 1% (v/v).

Results

Isolation of a Ca^{2+} or Mg^{2+}-activated ATPase

The plasma membrane fraction (PM) isolated after isotonic homogenization of brain tissue is rich in membrane markers like acetylcholinesterase and 5'-nucleotidase [23]. To probe for ecto-ATPase, activity of Ca^{2+}-ATPase was measured in order to exclude a potential contribution of Mg^{2+}-dependent, Ca^{2+}-stimulated ATPase. The activities of the fraction in Ca^{2+}-ATPase and in Mg^{2+}-ATPase were 78 ± 27 nmol P_i/min per mg protein and 180 ± 63 nmol P_i/min per mg protein, respectively (mean ± S.D., $n = 4$). Using 2% polydocanol 80% of the Ca^{2+}-ATPase activity could be solubilized. At the same time 65% of activity of 5'-nucleotidase and activity of hydrolysis of *p*-nitrophenylphosphate (non-specific phosphatases) stayed in the insoluble sediment. Extraction in polydocanol caused an increase in specific activity of Ca^{2+}-ATPase by a factor 1.6. Chromatography on Sephacryl S-300 lead to a removal of about 80% of the total protein from the Ca^{2+}-ATPase-enriched fractions and an 8-fold increase in specific activity. On DEAE ion-exchange chromatography activity of Ca^{2+}-ATPase can be eluted with 100 mM NaCl, resulting in a 55-fold increase in specific enzyme activity. On subsequent glycerol density gradient centrifugation activity of Ca^{2+}-ATPase sediments between 14% and 17% (w/v) glycerol. This step removes more than 95% of total protein from the active fractions resulting in specific enzyme activities of 6.3 ± 0.4 μmol/min per mg protein (Ca^{2+}-ATPase) and 10 ± 1.4 μmol/min per mg protein (Mg^{2+}-ATPase). Regarding the reduction of enzyme activity during the isolation procedure this corresponds to an approx. 200-fold enrichment of the enzyme.

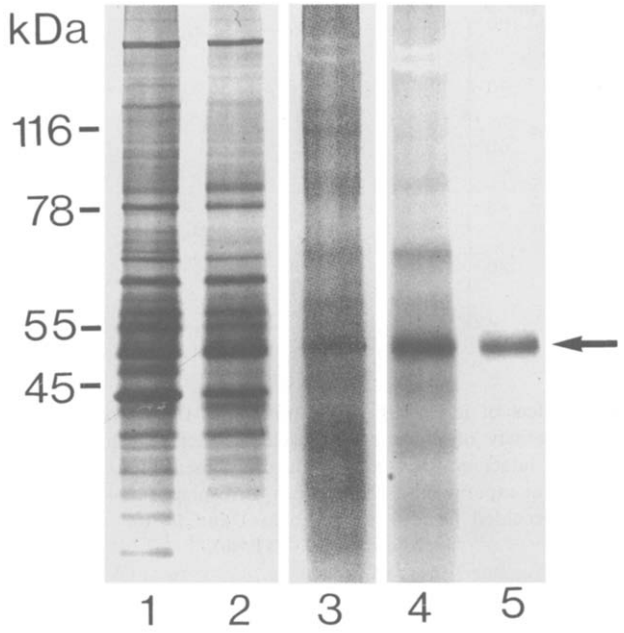

Fig. 1. Protein pattern of fractions obtained during purification of ecto-ATPase. Plasma membranes (1), supernatant fraction after solubilization of plasma membranes with polydocanol (2). Fraction enriched in Ca^{2+}-ATPase after chromatography on Sephacryl S-300 (3), after DEAE ion-exchange chromatography (4), or after glycerol density gradient centrifugation (5). Staining with Coomassie brilliant blue. Protein contents loaded per lane were 50 μg (1–3), 25 μg (4) or 5 μg (5).

An analyis of the protein pattern of the fraction enriched in activity of Ca^{2+}-ATPase leads to a successive enrichment of a protein band of 50 kDa (Fig. 1). Whereas the protein component is difficult to differentiate from other bands in the total plasma membrane fraction and after polydocanol solubilization it becomes enriched in the fractions enriched in enzyme activity after chromatography on Sephacryl S-300 and even more after DEAE ion-exchange chromatography. The fractions of highest ATPase activity obtained after glycerol density gradient centrifugation reveal only a single and strong protein band of 50 kDa. On two-dimensional PAGE the protein reveals a p*I* ranging from 5.3 to 5.9 with several individual spots (Fig. 2). These results suggest that the Ca^{2+}-ATPase activity measured is associated with a protein of an apparent molecular mass of 50 kDa.

Labelling of the 50-kDa protein with azido-GTP and azido-ATP

When intact synaptosomes were exposed to [α-^{32}P]8-azido-GTP under UV illumination, protein bands between 30 kDa and 66 kDa, including a 50-kDa component, were labelled (Fig. 3, lane 1). The incubation medium contained 5 mM ATP in order to lower the background of proteins carrying specific ATP-binding activity. Labelling was identical in the presence of

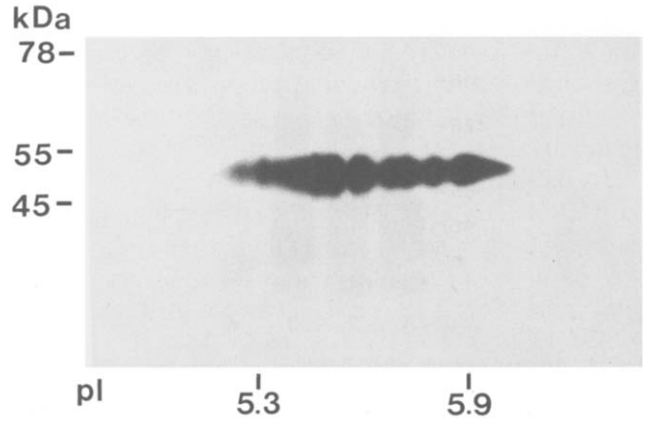

Fig. 2. Protein pattern after two-dimensional gel electrophoresis of the fraction derived after glycerol density gradient centrifugation (comp. Fig. 1, lane 5). Silver staining, 20 μg of protein loaded per lane.

$CaCl_2$ and $MgCl_2$. When synaptosomes were lyzed by Triton X-100 there was a strong enhancement of the 66-kDa component with the 50-kDa band unchanged (Fig. 3, lane 2).

For labelling with [α-^{32}P]8-azido-ATP Ca^{2+}-ATPase-enriched fractions of varying purity were used (Fig. 4). The plasma membrane fraction, the polydocanol extract and the fractions enriched in enzyme activity from the Sephacryl S-300 column contain a number of labelled protein bands, including a band of 50 kDa. This component represents the major labelled protein band on isolation of Ca^{2+}-ATPase using DEAE-MemSep chromatography.

Immunochemical detection of the protein

A monospecific antibody against the 50-kDa protein selectively recognizes the protein in Western blots of a variety of crude fractions, including a total tissue ex-

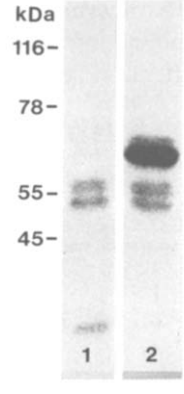

Fig. 3. Autoradiograph after labelling of synaptosomes with [α-^{32}P]8-azido-GTP and subsequent SDS-PAGE. Lane 1, after incubation of intact synaptosomes in the presence of $CaCl_2$, ATP and [α-^{32}P]8-azido-GTP. Lane 2, after incubation as for lane 1 but with the additional presence of 1% Triton X-100. The arrow marks the position of a 50-kDa protein. 50 μg of protein loaded per lane.

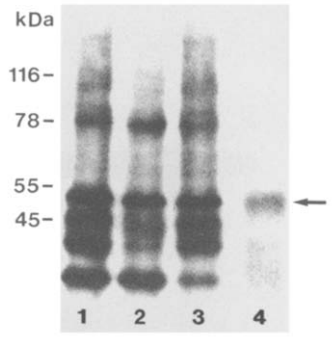

Fig. 4. Autoradiograph after labelling of synaptosomes with [α-^{32}P]8-azido-ATP and subsequent SDS-PAGE. Incubation in the presence of $CaCl_2$ of isolated plasma membranes (1), protein solubilized from plasma membranes by polydocanol (2) and fractions enriched in Ca^{2+}-ATPase after chromatography on Sephacryl S-300 (3), or after DEAE ion-exchange chromatography (4). The arrow marks the position of a 50-kDa protein. 70 μg of protein loaded in lanes 1–3 and 25 μg in lane 4.

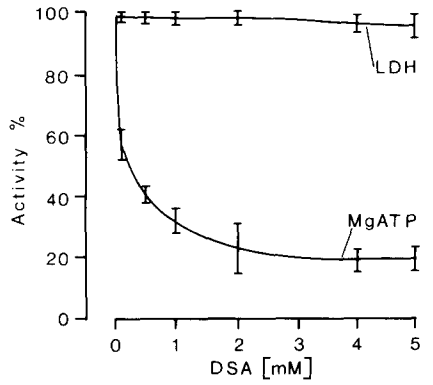

Fig. 6. Effect of increasing concentrations of diazotized sulfanilic acid on activity of ATPase and occluded lactate dehydrogenase (LDH) of intact synaptosomes. Values are means ± S.D. of three independent experiments. 100% values correspond to 140 U/mg of protein (occulded lactate dehydrogenase) and to 102 mU/mg of protein (Mg^{2+}-ATPase).

tract from bovine cerebral cortex, total synaptosomes, plasma membranes and fractions obtained after the chromatographical procedures (Fig. 5). However, the antibody had no influence on enzyme activity and it did not bind in immunocytochemical analyses of bovine tissues. Thus, it could not be used for a direct demonstration of the subcellular localization of its antigen.

Surface location of the protein

The activity of ectoenzymes can be blocked in the absence of any effect on cytoplasmic enzymes by diazotized sulfanilic acid, a low molecular weight, slowly permeating inhibitor of enzyme activity. When fractions of intact synaptosomes are incubated with DSA there is a concentration-dependent inhibition of activity of ATPase without a significant effect on cytosolic lactate dehydrogenase (Fig. 6). Maximal inhibition of ATPase activity is about 80%. A similar effect is achieved when the (ectoenzyme) acetylcholinesterase is analyzed (Fig. 8). Lysis of synaptosomes with Triton X-100 leads to a doubling of Mg^{2+}-ATPase activity without a significant effect on activity of Ca^{2+}-ATPase, acetylcholinesterase or 5′-nucleotidase. This suggests the existence of a pool of cytoplasmic Mg^{2+}-ATPase not accessible to extracellularly applied substrate. Addition of DSA to the lyzed synaptosome preparation causes inhibition of all enzyme activities analyzed.

If the Ca^{2+}- or Mg^{2+}-ATPase isolated had a surface location its inhibition should be maintained on enzyme purification after DSA treatment. Furthermore, the covalent binding of DSA molecules to the protein could be expected to alter the apparent molecular mass of the 50-kDa protein. In a series of parallel experiments intact synaptosomes were either treated with DSA or remained untreated. The addition of a large

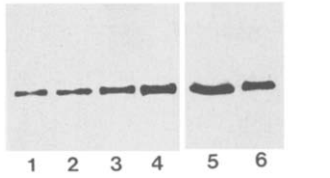

Fig. 5. Binding of monospecific anti-ecto-ATPase antibody to brain cortex homogenate and fractions derived from it. Homogenate of bovine cerebral cortex (1); synaptosomes (2); synaptic plasma membranes (3); fraction enriched in Ca^{2+}-ATPase after chromatography on Sephacryl S-300 (4), after DEAE ion-exchange chromatography (5), or after glycerol density gradient centrifugation (6). Protein contents loaded per lane were 70 μg (1–3), 50 μg (4), 25 μg (5) and 5 μg (6).

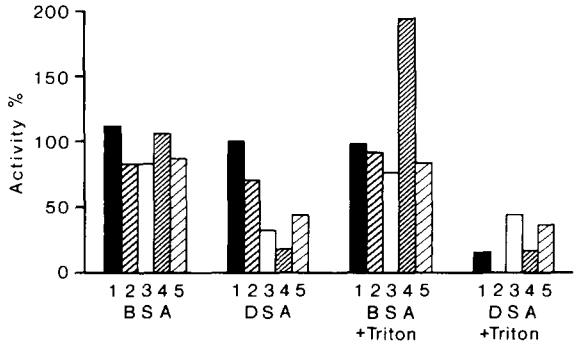

Fig. 7. Effect of diazotized sulfanilic acid on synaptosomal enzyme activities. Synaptosomes were incubated in the presence of either bovine serum albumin (BSA) or diazotized sulfanilic acid (DSA) and (in two experimental protocols) in the additional presence of 1% Triton X-100. Activities analyzed were lactate dehydrogenase occluded in synaptosomes (1), cytochrome-*c* oxidase (2), acetylcholinesterase (3), Mg^{2+}-ATPase (4) and 5′-nucleotidase (5). Enzyme activities of the intact synaptosome fraction stored on ice before incubation were taken as 100%. These correspond (related to mg of protein) to 166 U (occluded lactate dehydrogenase), 0.7 U (cytochrome-*c* oxidase), 17 U (acetylcholinesterase), 91 mU (Mg^{2+}-ATPase) and 49 mU (5′-nucleotidase). Values are from a representative experiment.

surplus of BSA after the end of incubation prevented any further effect of DSA. Presynaptic plasma membranes were isolated from either preparation and subjected to polydocanol solubilization. The solubilized fraction revealed the same degree of ATPase inhibition (80%) as previously observed with intact synaptosomes. When the soluble extract was subjected to DEAE ion-exchange chromatography the enzyme activity in the eluate after DSA treatment was still inhibited by 80% as compared to the eluate derived from control synaptosomes.

Analysis of the protein pattern after DEAE isolation of the Ca^{2+}-ATPase-enriched fractions revealed a considerable enrichment of the 50-kDa protein band (Fig. 8). Moreover, after DSA treatment this protein band was slightly shifted to higher molecular mass. This can also be shown in experiments where the proteins are detected by Western blot analysis using the monospecific anti-50-kDa antibody. When the same fractions are analyzed with a crude antibody (not immuno-affinity purified and recognizing additional protein bands) a significant shift can be observed also in one additional protein band (100 kDa) which is also a candidate for a surfcae located protein. In contrast, another protein (65 kDa) reveals the same molecular mass without and with DSA treatment and might not have a surface location.

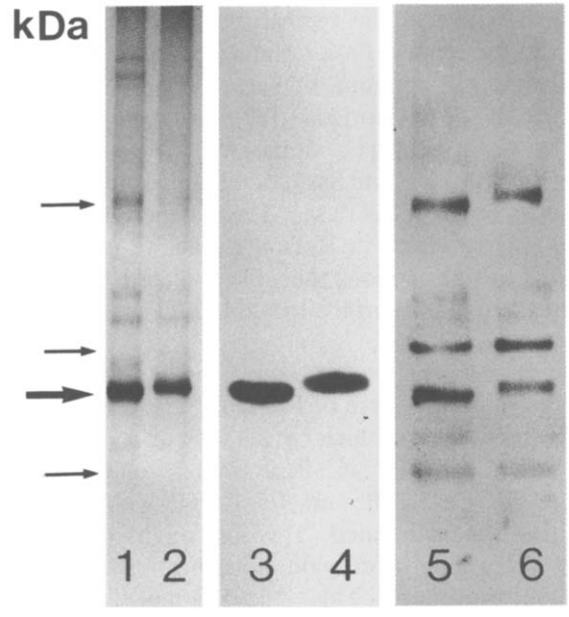

Fig. 8. Shift in molecular mass of 50-kDa protein after treatment of intact synaptosomes with diazotized sulfanilic acid. Synaptosomes were incubated in the absence (1,3,5) or presence (2,4,6) of diazotized sulfanilic acid. After preparation of plasma membranes and solubilization, ecto-ATPase was enriched by DEAE ion-exchange chromatography. (1,2) After staining with Coomassie brilliant blue; (3,4) immunotransfer blot of identical fractions using the monospecific anti-ecto-ATPase antibody and revealing a single band at 50 kDa (large arrow); (5,6) Western blot of identical fractions using an anti-ecto-ATPase antibody that was not immuno-affinity purified and recognized additional proteins of 30, 65 and 100 kDa (small arrows). Note that the apparent molecular masses of proteins of 50 kDa and 100 kDa, but not of a 65 kDa protein, are increased after preincubation in the presence of diazotized sulfanilic acid. Protein contents loaded per lane were 18 μg (1,2) and 25 μg (3–6).

TABLE I

Substrate specificity of Mg^{2+}- and Ca^{2+}-ATPase during the course of purification

Intact synaptosomes and fractions derived thereof were incubated in the presence of potential substrates in the presence of either $MgCl_2$ or $CaCl_2$. Results (production of P_i) are means of three independent experiments ± S.D. PEP, phospho*enol*pyruvate; pNPP, *p*-nitrophenyl phosphate; G6P, glucose 6-phosphate.

Substrate	Intact synaptosomes (nmol/min per mg)	SPM polydocanol-soluble (nmol/min per mg)	SPM polydocanol-insoluble (nmol/min per mg)	Glycerol gradient (μmol/min per mg)
Mg^{2+} salt				
ATP	193 ±22	157 ±27.8	74.4±9.5	10.3±1.4
ADP	49.4± 6.3	20 ± 1.3	3.3± 0.7	0
AMP	92.6± 9.3	9 ± 3.4	295 ±27.8	0
GTP	43 ± 5.3	200 ±26.4	90.6±19.9	8.5±0.4
GDP	16.2± 1.8	28 ± 4	12 ± 0.6	0
UTP	69 ± 8.3	100 ± 6.8	72.4± 4.3	1.1±0.2
ITP	74.3± 8.2	266 ±18.3	116 ± 9.3	6.7±0.5
CTP	92.9± 5	104 ± 9.3	68 ± 7.2	0.3±0.1
PEP	2.1± 0.3	4 ± 0.9	2.8± 1	0
pNPP	11.3± 2	9 ± 2.3	27.3± 4.1	0
G6P	1.7± 0.1	1.6± 0.2	0.9± 0.2	0
Ca^{2+} salt				
ATP	58.6± 7.2	135 ±13	59.4±13	6 ±0.4
ADP	25 ± 1.8	10.6± 1.7	3 ± 0.7	0
GTP	31 ± 3.2	66 ±11.7	22.8± 3.7	0.9±0.2
UTP	74.8±10.3	38.4± 1.7	14.4± 0.3	0.4±0.1
ITP	33 ± 1.2	64.8± 8.3	26.1± 4	1.3±0.1
CTP	92.9± 5	118 ± 6.3	49 ± 8.2	0.9±0.1

Substrate specificity of the purified enzyme

The rate of hydrolysis of a variety of substrates were determined in intact fractions of cortical synaptosomes, in the fraction solubilized by polydocanol and in the fraction of highest purity of Ca^{2+}-ATPase obtained after final glycerol density gradient centrifugation (Table I). In the intact synaptome fraction, the rate is highest for Mg^{2+}-ATP with UTP, ITP and CTP revealing about half the rate. Activation by Ca^{2+} is about three time lower than by Mg^{2+}. Activity for hydrolysis of ADP is considerably lower than that for the trinucleotides and also lower than activity of 5′-nucleotidase. The virtual lack of hydrolysis of phospho*enol*pyruvate, *p*-nitrophenyl phosphate and glucose 6-phosphate suggests that non-specific phosphatases do not significantly contribute to the results obtained.

The picture is considerably altered after solubilization. Now the preponderance ATP hydrolysis over the other trinucleotides in the presence of Mg^{2+} is lost. In

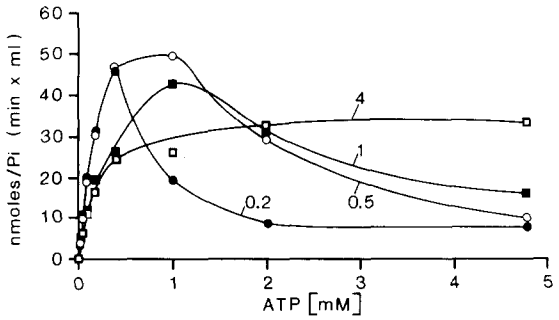

Fig. 9. Effect of varying ratios of substrate and cosubstrate on activity of ecto-ATPase. The fraction derived after glycerol density gradient centrifugation was in incubated with increasing concentrations of ATP (Na salt) in the presence of 0.2, 0.5, 1 and 4 mM $MgCl_2$.

addition activity of Ca^{2+}-ATPase is only about 20% smaller than that of Mg^{2+}-ATPase. Most of the 5'nucleotidase activity remains within the pellet of non-solubilized material. The most significant observation is that activity of ADPase is essentially lost on solubilization. Fractions of highest specific ATPase activity as obtained after glycerol gradient centrifugation and in the presence of Mg^{2+} also contain high activity of hydrolysis of GTP and ITP, while that of UTP and CTP is lost. Activity of Ca^{2+}-ATPase remains high but activation of the other trinucleotides by Ca^{2+} is lower.

When a variety of co-substrates were tested on the glycerol gradient purified enzyme the following grading for stimulation of ATPase activity could be derived: $Mg^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+} > Ni^{2+} > Ce^{3+} > Fe^{3+} > La^{3+} > Sr^{2+} > Zn^{2+} > Cu^{2+} > Li^{+} > Sn^{2+}$.

Kinetic properties

K_m values of the purified enzyme for ATP were 153 μM in the presence of Mg^{2+} and 127 μM in the presence of Ca^{2+}. The molar ratio of metal cation to ATP needs to be at least 1:1 to maintain maximal enzyme activity (Fig. 9). Lower ratios lead to a reduction in rate, suggesting that the substrate is a complex of the metal cation and ATP.

Further biochemical properties

The optimal pH for Mg^{2+}-ATPase activity of the purified enzyme is between 7.5 and 8.5. Higher and lower pH values are highly inhibitory. Of a large variety of potential inhibitors of ATPase activities (ouabain, 2 mM; oligomycin, 80 μg/ml; *o*-vanadate, 300 μM; DCCD, 500 μM; NEM, 10 μM; NBDL-Cl, 100 μM) none showed a significant inhibitory action on the Mg^{2+}-ATPase activity of the purified enzyme.

Discussion

The present study confirms the association of a surface located activity of ATPase with fractions of isolated synaptosomes [11,12,30–32]. The ecto-ATPase belongs to the increasing number of enzymes that are situated in the plasma membrane with their active site facing the extracellular medium. Ectoenzmyes have been described not only for the hydrolysis of nucleotides but also of acetylcholine, butyrylcholine and peptides, and for the glycosylation, deglycosylation and phosphorylation of proteins [33]. Whereas some of these proteins like 5'-nucleotidase [34] and certain splicing variants of acetylcholinesterase [35] are anchored to the membrane via a glycosyl phosphatityl inositol (GPI) anchor, others insert into the membrane by hydrophobic interaction. GPI-anchored proteins can be solubilized effectively solely with detergents of low critical micellar concentration. Our results demonstrate that activity of ecto-ATPase behaves different on solubilization from the GPI-anchored 5'-nucleotidase, suggesting that ecto-ATPase is not GPI-anchored. This is in accordance with observations on chromaffin cells in culture where phosphatidyl inositol-specific phospholipase C releases 5'-nucleotidase but not activity of ecto-ATPase or ecto-ADPase from the cell surface [36].

The ATPase purified from synaptosomal plasma membranes shares the typical biochemical properties reported for ecto-ATPase in intact cells. These include an alkaline pH optimum between 7.5 and 8.5 (similar also to ecto-5'-nucleotidase [37]), comparable activation by Ca^{2+} or Mg^{2+} alone, $K_{m,ATP}$ values in the order of 100 μM, and lack of effective inhibition by inhibitors of other ATPases. These general properties (previously reviewed in Refs. 11,12,38,39) give further support to the notion that the isolated enzyme is identical to the surface-located ATPase in intact synaptosomes.

There are a number of alterations in enzyme properties during the course of isolation. There is a considerable reduction or loss of activity of hydrolysis of Mg^{2+}-UTP and Mg^{2+}-CTP, as well as of Ca^{2+}-GTP, Ca^{2+}-UTP, Ca^{2+}-ITP and Ca^{2+}-CTP while ATPase activities are maintained. This loss might result from slight alterations in enzyme configuration during the isolation procedure. This study cannot resolve the question whether the isolated protein is an apyrase and thus would contain both, activity of ATPase and ADPase. The ability of the preparation to hydrolyze ADP is entirely lost on detergent solubilization. In agreement with previous results [12] it is noteworthy that the rate of ADP hydrolysis of isolated mammalian synaptosomes is considerably lower that that of ATP hydrolysis. The possibility that a specific ecto-ATPase (or ecto-nucleoside triphosphatase) exists in addition to an ecto-apyrase [22] should not be excluded.

During the isolation procedure a band of 50 kDa typically appears in the fractions enriched in ATPase activity and is the only protein band after glycerol

density gradient centrifugation. This suggests that the protein band corresponds to the ATPase. Our results further suggest that this protein band is derived from the plasma membrane and that its active center faces the extracellular space. The activity of the ATPase is inhibited when DSA is applied extracellularly to intact synaptosomes under conditions that do not affect intracellular enzyme activities, suggesting that the active site of the enzyme faces the extracellular space. Note that the loss of activity is maintained when the ATPase is isolated. The small shift of the 50-kDa band after DSA incubation of synaptosomes supports the notion that part of the polypeptide chain of the protein faces the extracellular medium and, thus, can bind externally applied DSA.

Labelling with azido-ATP and azido-GTP would be expected for a protein capable of hydrolyzing either ATP or GTP. In order to exclude the possible identity of the isolated protein with known nucleotide-binding proteins of similar molecular mass Western blots were performed using antibodies against β-tubulin and the α-subunit of trimeric G-proteins (anti-α-common antibody). None of the antibodies bound to the 50-kDa band (unpublished results). Taken together our results suggest that the isolated protein of 50-kDa represents a synaptosomal surface-located ATPase.

Proteins that could represent ecto-ATPase have been isolated from other tissues. To date no general picture concerning either molecular mass or biochemical properties can be derived. Either the molecular properties of ecto-ATPases vary between tissues or several ecto-ATPases with differing molecular properties might exist. The deduced amino-acid sequence of an ecto-ATPase (100 kDa) from rat liver plasma membranes was found to be identical to cell-CAM 105, a well-characterized cell adhesion molecule and member of the immunoglobulin superfamily [40]. The same hepatocyte protein is also a substrate for tyrosine kinase activity of the insulin receptor [41]. Activity of ATPase could not be detected in association with isolated liver cell-CAM 105 by others [42] and has not yet been reported for other members of the immunoglobulin superfamily. A polyclonal antibody directed against liver ecto-ATPase detects a protein of identical molecular mass in plasma membranes from rat parotid gland [43]. Cell-CAM 105 is not expressed in nervous tissue [42]. Other tissue sources from which the purification of potential ecto-ATPases with differing molecular properties have been reported include oviductal secretions [44], sheep kidney medulla plasma membranes [45], rabbit skeletal muscle transverse tubules [46,47], human oat cell membranes [48] and rat heart plasma membranes [49–51]. One of the ATPases from heart plasma membranes is a dimer of 90 kDa and does not hydrolyze ADP. The enzyme preparation reveals low and high affinitiy binding of ATP-γ-^{32}S and is enriched in phospholipids and cholesterol. The Ca^{2+}-ATPase from rabbit skeletal muscle transverse tubules has major subunits of 107 kDa and 30 kDa, that of the sheep kidney exhibits two peptides of 150 kDa and 77 kDa and the enzyme from oat cell membranes has 30 kDa.

An ATP diphosphohydrolase (apyrase) from bovine aorta has estimated molecular masses of 110 kDa [52] or 189 kDa [53]. Apyrase preparations from rat placenta and pig pancreas preparations reveal molecular masses of 67 kDa and 65 kDa, respectively, similar to that of a potato microsomal apyrase (50 kDa) [19,54]. Reliable statements on the relation between the various enzymes isolated can only be made when sequence information is available.

The antibody prepared against the denatured synaptosomal ecto-ATPase did not bind the antigen in formaldehyde-fixed tissue sections. The ecto-ATPase may not be neuron-specific but also be associated with glial and endothelial cell. Cultured C6 rat glioma cells [55] or cultured rat brain astrocytes [56] contain activity of ecto-ATPase, but like 5′-nucleotidase [57] ecto-ATPase might not have a ubiquitous distribution in brain. The cellular distribution of ecto-ATPase in brain is expected to reflect the pattern of physiological activity of extracellular ATP in this tissue.

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References

1 Pizzo, P., Murgia, M., Zambon, A., Zanovello, P., Bronte, V., Pietrobon, D. and Divirgilio, F. (1992) J. Immunol. 149, 3372–3378.
2 Ehrlich, Y.H., Davis, T.B., Bock, E., Kornecki, E. and Lenox, R.H. (1986) Nature 320, 67–70.
3 Kübler, D., Reinhardt, D., Reed, J., Pyerin, W. and Kinzel, V. (1992) Eur. J. Biochem. 206, 179–186.
4 Fiedler, J.L., Pollard, H.B. and Rojas, E. (1992) J. Membrane. Biol. 127, 21–33.
5 Edwards, F.A., Gibb, A.J. and Colquhoun, D. (1992) Nature 359, 144–147.
6 Evans, R.J., Derkach, V. and Surprenant, A. (1992) Nature 357, 503–505.
7 Silinsky, E.M., Gerzanich, V. and Vanner, S.M. (1992) Br. J. Pharmacol. 106, 762–763.
8 Smith, D.O. (1991) J. Physiol. 432, 343–354.
9 Vizi, E.S., Sperlágh, B. and Baranyi, M. (1992) Neuroscience 50, 455–465.
10 Abraham, E.H., Prat, A.G., Gerweck, L., Seneveratne, T., Arceci, R.J., Kramer, R., Guidotti, G. and Cantiello, H.F. (1993) Proc. Natl. Acad. Sci. USA 90, 312–316.
11 Grondal, E.J.M. and Zimmermann, H. (1986) J. Neurochem. 47, 871–881.

12 Nagy, A.K., Shuster, T.A. and Delgado-Escueta, A.V. (1986) J. Neurochem. 47, 976–986.
13 Zimmermann, H. (1992) Biochem. J. 285, 345–365.
14 Schadeck, R.J.G., Sarkis, J.J.F., Dias, R.D., Araujo, H.M.M. and Souza, D.O.G. (1989) Brazilian J. Med. Biol. Res. 22, 303–314.
15 Valenzuela, M.A., López, J., Depix, M., Mancilla, M., Kettlun, A.M., Catalán, L., Chiong, M., Garrido, J. and Traverso-Cori, A. (1989) Comp. Biochem. Physiol. B. 93, 911–919.
16 Battastini, A.M.O., Da Rocha, J.B.T., Barcellos, C.K., Dias, R.D. and Sarkis, J.J.F. (1991) Neurochem. Res. 16, 1303–1310.
17 Sarkis, J.J.F. and Salto, C. (1991) Brain Res. Bull. 26, 871–876.
18 Yagi, K., Shinbo, M., Hashizume, M., Shimba, L.S., Kurimura, S. and Miura, Y. (1991) Biochem. Biophys. Res. Commun. 180, 1200–1206.
19 Pieber, M., Valenzuela, M.A., Kettlun, A.M., Mancilla, M., Aranda, E., Collados, L. and Traversocori, A. (1991) Comp. Biochem. Physiol. B. 100, 281–285.
20 Côté, Y.P., Filip, J.G., Battistini, B., Gauvreau, J., Sirois, P. and Beaudoin, A.R. (1992) Biochim. Biophys. Acta 1139, 133–142.
21 Nagy, A.K., Shuster, T.A. and Delgado-Escueta, V. (1989) J. Neurochem. 53, 1166–1172.
22 Magocsi, M. and Penniston, J.T. (1991) Biochim. Biophys. Acta 1070, 163–172.
23 Henkel, A. and Zimmermann, H. (1992) Neurochem. Int. 20, 55–59.
24 Laemmli, U.K. (1970) Nature 227, 680–685.
25 O'Farell, P.Z. and Goodman, H.M. (1977) Cell 12, 1133–1142.
26 Koepsell, H., Korn, K., Ferguson, D., Menhr, H., Ollig, D. and Haase, W. (1984) J. Biol. Chem. 259, 6548–6558.
27 Towbin, H., Staehelin, H. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
28 Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) Anal. Biochem. 100, 95–97.
29 Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
30 Lin, S.C. and Way, E.L. (1984) J. Neurochem. 42, 1697–1706.
31 Terrian, D.M., Hernandez, P.G., Rea, M.A. and Peters, R.I. (1989) J. Neurochem. 53, 1390–1399.
32 James, S. and Richardson, P.J. (1993) J. Neurochem. 60, 219–227.
33 Kenny, A.J. and Turner, A.J. (eds.) (1987) in Mammalian Ectoenzymes, pp. 329–352, Elsevier, Amsterdam.
34 Vogel, M., Kowalewski, H., Zimmermann, H., Hooper, N.M. and Turner, A.J. (1992) Biochem. J. 284, 621–624.
35 Legay, C., Bon, S. and Massoulié, J. (1993) FEBS Lett. 315, 163–166.
36 Torres, M., Pintor, J. and Miras-Portugal, M.T. (1990) Arch. Biochem. Biophys. 279, 37–44.
37 Grondal, E.J.M. and Zimmermann, H. (1987) Biochem. J. 245, 805–810.
38 Dhalla, N.S. and Zhao, D. (1988) Prog. Biophys. Mol. Biol. 52, 1–37.
39 Grondal, E.J.M. and Zimmermann, H. (1988) in Cellular and Molecular Basis of Synaptic Transmission (Zimmermann, H., ed.), pp. 395–410, Springer, Heidelberg.
40 Lin, S.H., Culic, O., Flanagan, D. and Hixson, D.C. (1991) Biochem. J. 278, 155–161.
41 Najjar, S.M., Accili, D., Philippe, N., Jernberg, J., Margolis, R. and Taylor, S.I. (1993) J. Biol. Chem. 268, 1201–1206.
42 Öbrink, B. (1991) Bioessays 13, 227–234.
43 Cheung, P.H., Dowd, F.J., Porter, J.E. and Lin-Sheng, L. (1992) Cell. Signal. 4, 25–35.
44 Rosenberg, M.D., Gusovsky, T., Cutler, B., Berliner, A.F. and Anderson, B. (1977) Biochim. Biophys. Acta 482, 197–212.
45 Gantzer, M.L. and Grisham, L.M. (1979) Arch. Biochem. Biophys. 198, 263–269.
46 Hidalgo, C., Gonzalez, M.E. and Lagos, R. (1983) J. Biol. Chem. 258, 13937–13945.
47 Kirley, T.L. (1988) J. Biol. Chem. 263, 12682–12689.
48 Knowles, A.F. and Leng, L. (1983) Biochim. Biophys. Acta 731, 88–96.
49 Tuana, B.S. and Dhalla, N.S. (1988) Mol. Cell. Biochem. 81, 75–88.
50 Zhao, D. and Dhalla, N.S. (1991) Mol. Cell Biochem. 107, 135–149.
51 Zhao, D., Elimban, V. and Dhalla, N.S. (1991) Mol. Cell Biochem. 107, 151–160.
52 Yagi, K., Arai, Y., Kato, N., Hirota, K. and Miura, Y. (1989) Eur. J. Biochem. 180, 509–513.
53 Cote, Y.P., Picher, M., Stjean, P., Beliveau, R., Potier, M. and Beaudoin, A.R. (1991) Biochim. Biophys. Acta 1078, 187–191.
54 LeBel, D., Poirier, G.G., Phaneuf, S., St.-Jean, P., Laliberté, J.F. and Beaudoin, A.R. (1980) J. Biol. Chem. 255, 1227–1233.
55 Stefanovic, V., Mandel, P. and Rosenberg, A. (1976) J. Biol. Chem. 251, 3900–3905.
56 Lai, K.M. and Wong, P.C.L. (1991) J. Neurochem. 57, 1510–1515.
57 Zimmermann, H., Vogel, M. and Laube, U. (1993) Neuroscience 55, 105–112.