Demonstration of an Extracellular ATP-Binding Site in NCAM: Functional Implications of Nucleotide Binding[†]

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ABSTRACT: A minor fraction of the total ecto-type (E-type) ATPase activity of rat synaptosomes has been detected in immunoprecipitates of the neural cell adhesion molecule, NCAM, indicating that this either is an intrinsic enzymatic activity of NCAM or of an ATPase tightly associated to NCAM [Dzhandzhugazyan & Bock (1993) *FEBS Lett. 336*, 279−283]. We here demonstrate ATPase activity in preparations of the lipid-anchored as well as the transmembrane NCAM isoforms immunoisolated from transfected L-cells. A fraction of the E-type ATPase activity is spontaneously released from synaptosomes. Released material was fractionated by various chromatographic procedures and an extracellular fragment of NCAM was shown to co-elute with the major part of the enzymatic activity. Furthermore, it was shown that agarose-coupled NCAM-antibodies retained 85% of the ATPase activity released from synaptosomes after treatment with phosphatidylinositol-specific phospholipase C. These findings restricted the association or expression of the enzymatic activity to the extracellular part of NCAM. An affinity reagent, 5′-*p*-fluorosulfonylbenzoyl adenosine, FSBA, was shown to inhibit ATPase activity of immunoisolated NCAM, and incorporation of FSBA was detected in all three major NCAM isoforms (A, B, and C). An excess of ATP prevented both inactivation of the enzyme and affinity labeling of NCAM. Thus, NCAM contains an ATP-binding site, and this site is localized extracellularly and probably has the catalytic function. Binding of the substrate or FSBA protected a proteolytic cleavage site in NCAM localized close to the membrane presumably by induction of a local conformational change in NCAM, indicating a mechanism by which ATP may regulate NCAM adhesion and adhesion-triggered processes. A possible role of this mechanism in synaptic plasticity and memory consolidation is proposed.

Extracellular ATP is universally employed in cell−cell communication, particularly in synaptic transmission (*1*), and acts via numerous purinergic receptors and ecto-ATP hydrolyzing enzymes (*2*). Ca^{2+}- or Mg^{2+}-dependent ecto-ATPases and ecto-ATP diphosphohydrolases (ATPDases or apyrases) are E-type nucleotide hydrolyzing enzymes that have been demonstrated in viruses, protozoa, plants, and vertebrate tissues (for review, see ref 3). A broad variety of enzymatic characteristics (*3*−*5*) seems to indicate that the enzymes are distinct proteins. However, recent cloning and sequencing of a potato apyrase (*6*) has revealed structural relationships between a number of intra- and extracellular nucleotide-hydrolyzing enzymes, including a mammalian ecto-ATPDase (*7*, *8*), identical to the activation marker CD39 expressed by immunocompetent cells (*9*) and a variety of highly vascularized tissues (*8*). This group has "apyrase-conserved regions" in the structure and extremely high turnover rates (*5*, *6*), consistent with a role in regulation of purinergic transmission and salvage of purines. In brain, numerous synaptosomal E-type ATP/ATPDases (or enzymatic isoforms) differing in chromatographic properties, pH-optima, sensitivity to azide and to affinity-modifying ATP analogues (*10*, *11*) contribute to the extracellular ATP hydrolysis. Immunocytochemical crossreactivity between potato apyrase, some related ATP/ATPDases, and solubilized synaptosomes (*12*) indicates that some of the synaptosomal E-type enzymes might belong to CD39-related family; however, RNA hybridization experiments do not support this hypothesis (*8*). A minor fraction of the synaptosomal E-type ATPase activity has been shown to be associated with the neural cell adhesion molecule, NCAM (*11, 13*).[1]

Ecto-ATPase activity is enriched at cell−cell contacts, and a high activity in malignant cells seems to be associated with the ability to overcome contact inhibition (*14*). The amino acid sequence deduced from a cDNA of a rat hepatocyte ecto-ATPase was reported to be homologous to that of the human biliary glycoprotein (*15*) and identical to the amino acid sequence of rat liver Cell-CAM, an adhesion molecule of the immunoglobulin superfamily (*16*). However, the ATP-hydrolyzing function of Cell-CAM is a matter of

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[1] Abbreviations: NCAM, neural cell adhesion molecule; PI-PLC, phosphatidylinositol-specific phospholipase C; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DMSO, dimethyl sulfoxide; T-TBS, Tween-Tris buffered saline; oATP, oxidized ATP; FSBA, 5′-*p*-fluorosulfonylbenzoyl adenosine; FSB-OMe, fluorosulfonylbenzoic acid methyl ester; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; AP, alkaline phosphatase; GPI, glycosylphosphatidylinositol; ELISA, enzyme-linked immunosorbent assay; TLC, thin-layer chromatography; LTP, long-term potentiation.

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controversy. Thus, an isolated, highly active liver E-type ATPase differs in molecular mass from Cell-CAM (*4*). An attempt to clone a human liver E-type ATPase using Cell-CAM cDNA as a probe led to the isolation of the cDNA of the biliary glycoprotein, and since no enzymatic activity has been demonstrated directly in the biliary glycoprotein, it was concluded that the employed probe coded for an adhesion molecule, not for an ATPase (*17*). On the other hand, cells transfected with the biliary glycoprotein achieve a temperature and divalent cation dependent adhesiveness which can be inhibited by addition of ATP (*18*), and phosphorylation of Cell-CAM increases the ecto-ATPase activity in Cell-CAM-transfected cells, but not in control cells, indicating at least a functional association of the enzyme and the adhesion molecule (*19*). A similar example is provided by a well-characterized membrane endo-ATPase: the adhesion molecule on glia, AMOG, which is the rodent β_2-isoform of the β-subunit of the sodium pump. AMOG forms a functionally active complex with the catalytic α-subunit, and antibodies to AMOG have been shown to affect the transport activity of the Na,K-ATPase (*20*). Finally, the ecto-enzyme 5′-nucleotidase, CD73, which is a member of the cascade hydrolyzing ATP to adenosine, recently has been shown to be an adhesion molecule (*21*).

The NCAM is a member of the immunoglobulin superfamily operating in cell adhesion and adhesion-triggered signal transduction via homophilic, assisted homophilic and heterophilic mechanisms. There is one gene for NCAM, but due to alternative splicing several isoforms are produced. In adult rodent brain, three major NCAM isoforms of 190, 135, and 115 kDa, designated NCAM-A, -B, and -C, are expressed. Extracellularly, all isoforms consist of five Ig-like domains at the N-terminus followed by two fibronectin-type III domains. NCAM-A and NCAM-B are transmembrane proteins, whereas NCAM-C is linked to the membrane via a glycosylphosphatidylinositol, GPI, anchor. NCAM plays a pivotal role in neural development, neural regeneration, synaptic plasticity and tumor invasion (for reviews, see refs 22 and 23). We have demonstrated an E-type ATP- and, to a lower extent, ADP-hydrolyzing activity in immunoisolated preparations of NCAM and proposed that this either was an intrinsic enzymatic activity of NCAM or that a tight functional association between an ecto-ATPase and NCAM existed (*13*). We here focus on the NCAM isoform-specificity of this activity, on the identification of the catalytic component using various chromatographic techniques, immunoprecipitation and affinity modification, and on the elucidation of the effects produced by binding of substrate or substrate analogues. Since ATP is a preferential substrate and since ADP hydrolysis was not studied in the present work, the term ATPase activity is used throughout this article.

MATERIALS AND METHODS

Materials. Phosphatidylinositol-specific phospholipase C, PI-PLC, from *Bacillus cereus*, 5′-fluorosulfonylbenzoyl adenosine, FSBA, and polyclonal rabbit antibodies against FSBA were from Boehringer-Mannheim (Mannheim, Germany). Mono Q HR 5/5, protein G-Sepharose Fast Flow and lysine-Sepharose were from Pharmacia (Uppsala, Sweden). Tween-20 (EIA grade), acrylamide and bisacrylamide were from Bio-Rad Laboratories (Richmond, CA). Secondary horseradish peroxidase, HRP, or alkaline phosphatase, AP, -conjugated swine antibodies against rabbit immunoglobulins and AP-conjugated streptavidin were from Dako (Glostrup, Denmark). Nitrocellulose sheets BA85 were from Schleicher & Schuell (Dassel, Germany). The SuperSignal CL-HRP substrate system and the bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). Silica gel precoated sheets (Kieselgel 60 F_{254}) were from Merck (Darmstadt, Germany). Monoclonal antibodies to the cytoplasmic part of NCAM, OB11, and all other reagents were supplied by Sigma Chemical Co. (St. Louis, MO).

Membrane Fractions. Synaptosomal fractions were isolated essentially as described previously (*13*). Brains of 40-day-old Wistar rats were homogenized in a buffer consisting of 0.32 M sucrose, 30 mM histidine, 1 mM EDTA, pH 7.5. The supernatant obtained after two differential centrifugations (1000*g* for 10 min followed by 4000*g* for 15 min) was applied on a cushion of 16% v/v Percoll in homogenization buffer, adjusted to pH 7.5 and centrifuged at 30000*g* for 60 min, allowing the sedimentation of mitochondria (*24*). The fraction concentrated at the top of 16% v/v Percoll was collected, diluted 3-fold with homogenization buffer and pelleted at 30000*g* for 60 min. The pellet was resuspended in homogenization buffer at a protein concentration of 5−8 mg/mL. The amount of NCAM (the combined three isoforms) was quantified by ELISA and found to be 7 μg per mg of membrane protein. By determining both accessible and latent Na,K-ATPase activities (*25*) the obtained membrane fraction, in the following referred to as the synaptosomal fraction, was shown to contain approximately 80% tightly sealed synaptosomes and 20% leaky membrane fragments. The preparation was stored in aliquots at −20 °C.

Postnuclear membrane fractions were prepared from L-cells stably transfected with human NCAM-B or -C (*26*, *27*). The cells were grown in Dulbecco's modified Eagle's medium, DMEM (Gibco, Paisley, U.K.), supplemented with 10% fetal calf serum. The following clones were employed: wild-type L-cells, L0; two vector-transfected clones, LVN-101 and LVN-212; two NCAM-B-transfected clones, LBN-106 and LBN-110; and two NCAM-C-transfected clones, LCN-202 and LCN-212. Confluent monolayers were washed and harvested in 0.1 M sucrose, 30 mM histidine, 1 mM EDTA, pH 7.5, followed by homogenization in a Teflon−glass homogenizer using the same buffer supplemented with 0.1 mM phenylmethylsulfonyl fluoride, PMSF, and aprotinin, 100 units/mL. The homogenate was centrifuged at 3000*g* for 15 min, and the resulting supernatant was centrifuged at 30000*g* for 60 min. The pellet was suspended and stored in homogenization buffer in aliquots at −20 °C.

Solubilization. The procedure has briefly been described previously (*13*). Membrane fractions were diluted to 1.4−3 mg of protein/mL in a medium containing 25 mM HEPES, 0.1 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 0.5 mM PMSF and 25% w/v glycerol, pH 7.5. 1% w/v 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS, was freshly prepared in the same buffer. Both mixtures were kept on ice for 10 min. An equal volume of CHAPS solution was added slowly with gentle homogenization to the diluted membrane fraction giving a final concentration of 0.5% w/v CHAPS. The mixture was kept on ice for 30 min, and insoluble material was removed by centrifugation at 100000*g* for 45 min.

Immunoadsorption of NCAM. Antibodies were coupled to protein G-Sepharose according to Harlow and Lane (*28*).

Preparations of rabbit polyclonal antibodies were isolated (*29*) either from immune serum of rabbits immunized with rat NCAM (NCAM antibodies) or from pooled serum obtained prior to immunization of rabbits (control antibodies). No interaction of control antibodies with synaptosomal proteins was observed. Antibodies against rat NCAM or control antibodies were gently mixed with protein G-Sepharose in 20 mM HEPES, 150 mM NaCl, pH 7.5, overnight at 4 °C (20 mg of protein per 1 mL of Sepharose suspended in a final volume of 5 mL). The agarose was sedimented at 3000*g* for 5 min and washed twice with 10 mL of the same buffer. Freshly prepared 20 mM dimethylpimelimidate in 10 mL of 0.2 M sodium borate, pH 9.0, was added to the sedimented agarose. After being mixed for 1 h at room temperature, the agarose was sedimented and suspended in 10 mL of 0.2 M ethanolamine, pH 8.2, for 2 h at room temperature, and then washed and resuspended in 25 mM HEPES, 150 mM NaCl, pH 7.5, containing 0.02% w/v sodium azide.

NCAM- or control-immunosorbent corresponding to a 50 μL bed volume suspended in 20 mM HEPES, pH 7.5, was mixed routinely with 0.2 or 0.5 mL of supernatants from membranes treated with CHAPS or PI-PLC, respectively. The mixture was incubated with gentle mixing at room temperature for 30 min after which incubation was continued at 4 °C for additional 6 h. The immunosorbent was sedimented and 0.5 mL of 1% w/v ovalbumin, 0.25% w/v CHAPS in washing buffer, 20 mM HEPES, 10% w/v glycerol, pH 7.5, was added and the mixture was incubated overnight at 4 °C. A cushion of 15% w/v sucrose in washing buffer containing 0.1% w/v CHAPS was placed in a tube, and the immunosorbent was again sedimented by centrifugation and transferred to a new tube in 5 mL washing buffer containing 0.1% w/v CHAPS and washed for 6 h at 4 °C. Subsequently the immunosorbent was sedimented, resuspended in 10 mL of washing buffer containing 0.03% w/v CHAPS and washed overnight. The presence of ovalbumin during immunoisolation not only reduced unspecific binding to the sorbent, but was also important for elimination of low amounts of Na,K-ATPase [3−4% of the immunoisolated NCAM protein (*30*)]. This was the only non-E-type membrane ATPase otherwise co-isolated with NCAM (*11*, *30*) because of the binding to NCAM *via* an oligomannosidic chain in the β_2-isoform of the β-subunit of brain Na,K-ATPase (*31*). Before affinity modification of immunoadsorbed NCAM preparations the sorbent was washed with 20 mM HEPES, pH 7.5. Before ATPase assay the immunosorbent was washed with the ATPase assay medium without ATP (see below). Immunoisolation of NCAM from FSBA-modified synaptosomes was performed according to the same protocol. Human NCAM was immunoisolated from postnuclear membrane fractions of transfected L-cells using anti-human NCAM rabbit polyclonal antibodies obtained according to Ibsen et al. (*32*).

Affinity Modification. Oxidized ATP, oATP, was synthesised as described previously (*33*), stored at −70 °C and used within 1 week after synthesis. FSBA was freshly prepared as a 30 mM stock solution in dimethyl sulfoxide, DMSO. Flurosulfonylbenzoic acid methyl ester, FSB-OMe, was prepared by methanolysis of fluorosulfonylbenzoyl chloride overnight at room temperature (*34*). Affinity modification of NCAM bound to immunosorbent or of synaptosomes was performed for 1 h at 37 °C in 20 mM HEPES, pH 7.5, containing 0.2 mM $MgCl_2$ and varying concentrations of oATP or FSBA. In some experiments ATP was added in concentrations indicated in the text. Controls contained all compounds in amounts identical to those added to the affinity-modified samples, including DMSO and/or ATP, but no ATP analogue. When employing a two-step modification, the sample was suspended in 20 mM HEPES, pH 7.5, in the presence or absence of ATP, FSB-OMe from a stock solution in DMSO was added, and the mixture was incubated at 22 °C for 1 h. The sample was then 2-fold diluted, centrifuged through a cushion of 15% w/v sucrose in 20 mM HEPES, pH 7.5, and modified with FSBA as described above. All experiments were repeated at least five times. For optimization of FSBA detection, Na,K-ATPase isolated in membrane-bound form from pig kidney (*35*) was affinity modified by 1 mM FSBA in 20 mM HEPES, 1 mM $MgCl_2$, and 150 mM NaCl, pH 7.5, at 37 °C for 1 h.

PI-PLC-Mediated and Spontaneous Release of NCAM. Synaptosomes were treated with PI-PLC for 3 h at 37 °C in 25 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, using 2 U PI-PLC per mg synaptosomal protein in a volume of 200 μL at a concentration of approximately 2.5−3 mg of protein/mL. The released fraction was separated from the synaptosomes by centrifugation at 100000*g* for 1 h. For spontaneous release of proteins, synaptosomes were suspended in the same buffer as above, but without PI-PLC, at a protein concentration of 0.7−1 mg/mL and incubated at 37 °C for 5−8 h. The membrane material was removed by centrifugation at 100000*g* for 1 h.

The amount of synaptosomal protein was determined by a modified Lowry procedure (*36*) after precipitation with 5% w/v trichloroacetic acid. The ratio of released protein to total synaptosomal protein was determined using the bicinchoninic acid protein assay reagent according to the procedure given by the manufacturer.

Chromatography on Mono Q and Lysine-Sepharose Columns. Fractionation was performed at 4 °C using an FPLC system (Pharmacia, Uppsala, Sweden). A Mono Q column with a bed volume of 1 mL was equilibrated with 20 mM imidazole-HCl, 0.1 mM EDTA, 10% w/v glycerol, and 0.25% w/v CHAPS, pH 7.5, at a flow rate of 0.5 mL/min. To proteins spontaneously released from a synaptosomal preparation containing 4 mg of membrane protein was added CHAPS to a final concentration of 0.25% w/v. The mixture was diluted 2.5-fold with equilibration buffer, applied on the column, and eluted with a linear gradient of 0−0.45 M NaCl in equilibration buffer. Fractions of 1 mL were collected and E-type ATPase activity and P_i-background were measured using aliquots of the individual fractions. The concentration of NaCl was determined by conductometry using serial dilutions of 1 M NaCl in equilibration buffer as a standard. In a second series of experiments the Mono Q column was equilibrated with 10 mM Tris-HCl, 2 mM $MgCl_2$ and 0.25% w/v CHAPS, pH 8.2. The same sample as above was diluted by adding 1.5 vol distilled water adjusted to pH 9.5 by Tris-base. The mixture was supplemented with $MgCl_2$ and CHAPS to final concentrations of 2 mM and 0.25% w/v, respectively, and pH was adjusted to pH 8.2 by 0.1 M Tris-HCl, pH 9.5. The sample was applied on the column and fractionated as above.

A Lysine-Sepharose column, with a bed volume of 4 mL, was equilibrated with 25 mM Tris-HCl and 0.25% w/v CHAPS, pH 7.4, at a flow rate of 0.1 mL/min. To the

fraction of proteins spontaneously released from a preparation of synaptosomes corresponding to 4 mg of membrane protein, CHAPS to a final concentration of 0.25% w/v was added. This mixture was diluted 2.5-fold with equilibration buffer, applied on the column and eluted by a linear gradient of 0−0.35 M KCl in equilibration buffer. Fractions of 1 mL were collected and analyzed as described above. The concentration of KCl in the various fractions was determined by conductometry using serial dilutions of 1 M KCl as a standard.

Determination of ATPase Activity. E-type ATPase activity was determined as the enzymatic release of P_i from ATP into a medium consisting of 20 mM HEPES, 50 mM NaCl, 1 mM $CaCl_2$, and 1 mM ATP, pH 7.5, in the presence of the membrane endo-ATPase inhibitors: 1 mM ouabain, 10 mM sodium azide, and 100 μM sodium orthovanadate. For determination of the enzymatic P_i release, the nonenzymatic P_i release from ATP taking place during incubation in assay medium without enzyme was measured. Likewise, the P_i content in the enzyme-containing samples without addition of ATP and without incubation was determined, and these two values were subtracted from the total P_i released during the enzymatic reaction. KH_2PO_4 was used as P_i standard. When intact cells were assayed, the medium additionally contained 10% w/v sucrose and 10 mM glucose. Routinely, duplicate samples of synaptosomes were incubated for 15−30 min at 37 °C after addition of ATP. P_i was determined mixing equal volumes of reaction mixture with ice-cold P_i reagent consisting of 1% w/v ammonium heptamolybdate, 1% w/v SDS and 0.2% w/v ascorbic acid in 3% v/v H_2SO_4 (*13*, *37*). After color development for 15 min at 37 °C the absorbance at 820 nm was measured (procedure A). ATPase activity of proteins released from synaptosomes and of material bound to the immunosorbent was determined similarly, but terminating the reaction after 60−90 min. P_i release from immunoadsorbed NCAM preparations was, as previously shown, linear in this period (*13*). The released P_i was determined colorimetrically in aliquots of reaction mixture using both procedure A and a modified, more sensitive procedure, procedure B, in which a malachite green color reagent was used. After a 5−10-fold dilution of the samples with distilled water, 120 μL aliquots were mixed in wells of a 96-well microtiter plate (Greiner, Frickenhausen, Germany) with 30 μL of malachite green reagent prepared according Baykov et al. (*38*). One minute later 15 μL of quenching solution consisting of 34% w/v sodium citrate dihydrate was added (*39*), and after 30 min at room temperature the absorbance at 620 nm was measured. Since the results obtained by the two colorimetric procedures were identical, mean values were calculated. When we assayed very dilute samples (e.g. chromatographic fractions), they were mixed with 2-fold concentrated ATPase assay medium, and after 60 min at 37 °C 120 μL of the reaction mixture was withdrawn, and P_i was determined without dilution of the sample using procedure B. In some experiments, the remaining reaction mixture was incubated additionally for 90 min, and the released P_i was determined according to procedure A. This resulted in approximately 25% lower ATPase activity values, but the distribution of activity after fractionation was identical to that obtained with procedure B. Finally, the P_i content in samples, to which no ATP was added, was determined for the individual chromatographic fractions. Na,K-ATPase was measured as the difference in enzymatic P_i release into assay media, containing 30 mM histidine/HCl, pH 7.5, 3 mM $MgCl_2$, 130 mM NaCl, 20 mM KCl, and 3 mM ATP in the absence or presence of 1 mM ouabain (*25*, *33*, *35*).

Gel Electrophoresis, Immunoblotting, Enzyme-Linked Immunosorbent Assay (ELISA) and Thin-Layer Chromatography (TLC). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis, SDS−PAGE, was performed in 5% or 7.5% w/v polyacrylamide gels according to Laemmli (*40*). Samples were dissolved in SDS sample buffer (2% w/v SDS, 10 mM dithiothreitol, 5 mM EDTA, 1 mM PMSF) and incubated for 3 min in boiling water. FSBA-modified samples were treated for 20 min at 50 °C in sample buffer, and PAGE was performed at ca. 15 °C. As a positive control Na,K-ATPase isolated from pig kidney was employed. It was modified by FSBA as described above or biotinylated by a 10-fold molar excess of biotinamidocaproate *N*-hydroxysuccinimide ester over protein, washed, and solubilized using a 6-fold excess (w/w) over protein of the nonionic detergent octaethylene glycol dodecyl monoether, $C_{12}E_8$. The solubilized biotinylated preparation was precipitated by 70% v/v methanol overnight at −20 °C and separated by SDS−PAGE. Electroblotting onto nitrocellulose sheets was performed according to Bjerrum and Schafer-Nielsen (*41*). Binding of FSBA was detected using rabbit polyclonal antibodies against FSBA. Blots were incubated for 30 min in 2% w/v gelatin dissolved in 50 mM Tris, 150 mM NaCl, pH 7.4 (TBS), then additionally blocked in 2% w/v Tween-20 in TBS for 5 min, and, after washing in 0.1% w/v Tween-20 in TBS (T-TBS), incubated for 2 h with anti-FSBA antibodies diluted 1:500 in T-TBS. For detection of NCAM, blots were blocked for 5 min by 2% w/v Tween-20 in TBS, washed in T-TBS and incubated with rabbit antibodies against rat brain NCAM diluted with T-TBS 1:1000. Bound primary anti-FSBA or anti-NCAM antibodies were visualized by incubation with HRP-conjugated swine antibodies against rabbit immunoglobulins followed by ECL detection using the SuperSignal CL-ECL substrate system and Kodak BioMax MR-50 film. The relative intensity of immunoblotted polypeptide bands was estimated by scanning the films after ECL detection followed by quantification using Molecular Dynamics Image Quant software. Relevant controls were subtracted from the obtained values. Blots of biotinylated or FSBA-modified Na,K-ATPase were incubated with AP-conjugated streptavidin or AP-conjugated secondary antibodies, respectively, using a chromogenic reaction for enzyme detection.

TLC of nucleotides was performed according to Bronnikov and Zakharov (*42*). Samples were concentrated by freeze-drying and separated by TLC on silica gel containing a fluorescent indicator.

ELISA of NCAM was performed as previously described (*32*).

RESULTS

Immunoisolation of NCAM from NCAM-Transfected L-Cells

Immunoisolated NCAM has previously been shown to retain ca. 15% of the total E-type ATPase activity (13) of CHAPS-solubilized synaptosomes, while only 0.7% of the membrane protein is constituted by NCAM. In order to determine whether ecto-ATPase activity of NCAM was tightly associated with NCAM or an integral function of one

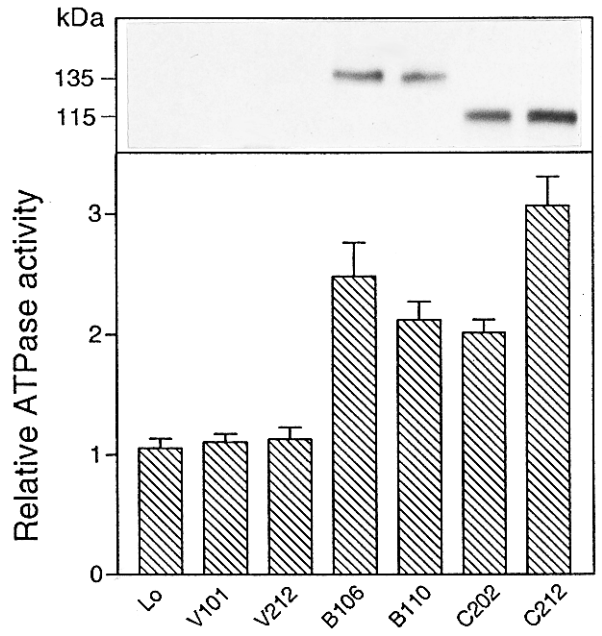

FIGURE 1: E-type ATPase activity immunoisolated from human NCAM-transfected L-cells. Membrane fractions from parent L0, vector- (V101, V212), NCAM-B- (B106, B110), and NCAM-C- (C202, C212) transfected cells were solubilized with 0.5% CHAPS. Equal aliquots of the supernatants were incubated with human NCAM- or control-immunosorbent. The ratio of ATPase activities bound to NCAM-immunosorbent over control-immunosorbent is shown. Immunoblotting by human NCAM antibodies of the immunoadsorbed NCAM preparations is shown in the insert. Bars on the left-hand side indicate positions of NCAM-B (135 kDa) and NCAM-C (115 kDa).

or more NCAM isoforms, we employed mouse fibroblastoid cells (L-cells) stably transfected with vector alone or with cDNA encoding the human transmembrane NCAM-B or the lipid-anchored NCAM-C isoforms. The NCAM-transfected cell lines expressed the protein at a level of approximately 0.1 pg of NCAM per cell (*26*, *27*). Ecto-ATPase activity was determined in two clones corresponding to each construct. The level expressed by vector-transfected cells was 0.73 ± 0.16 milliunits/10^6 cells (mean $\pm$ SEM), and by NCAM-B and -C-transfected clones 0.98 ± 0.09 and 1.14 ± 0.35 milliunits/10^6 cells, respectively. The increase in enzyme activity in the NCAM transfectants was not statistically significant. The specific activity of the NCAM-associated ATPase has previously been determined to be 4.5 units/mg of NCAM protein (*13*), and the expression of approximately 0.1 pg of NCAM per cell can therefore be calculated to add an activity corresponding to 0.45 milliunits/10^6 cells, which is of the same size as the observed variation in the employed cell lines. NCAM was therefore immunoisolated from membrane fractions prepared from the wild-type cells or the transfected cell lines, and the amount of NCAM was determined by ELISA and found to be 1.1 ± 0.09 and 1.0 ± 0.06 μg of NCAM per mg of membrane protein for the two NCAM-B-transfected (B106 and B110), and 0.8 ± 0.08 and 1.2 ± 0.13 μg of NCAM per mg of membrane protein for the two NCAM-C-transfected clones (C202 and C212). No NCAM could be detected in wild type L-cells (L0) or vector-transfected clones (V101 and V212). NCAM-B and NCAM-C were immunoisolated from CHAPS-solubilized membrane fractions of the corresponding clones, as visualized by immunoblotting (Figure 1, insert). No NCAM immunoreactivity was detected in the wild-type or the vector-transfected clones. The ATPase activity in fractions isolated by means of NCAM-specific antibodies relative to the activity in fractions isolated by means of control-antibodies appears from Figure 1. The relative activity was increased in all four NCAM-positive clones, whereas no such increase was observed in the three control-clones. We therefore conclude that lipid-anchored NCAM-C as well as transmembrane NCAM-B were coisolated with an E-type ATPase activity in NCAM-transfected L-cell.

Analysis of Soluble Forms of NCAM

In order to obtain the extracellular part of NCAM in a soluble form, two procedures were used: (i) incubation of synaptosomes for 5−8 h at 37 °C in 25 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, resulting in a spontaneous proteolytic cleavage of NCAM at a site close to the extracellular surface of the membrane (*43*, *44*); (ii) treatment of rat synaptosomes by PI-PLC for 3 h at 37 °C resulting in the release of NCAM-C from the membranes. The proteolytically released fragment did, as expected, not react on immunoblots with a monoclonal antibody, OB11, which recognizes the cytoplasmic part of transmembrane isoforms of NCAM (not shown). Treatment of synaptosomes with PI-PLC released ca. 5% of the total E-type ATPase activity and 7% of the total protein of the membrane. Spontaneous proteolysis released ca. 6−8% of the total enzyme activity and up to 12% of the total protein when incubating for 5−8 h at 37 °C. When incubating for 8 h the total activity was reduced by 40% and longer incubations did not increase the recovery of enzyme in the soluble fraction. Even though the treatment did not increase the specific activity of E-type Ca^{2+}-dependent ATPase in the supernatant, the spectrum of membrane ATPases recovered in the supernatant differed drastically from the original pattern observed in synaptosomes and in the mixture before sedimentation. This is exemplified by one of the major transport ATPases of synaptosomes, Na,K-ATPase, whose spatial organization including several transmembrane segments prevents membrane release of the enzyme in an active form (see e.g. ref 25). After treatment, Na,K-ATPase, in contrast to the E-type ATPase activity, remained in the sedimented membrane fraction, and only trace amounts, not exceeding 0.5% of the ATPase activity present in the mixture before sedimentation, were detectable in the supernatant (not shown). This indicated that the major part of the of E-type activity measured in the supernatant did not originate from small membrane fragments not sedimented by high-speed centrifugation.

In Figure 2A and B are shown the results of ion-exchange chromatography on a Mono Q column of spontaneously released material containing 18 milliunits of E-type ATPase activity obtained by procedure i using two different fractionation buffers (see legend to Figure 2), which both contained 0.25% CHAPS. At pH 7.0 in the presence of 0.1 mM EDTA all applied activity was bound to the Mono Q column, and the major peak of ATPase activity coeluted with fractions containing NCAM using a linear (0−0.45 M) gradient of NaCl (Figure 2A). For comparison, another buffer system containing 2 mM $MgCl_2$ in 10 mM Tris-HCl, pH 8.2, was used, since it has been suggested that this buffer system was capable of separating ATPase activity from NCAM (*45*). An almost identical enzymatic activity was eluted, and also in this buffer system the major peak of

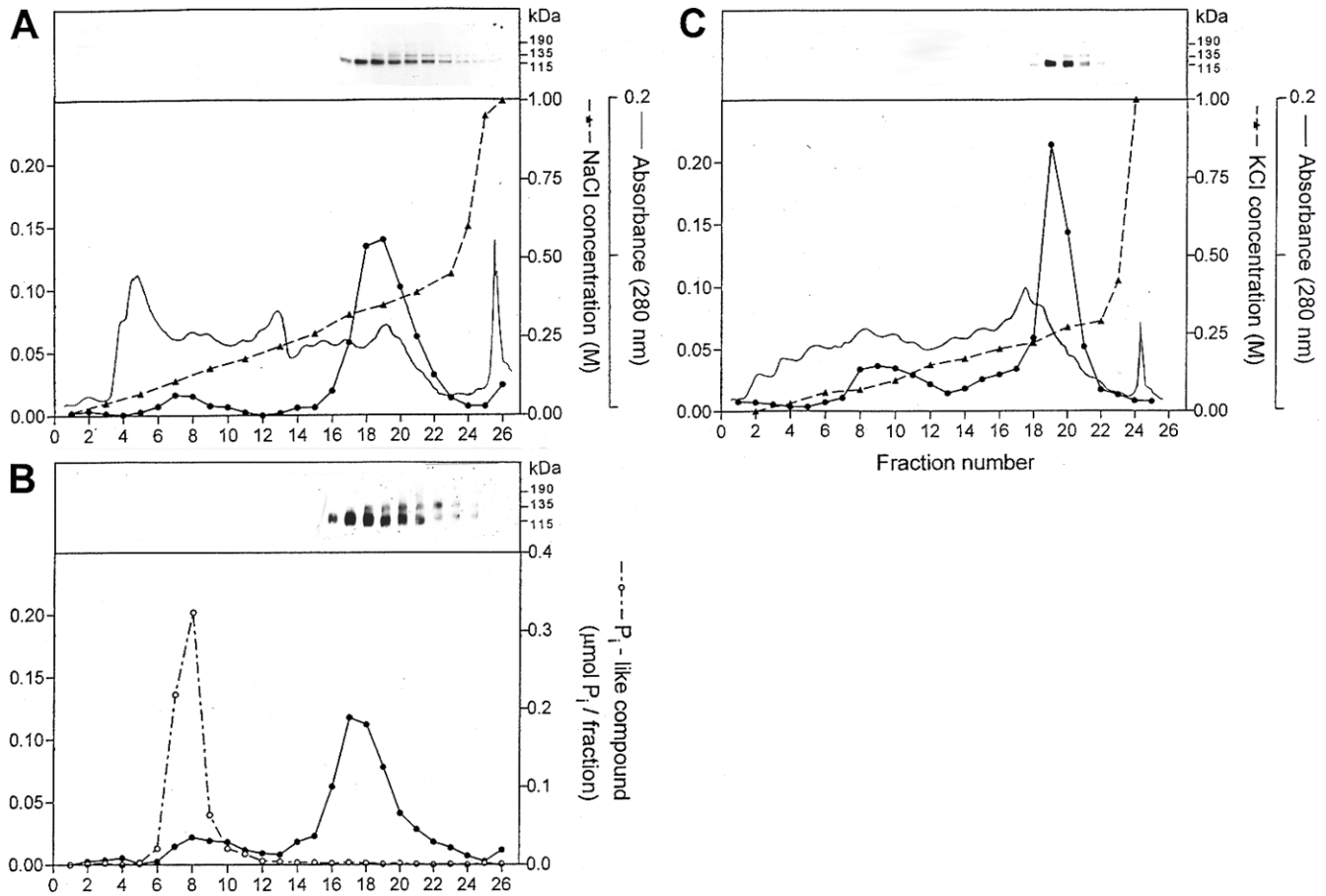

FIGURE 2: Chromatography of the soluble fractions spontaneously released from rat synaptosomes. The left *y*-axes indicate the ATPase activity (−●−) in μmoles of P_i/fraction/hour. Synaptosomes (4 mg of protein) were incubated in 25 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, for 8 h at 37 °C and then sedimented. Supernatants, each containing 18 milliunits of E-type ATPase activity, were diluted as described below for the individual columns, applied on the columns and fractionated (1 mL/fraction) by elution with a linear gradient of 0−0.45 M NaCl (A, B) or 0−0.35 M KCl (C) in equilibrations buffers. Separation was monitored by absorbance at 280 nm. E-type ATPase activity was measured as the enzymatic release of P_i from ATP determined colorimetrically, subtracting nonenzymatic ATP hydrolysis and P_i background in the individual fractions (see panel B). The concentration of NaCl and KCl was determined by conductometry. (A) A Mono Q column (HR 5/5) was equilibrated with 20 mM imidazole-HCl, 0.1 mM EDTA, 10% glycerol, 0.25% CHAPS, pH 7.5. CHAPS to a final concentration 0.25% was added to the supernatant sample. The mixture was diluted 2.5-fold with the equilibration buffer and applied on the column. (B) A Mono Q column was equilibrated with 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.25% CHAPS, pH 8.2. The supernatant was diluted 2.5-fold with distilled water, supplemented with $MgCl_2$ and CHAPS giving final concentrations of 2 mM and 0.25%, respectively. pH was adjusted to pH 8.2 with Tris-HCl, and the mixture was applied on the column. The results of the colorimetric determination of P_i (or P_i-like compounds) in the individual chromatographic fractions are plotted as P_i-content per fraction. (C) A lysine-Sepharose column, 4 mL bed volume, was equilibrated with 25 mM Tris-HCl, 0.25% CHAPS, pH 7.4. CHAPS was added to the supernatant sample to a final concentration of 0.25%, and the mixture was diluted 2.5-fold with equilibration buffer and applied on the column. Immunoblotting with NCAM antibodies of aliquots of fractions from the chromatographies A, B, and C, respectively, are shown at the top of each panel. Bars on the right-hand side indicate positions of intact NCAM-A (190 kDa), NCAM-B (135 kDa), and NCAM-C (115 kDa).

ATPase activity coeluted with NCAM, see Figure 2B. Two blanks were subtracted from all enzyme determinations: the P_i amounts released by nonenzymatic hydrolysis of ATP under the chosen conditions, and the P_i amounts in aliquots of the chromatographic fractions determined in ATPase assay medium without addition of ATP. The importance of the latter control appears from Figure 2B. Fractions 6−10 eluted from the Mono Q column at pH 8.2 gave a strong P_i-like reaction, whereas this compound at pH 7.0 was found in the effluent of the Mono Q column (not shown). This P_i-reactivity presumably mainly originated from the brain membranes rather than from the reagents used for the chromatography, since it could be detected in the soluble material obtained according to procedure i. The nature of this product was not further analysed, but since two different colorimetric procedures were employed, it probably was not an artifact of the determination procedure, indicating that if the background P_i content in the chromatographic fractions is not controlled, this peak might be misinterpreted as reflecting the elution of ATPase activity.

Lysine-Sepharose has previously been employed successfully for isolation of NCAM (*29*). When a preparation of spontaneously released proteins was applied on lysine-Sepharose which combines the characteristics of an ion-exchange and a group-specific adsorbent matrix, a highly concentrated peak of ATPase activity was eluted from the column using a 0−0.35 M linear gradient of KCl (Figure 2C). As shown in Figure 2C, insert, NCAM was again eluted in the same fractions as the major part of the ATPase activity. In all three chromatograms the dominant component in the NCAM fractions was the extracellular fragment of NCAM, which almost coincides in electrophoretic mobility with NCAM-C, followed by minor amounts of the NCAM-A and -B isoforms, which are known to be shed in intact forms from the membrane (*44*). A small, broad peak of E-type ATPase activity observed in all three chromatograms in

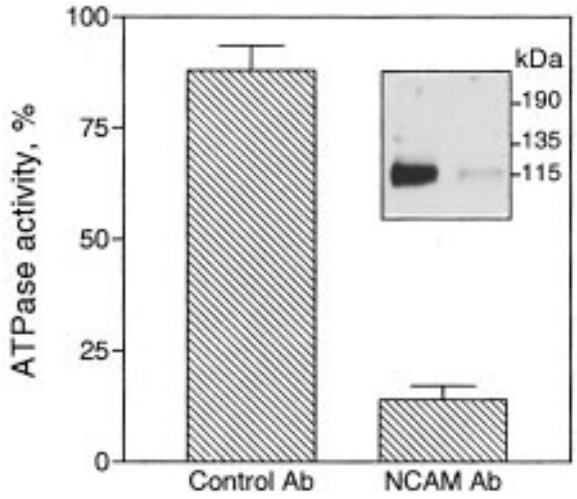

FIGURE 3: Depletion of PI-PLC released ATPase activity and NCAM by immunoadsorption. Synaptosomes were treated with PI-PLC for 3 h at 37 °C, and aliquots of the supernatant were incubated with immunosorbents coupled with control antibodies or NCAM antibodies. The E-type ATPase activity of the supernatant before immunoadsorption was taken as 100%. The ATPase activity remaining in the supernatant after sedimentation of the control- or NCAM-immunosorbent is expressed as a mean value ± SEM. Immunoblotting of NCAM remaining in the respective supernatants after control- (left) and NCAM- (right) immunoadsorption is shown in the insert. Bars on the right-hand side of the insert indicate positions of NCAM-A (190 kDa), NCAM-B (135 kDa), and NCAM-C (115 kDa).

fractions 6−13 may be derived from trace amounts of unsedimented membrane fragments solubilized by CHAPS. At this position, ca. 80% of the E-type ATPase activity of solubilized synaptosomes has previously been shown to be eluted (*11*), whereas a later, minor peak containing the three intact isoforms of NCAM and enriched in NCAM-associated ATPase (*11*) coincides with the peak of the extracellular part of NCAM in the experiments presented here. In conclusion, the major peak of ATPase activity shed from brain membranes coeluted with the extracellular fragment of NCAM after chromatography on different matrices.

Treatment of synaptosomes with PI-PLC (procedure ii) provided another way of obtaining the extracellular part of NCAM, in this case by hydrolysis of the GPI-anchor, which connects NCAM-C to the membrane. As mentioned above, this treatment released ca. 5% of the total E-type ATPase activity. In order to test the possible association between the released activity and NCAM, immunoadsorption was employed. In Figure 3 is shown the enzyme activity in the supernatant after incubation with either anti-NCAM or control antibodies coupled to protein G-Sepharose. NCAM was almost completely removed from the supernatant by the NCAM-immunosorbent, but not by the control-immunosorbent, see Figure 3, insert. The NCAM-immunosorbent also removed almost 90% of the PI-PLC-released enzymatic activity, whereas ATPase activity in the supernatant decreased only slightly after incubation with control-immunosorbent.

Thus, the NCAM-associated ATPase activity accounted for the major part of the ATPase activity in PI-PLC released material. By means of different chromatographic procedures and immunoprecipitation of proteolytically and PI-PLC released ATPase activity and NCAM, independent results were obtained indicating that the catalytic capacity either was a function of the extracellular part of NCAM or reflected a tight functional association of this part of NCAM and an ecto-ATPase. Therefore, to identify the catalytic component, affinity modification was employed.

Affinity Modification

Two ATP analogues capable of modifying ATP-binding sites, FSBA and oATP, were tested for their inhibitory action on the NCAM-associated ATPase activity. Synaptosomes were solubilized by CHAPS, and NCAM was immunoisolated by NCAM-immunosorbent. Control-immunosorbent bound an ATPase activity corresponding to ca. 10% of that isolated by the specific antibodies. K_m for ATP of immunoisolated NCAM-associated ATPase activity was determined to be 110 μM (not shown), which is in the range determined for E-type ATPases of rodent synaptosomes [50−194 μM (*24*, *46*)]. Both oATP and FSBA were able to inactivate the NCAM-associated ATPase. oATP caused a strong and irreversible inactivation (Figure 4A). Addition of 5 mM $NaCNBH_3$ was without effect (not shown), indicating that a conjugated Schiff base or a morpholine-like compound, but not a simple Schiff base, were the most probable products of the modification. A morpholine-like product of the oATP modification has in fact been identified at the active sites of some transport ATPases (*33*, *47*). ATP protected the enzyme from inactivation, but even at a 20-fold excess of ATP over the reagent the protection was only partial, probably due to β-elimination in oATP, resulting in the accumulation of a highly active and less selectively modifying product during the incubation (*33*, *47*). FSBA also irreversibly inactivated the NCAM-immunoisolated ATPase. The inhibition was time-dependent (Figure 4B) and concentration-dependent (Figure 4C), and achieved a maximal level of approximately 90% at a concentration of 1.5 mM FSBA after incubation for 1 h at 37 °C (Figure 4B,C). Inactivation followed pseudo-first-order kinetics with a rate constant $k_{obs} = 0.04$ min^{-1} at 1.5 mM FSBA and with a $K_D = 1.8$ mM as determined from a double-reciprocal plot of $1/k_{obs}$ versus 1/[FSBA] in the range 0.15−1.5 mM FSBA (plots not shown). ATP added in a 10-fold molar excess over the reagent protected the ATPase activity almost completely (Figure 4C). A similar inactivation of the NCAM-associated ATPase and protection with ATP were observed, when the modification was performed on synaptosomes followed by immunoisolation of NCAM (see Figure 6 and below). Subsequently, the latter approach, which presumably preserves the native conformation of the protein during modification, was employed for detection of bound FSBA in immunoisolated NCAM.

Binding of FSBA to Immunoisolated NCAM

Covalently bound FSBA in samples of immunoisolated NCAM was demonstrated by immunoblotting using anti-FSBA antibodies. Since these antibodies recognize the adenosine moiety in the products of FSBA modification (*48*), care was taken to preserve the ester linkage between the benzoyl and nucleoside moieties of the reagent. Due to a decreased stability of this linkage at elevated temperatures and extreme pH values (*49*), boiling as well as high and low pH values were avoided, e.g. in connection with protein staining, and SDS−PAGE was performed at ca. 15 °C. First, the selectivity of the FSBA-immunoblotting procedure was tested on purified pig kidney Na,K-ATPase. This enzyme

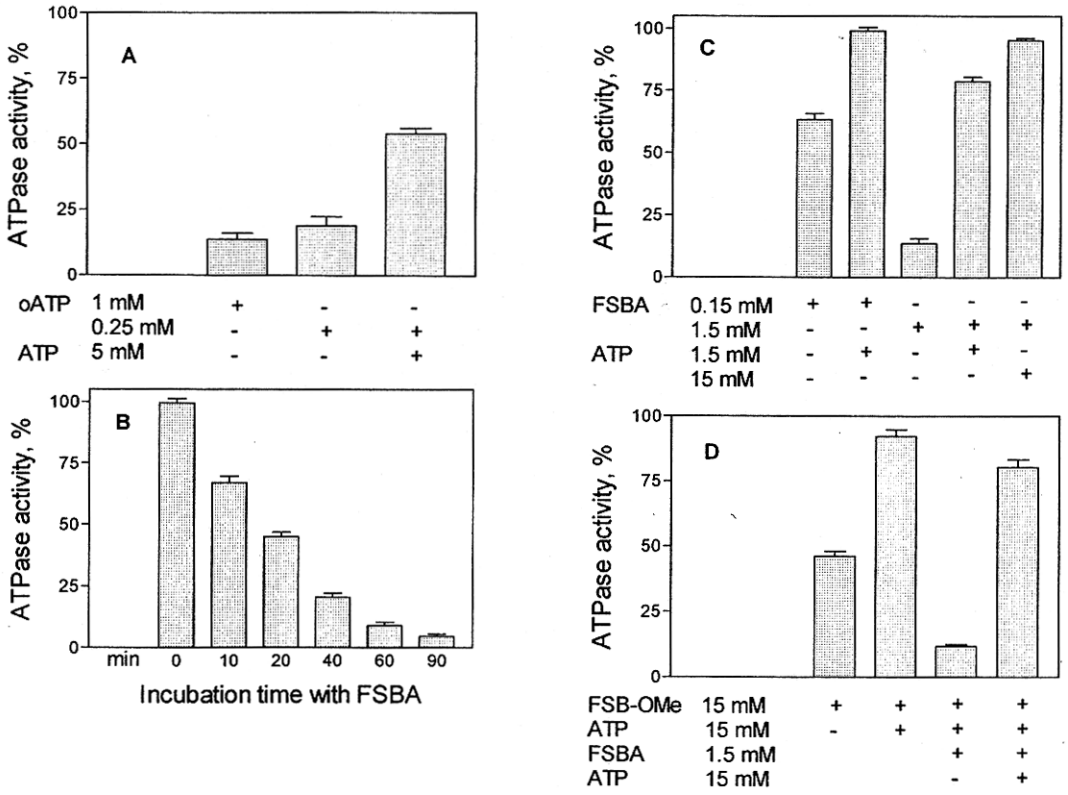

FIGURE 4: Inhibition of NCAM-immunoisolated ATPase activity by ATP analogues. NCAM was immunoisolated by NCAM-immunosorbent from supernatants of CHAPS-solubilized synaptosomes. Equal aliquots of the immunosorbent with bound NCAM were incubated in 20 mM HEPES, 0.2 mM $MgCl_2$, pH 7.5, at 37 °C for 1 h (A, C, D) or 0−90 min (B) with the affinity reagents in the presence or absence of ATP in the indicated concentrations. FSBA and FSB-OMe were added from stock solutions in DMSO, giving a final DMSO concentration of 5%. (A) Modification with oATP; (B) time-dependence of modification with 1.5 mM FSBA; (C) modification with 0.15 or 1.5 mM FSBA; (D) two-step modification with FSB-OMe followed by FSBA. First, the immunoisolated preparation was modified with FSB-OMe in the absence or presence of ATP in 20 mM HEPES, pH 7.5, for 1 h at 22 °C. Secondly, the preparation treated with FSB-OMe in the presence of ATP (column 2), after appropriate washing, was modified with FSBA in the absence (column 3) or presence (column 4) of ATP. The activity of control samples corresponding to each of the test samples, containing all added compounds except the modifying reagent was taken as 100%. Controls to samples treated with the reagents in the presence of ATP, contained both DMSO and ATP, and controls to samples without ATP contained DMSO only. The E-type ATPase activity remaining after modification was expressed as the mean values of percent activity of the corresponding control ± SEM.

consists of two types of tightly associated subunits, as seen in Figure 5A, lane 1, showing biotinylated Na,K-ATPase probed with AP-conjugated streptavidin. Only the catalytic α-subunit was recognized by the FSBA antibodies after modification despite the fact that the noncatalytic β-subunit is a membrane glycoprotein, containing exposed to the hydrophilic phase several chemical groups which might be unspecifically modified (Figure 5A, lanes 2−5). The modification resulted in an almost complete inactivation of the enzyme (not shown). The detection limit corresponded to 1.25 μg of the FSBA-inactivated Na,K-ATPase and to ~0.9 μg of the α-subunit when employing AP-conjugated secondary antibodies and chromogenic visualization. Therefore, to increase the sensitivity of the procedure HRP-coupled secondary antibodies and the ECL detection procedure were employed subsequently. Figure 5B shows an immunoblotting of a preparation of NCAM immunoisolated from 0.2 mL of CHAPS-solubilized synaptosomes containing 1.4 μg of NCAM (all isoforms) and modified with different concentrations of FSBA and probed with FSBA antibodies. In Figure 4C it can be seen that FSBA at a concentration of 0.15 mM reduced the activity of the immunoisolated enzyme by ca. 30%, and that the inhibition was completely prevented by addition of 1.5 mM ATP. In Figure 5B it is shown that the same ATP concentration nearly completely prevented incorporation of FSBA (compare lane 2 with 1), indicating that the modification predominantly was restricted to the substrate-binding site. At 1.5 mM FSBA the NCAM-associated ATPase was almost completely inactivated, but a 10-fold excess of ATP still protected the enzymatic activity from inactivation as demonstrated in Figure 4C. However, under these conditions, ATP only partially inhibited the binding of FSBA (compare lanes 1 and 2 with lanes 3 and 4 in Figure 5B), indicating that at higher FSBA concentrations an unspecific modification outside of the ATP-binding site probably took place. To decrease or prevent unspecific FSBA binding, FSB-OMe was employed. This compound, which contains the same modifying group as FSBA, but no "substrate-like" part, had hardly any inhibitory effect on the immunoisolated ATPase at a concentration of 1.5 mM (not shown). FSB-OMe at 15 mM partially inhibited the immunoisolated ATPase (Figure 4D), but addition of ATP in an equimolar amount eliminated the inactivation, indicating that FSB-OMe has access to, but not a high affinity for the catalytic site. If affinity modification with FSBA was performed after removal of FSB-OMe and ATP, a strong inhibition of the enzymatic activity was observed, and this inhibition was almost as effectively prevented by ATP as the effect of treatment with FSBA alone (compare Figure 4C and 4D). The difference between the two procedures appears when the level of unspecific incorporation of FSBA into immunoisolated NCAM preparations, i.e. in the presence

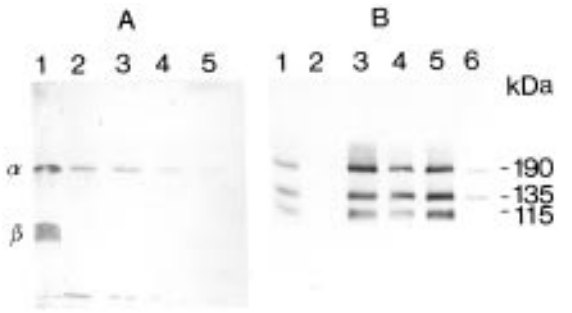

FIGURE 5: Selectivity and optimization of immunodetection of bound FSBA. (A) Purified biotinylated pig kidney Na,K-ATPase (2.5 μg of protein in lane 1) or FSBA-modified Na, K-ATPase (10, 5, 2.5, and 1.25 μg in lanes 2−5, respectively) were either boiled in SDS−PAGE sample buffer for 3 min (lanes 1 and 2) or incubated for 20 min at 50 °C (lanes 3−5), separated by SDS−PAGE in 7.5% polyacrylamide gel, electroblotted onto nitrocellulose and probed with streptavidin-AP (lane 1) or with anti-FSBA antibodies, followed by incubation with AP-conjugated secondary pig anti-rabbit immunoglobulin antibodies (lanes 2−5) and chromogenic detection. (B) NCAM preparations immunoisolated from 0.2 mL of CHAPS-solubilized synaptosomes containing, all together, 1.4 μg of NCAM-A, -B, and -C were modified as described in Figure 4B and C with 0.15 mM FSBA (lanes 1 and 2) or 1.5 mM FSBA (lanes 3−6) in the presence of ATP: 1.5 mM (lane 2) or 15 mM (lanes 4, 6). The preparations in lanes 5 and 6 were treated with 15 mM FSB-OMe in the presence of 15 mM ATP before modification with FSBA. Samples were incubated for 20 min at 50 °C in sample buffer and separated by SDS−PAGE in 5% gel followed by immunoblotting using anti-FSBA antibodies. HRP-conjugated secondary antibodies and the ECL detection procedure using the SuperSignal CL-HRP substrate system were employed. Bars on the right side indicate the position of NCAM-A (190 kDa), NCAM-B (135 kDa), and NCAM-C (115 kDa).

of ATP, is compared (see lanes 3 and 4 vs lanes 5 and 6 in Figure 5B). The comparison shows that the introduction of the two-step modification allowed the optimization of the procedure yielding a remarkable increase in the selectivity of the FSBA incorporation at the ATP-binding site. Obviously, a hallmark of the procedure is that the FSBA-antibodies recognize an adenosine moiety absent in FSB-OMe.

The two-step modification was therefore employed in the experiments shown in Figure 6A and B. Synaptosomes were initially treated with FSB-OMe in the presence of ATP and, after removing FSB-OMe and the protective ligand by washing, equal amounts of synaptosomes were subsequently modified with FSBA in the presence of increasing ATP concentrations. NCAM was immunoisolated from the modified membranes, and immunoisolated ATPase activity was measured. The activity of the immunoisolated preparation after treatment with FSB-OMe in the presence of ATP was regarded as 100%, and the degree of inactivation by FSBA was determined and indicated at the top of Figure 6A. Treatment of the modified synaptosomes with 10 mM dithiothreitol did not reverse inactivation (not shown). Subsequently, immunoblotting of two identical aliquots of each sample was performed using antibodies against FSBA (Figure 6A, lanes 1−5) or against NCAM (Figure 6A, lanes 6−10). As seen in Figure 6A, lanes 6−9, and Figure 6B, the immunoprecipitates contained nearly identical amounts of NCAM. Taking the relative expression of the NCAM A, B, and C isoforms in adult rat brain as 24, 43, and 25% (*50*) and the molecular masses of the polypeptides as 120, 90, and 80 kDa (Swiss-Prot), 1.7 μg of the applied NCAM

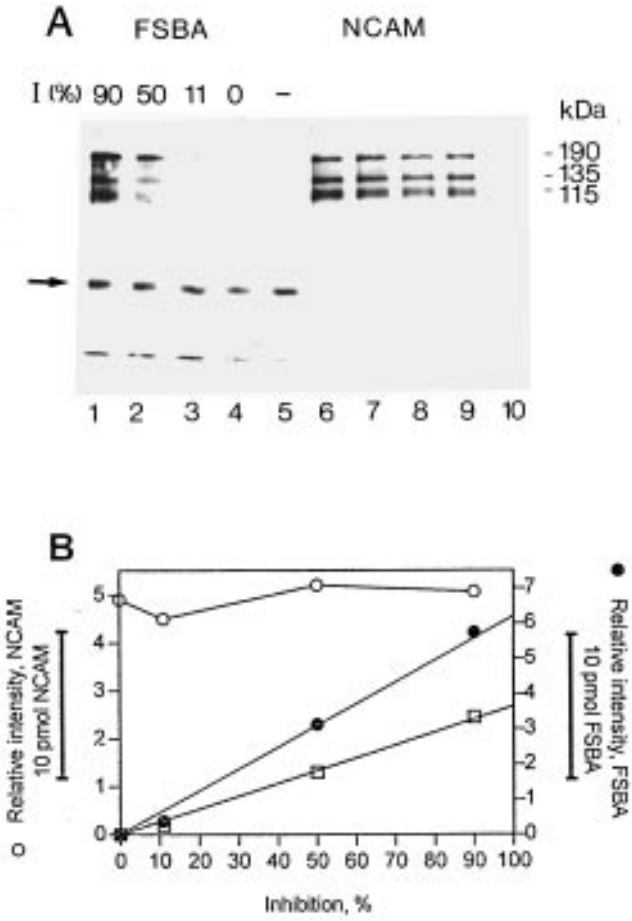

FIGURE 6: Detection of FSBA in immunoisolated NCAM. (A) Synaptosomes were first modified with 15 mM FSB-OMe in the presence of 15 mM ATP, washed and then modified with 1.5 mM FSBA in the absence (lanes 1 and 6) or in the presence of 5 mM ATP (lanes 2 and 7) or 15 mM ATP (lanes 3 and 8). Subsequently, NCAM was immunoisolated from the synaptosomes, treated as described in Figure 5B, and two identical samples each containing 1.7 μg of NCAM were separated by SDS−PAGE. Control NCAM preparations without modification are shown in lanes 4 and 9. In lanes 5 and 10 is shown an SDS-eluate of the NCAM-immunosorbent after incubation in CHAPS-containing solubilization buffer alone. Lanes 1−5 were immunoblotted with FSBA antibodies, and lanes 6−10 with NCAM antibodies. The positions of NCAM-A (190 kDa), NCAM-B (135 kDa), and NCAM-C (115 kDa) are indicated on the right-hand side, and the arrow on the left-hand side indicates the position of immunoglobulin heavy chains. Percent inhibition of the immunoisolated ATPase activity is indicated at the top of the FSBA immunoblotting. (B) The relative intensities of identical areas containing the three NCAM bands were quantified by scanning and Image analysis. Open circles represent intensities in the NCAM immunoblot (lanes 6−9) from which the control in lane 10 was subtracted. Lanes 1−4 in the FSBA-immunoblot (filled circles) were quantified similarly, subtracting the unspecific binding in the control sample in lane 4. Calibration to pmoles of bound FSBA was performed using the α-subunit of FSBA-modified Na,K-ATPase as a standard and the stoichiometric value given in ref 51. Squares show FSBA binding normalized to an amount of 10 pmol of NCAM. Calibration bars corresponding to the size of the signal generated by either 10 pmol of NCAM or 10 pmol of FSBA are presented at the left- and right-hand sides of the graph.

was calculated to correspond to 3.4, 8.0, and 5.3 pmol of NCAM A, B, and C, respectively, or to 16.7 pmol of all three isoforms. A calibration bar indicating the size of the signal of 10 pmol NCAM is shown on the left hand side of Figure 6B. If the incubation of solubilized synaptosomes with immunosorbent was omitted, no NCAM, only traces of IgG heavy chains, were recognized by the secondary

antibodies (Figure 6A, lane 10). IgG heavy chains were seen in all the FSBA-probed samples (Figure 6A, lanes 1−5), because the FSBA-immunoblots were exposed for ca. 2 min, whereas the NCAM-immunoblots only were exposed for ca. 5−10 s. Covalent incorporation of FSBA was observed in all three isoforms of NCAM in the FSBA-modified preparations (lanes 1−3), but not in the unmodified control (lane 4), and the FSBA incorporation into all three NCAM isoforms as well as the inactivation of immunoisolated ATPase activity gradually decreased by addition of increasing ATP concentrations (lanes 1−3). FSBA-positive NCAM bands in lanes 1−4 in Figure 6A were quantified by scanning and the control sample (lane 4) was subtracted. By plotting the obtained values versus enzyme inhibition a linear interrelationship between the two values (Figure 6B) was revealed indicating a single type of ATP-protectable FSBA-binding sites in NCAM preparations. In order to evaluate the stoichiometry of FSBA incorporation, relative FSBA-band intensities were calibrated to the amount of the bound FSBA in pmoles as shown in Figure 6B. The size of the calibration bar, 10 pmol of FSBA on the right hand side of Figure 6B, was determined using a series of FSBA-modified samples of NCAM and Na,K-ATPase, inactivated up to 56% and 50%, respectively, and blotted from the same gel. A ratio of 0.6 mol of FSBA per mol of α-subunit of Na,K-ATPase at complete inactivation has previously been reported (*51*). Based on this, it could be calculated that approximately 15.3 pmol of FSBA bound to 16.7 pmol of NCAM at complete inactivation (Figure 6B). Likewise, plotting FSBA-binding values normalized by NCAM amounts resulted in a ratio of 0.9 mol of FSBA per mol of NCAM polypeptide at complete inactivation as an average value for all three isoforms (Figure 6B). Since the various NCAM isoforms in brain are subject to posttranslational modifications, of which some are isoform-specific (*22*, *23*), the contribution from the individual isoforms to nucleotide binding and enzymatic activity may vary. The obtained stoichiometry seems consistent with one ATP-protectable FSBA-binding site in NCAM.

Release of NCAM after FSBA Modification

When preparing spontaneously released NCAM from synaptosomes it was noted, that the FSBA treatment affected the NCAM release. As shown in Figure 7, the spontaneous proteolytic release of NCAM decreased dramatically, if the modification with FSBA was performed in the absence but not in the presence of ATP (compare lanes 1 and 2 in Figure 7). This was not due to a general perturbation of the membranes treated with FSBA, since the total amount of protein spontaneously released from membranes was unaffected by FSBA treatment both in the presence and absence of ATP, reaching 11.0 ± 1.7% of the total membrane protein after incubation at 37 °C for 8 h. This was almost identical to the release from untreated synaptosomes, which was 11.8 ± 1.4%. Furthermore, PI-PLC-mediated release of NCAM was not affected by FSBA modification of the same membrane preparations (compare lanes 3 and 4 in Figure 7), supporting the assumption that no general perturbation was introduced by the FSBA modification.

Using the two-step modification, blocking of unspecific binding sites by FSB-OMe in the presence of ATP caused a decrease of ca. 20% in the amount of spontaneously released total protein as compared to the release from untreated brain membranes. Subsequent FSBA treatment did not change this, neither in the presence nor in the absence of ATP. The spontaneous release of NCAM was remarkably reduced when FSBA treatment was carried out without ATP, while protection of the ATP-binding site(s) by ATP kept the level of NCAM release nearly identical to that of the control preparation, i.e. the level obtained after blocking unspecific binding sites with FSB-OMe (lanes 6 and 7 in Figure 7). Using the two-step procedure did not affect the PI-PLC mediated NCAM release, independent of whether the ATP-binding site(s) were blocked by FSBA or not (Figure 7, lanes 8−10). It can be seen that the electrophoretic mobility of spontaneously released NCAM slightly exceeded that of NCAM-C released by PI-PLC (compare e.g. lane 2 to lane 3 in Figure 7). This difference of ca. 5 kDa between soluble forms of NCAM generated by either proteolytic cleavage at an external site near the membrane or by enzymatic cleavage of the GPI anchor has been observed previously (*43*). Since the synaptosomes were washed after treatment, release of NCAM in all experiments occurred into the same medium. Therefore, the binding of the substrate analogue at the ATP-binding site of NCAM evidently reduced the spontaneous, proteolytic release of the adhesion molecule.

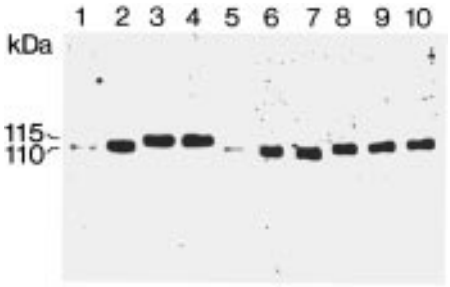

FIGURE 7: Effect of FSBA modification of synaptosomes on proteolytic and PI-PLC-mediated release of NCAM. Synaptosomes were modified by 1.5 mM FSBA (lanes 1−4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 15 mM ATP. Lanes 5−10 show synaptosomes which were modified by the two-step procedure: First with 15 mM FSB-OMe in the presence of 15 mM ATP (lanes 5−10) and subsequently with 1.5 mM FSBA (lanes 5, 6, 8, and 9) in the absence (lanes 5 and 8) or the presence (lanes 6 and 9) of 15 mM ATP. Samples only treated with FSB-OMe and ATP served as controls (lanes 7 and 10). After washing, equal amounts of suspended synaptosomes were either incubated at 37 °C for 8 h (lanes 1, 2, and 5−7) or treated with PI-PLC for 3 h at 37 °C (lanes 3, 4, and 8−10). Supernatants were analyzed by NCAM-immunoblotting. Note a slightly higher mobility of the spontaneously released extracellular part of NCAM compared to the PI-PLC released NCAM-C.

Effect of ATP and ADP on NCAM Release

In a following series of experiments ATP and ADP were added to synaptosomes suspended in 0.1 mM EDTA, 25 mM Tris-HCl, pH 7.5. In this medium hydrolysis of the substrates proceeded slowly despite a rather high protein concentration of 0.7 mg/mL. At concentrations of 5 mM ATP or 10 mM ADP, respectively, hydrolysis was not complete even after 8 h of incubation at 37 °C, as judged by TLC on silica gel of the incubation medium (not shown). From Figure 8A, it appears that both ATP and ADP reduced the spontaneous release of NCAM, ATP being the more effective (see lanes 1−3). If the ATP containing medium was removed after 5 h of incubation, and membranes were resuspended in an ATP-free medium and sedimented immediately after, no NCAM was released (lane 4) indicating

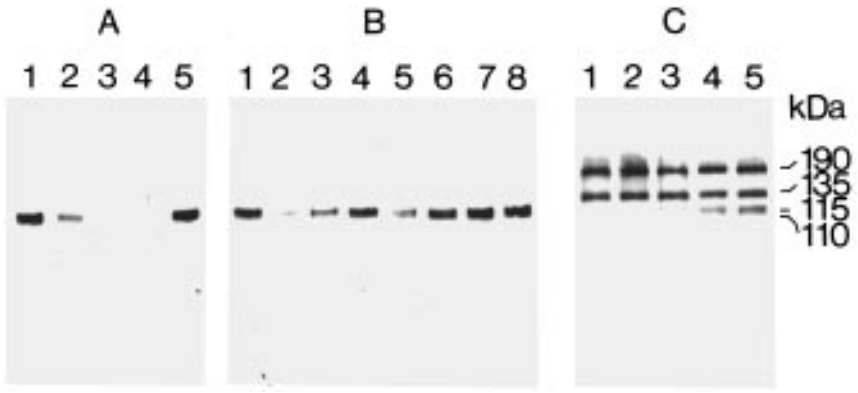

FIGURE 8: Effect of nucleotides on proteolytic release of NCAM. Synaptosomes were incubated in 25 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, at 37 °C for 5 h (A) and 8 h (B, C) with nucleotides added as indicated below. Supernatants (A, B) or pellets (C) obtained after sedimentation of synaptosomes (100000*g* for 1 h) were immunoblotted using NCAM antibodies. (A) Control-incubation without added nucleotides (lane 1), with 10 mM ADP (lane 2) or 5 mM ATP (lane 3). Synaptosomes, treated as in lane 3 and sedimented from the ATP-containing medium, were either washed with an ATP-free medium (lane 4) or incubated in ATP-free medium for additional 5 h at 37 °C (lane 5). (B) Control-medium from synaptosomes incubated without added nucleotides (lane 1) or with 5, 2.5, and 1.25 mM ATP (lanes 2−4), 10, 5, and 2.5 mM ADP (lanes 5−7) and 15 mM AMP (lane 8), respectively. (C) NCAM immunoblotting of the membrane-bound fractions. Pellets after PI-PLC-mediated release (lanes 1 and 2) or spontaneous release (lanes 3−5) were immunoblotted. Samples in lanes 1 and 2 were modified with 1.5 mM FSBA in the absence (lane 1) or presence (lane 2) of 15 mM ATP. The corresponding supernatants were shown in Figure 7, lanes 3 and 4. The membrane-bound material in lanes 3−5 was obtained in the absence of nucleotides (lane 3) or presence of 10 mM ADP (lane 4) or 5 mM ATP (lane 5). The corresponding supernatants are shown in panel B, lanes 1, 5, and 2. The positions of NCAM-A (190 kDa), NCAM-B (135 kDa), NCAM-C (115 kDa), and of the released extracellular part of NCAM (110 kDa) are marked on the right-hand side.

that ATP did not mediate an association between NCAM and an unknown component in the membrane, thereby preventing proteolytically cleaved NCAM from going into solution. Conversely, if the incubation was continued for additional 5 h after removal of ATP, NCAM was released into the supernatant (lane 5). When the concentration of ATP was decreased from 5 to 1.25 mM, the amounts of NCAM released into the medium gradually increased (Figure 8B, lanes 2−4). The same was observed, when the concentration of ADP was decreased from 10 to 2.5 mM (lanes 5−7). In samples with low substrate concentrations (lanes 4 and 7) only AMP and adenosine, but no ATP or ADP could be detected in the concentrated supernatants by TLC after 4 h incubation at 37 °C. Therefore, soluble NCAM was only released from synaptosomes by proteolysis when all substrates, ATP and ADP, of the NCAM-associated ATPase were converted into AMP and subsequently by the 5′-nucleotidase into adenosine. As shown in Figure 8B, lane 8, AMP even at a concentration of 15 mM, was unable to prevent a spontaneous release of NCAM of approximately the same magnitude as the release from the control, lane 1. Thus, binding of native substrate or an affinity modifier to the ATP-binding site present in all three NCAM isoforms, rather than derivatization *per se* protected the potential cleavage site near the membrane against proteolysis, most probably due to a conformational change in a part of the NCAM polypeptide chain close to the membrane.

Retention of NCAM-Isoforms on the Membrane by Treatment with ATP and ADP

Spontaneous release of the extracellular part of NCAM is, in principle, possible for each of the major NCAM isoforms (*44*). Figure 8C shows immunoblotting of the membrane-bound forms of NCAM after PI-PLC treatment or spontaneous NCAM release. Treatment by PI-PLC completely removed the C-isoform of NCAM from brain membranes modified by FSBA independent of addition of ATP (Figure 8C, lanes 1 and 2). Thus, binding of the ATP analogue does not affect hydrolysis at the GPI-anchor in NCAM. NCAM-C also appeared to be the major target of spontaneous proteolysis (lane 3), although it was not removed as completely as by PI-PLC treatment (compare lane 3 with lanes 1 and 2 in Figure 8C). A slight decrease in amounts of the two other isoforms, NCAM-A and -B, was also observed when comparing the samples in lanes 1 and 2 with lane 3. The demonstration of a preferential spontaneous release of NCAM-C over the two major transmembrane isoforms is in agreement with previous observations (*44*, *52*, *53*). The presence of substrates for the E-type ATPase activity of NCAM retained the adhesion molecule in a membrane-associated form. The effect was most pronounced for the C-isoform of NCAM. Thus, the observed effects of the nucleotides on membrane-association of NCAM and on release of the extracellular part of NCAM were mutually consistent. In conclusion, the results indicate that occupation of the nucleotide binding site in NCAM by native substrates or by covalently bound ATP analogue prevents the spontaneous release of NCAM from synaptosomal membranes, probably due to a local conformational change induced by substrate binding.

DISCUSSION

The demonstration of an E-type ATPase activity associated with immunoisolated NCAM preparations has raised a series of questions concerning the origin of this activity and its possible role in NCAM-mediated cell adhesion and adhesion-triggered processes (*11*, *13*). Different independent approaches were employed in the present study to answer these questions.

First, in order to elucidate the isoform-specificity of the phenomenon, separate NCAM isoforms were immunoisolated from cell lines stably transfected with GPI-anchored and transmembrane isoforms of NCAM. This approach had the additional advantage that unspecifically bound proteins in the NCAM preparations supposedly differed from those derived from neural tissue. The E-type ATPase activity

measured in the presence of orthovanadate, azide, and ouabain (inhibitors of the major membrane "endo"-ATPases and some phosphatases) was found to be associated with the transmembrane as well as the lipid-anchored isoforms of NCAM (Figure 1), indicating that the enzymatic activity—intrinsic or associated—was localized to the extracellular part of NCAM.

Next, we focused on the soluble forms of NCAM obtained either by a PI-PLC-mediated release of GPI-anchored NCAM-C or by spontaneous cleavage of NCAM at an extracellular site near the membrane. The latter procedure generates a slightly shorter extracellular NCAM fragment, which previously has been shown to have a preserved N-terminus (*43*, *53*). A major fraction of the E-type activity released by both procedures from synaptosomes together with NCAM could not be separated from the adhesion molecule. The enzymatic activity coeluted with NCAM when fractionated by different chromatographic procedures on different matrices (Figure 2). Furthermore, a major part of the ATPase activity released by PI-PLC was retained by the NCAM-immunosorbent (Figure 3). In contrast, no E-type ATPase activity could be released from chromaffin cells by PI-PLC treatment according to Torres et al. (*54*), in accordance with the observation that no NCAM-C was released by PI-PLC treatment of the closely related PC12 cells (*55*), and that chromaffin cells only express very low amounts (if any) of NCAM-C (*56*). If an NCAM-associated ATPase exists, it can neither be a transmembrane protein, nor GPI-anchored, since the enzyme activity in the here reported experiments followed both the spontaneously and the PI-PLC released NCAM. If it is a soluble, so called "exo"-ATPase (or a soluble fragment of an ATPase), it must be almost irreversibly associated to the extracellular part of NCAM. However, washing of synaptosomes with 2 M NaCl or 0.8 M NaI before solubilization and immunoisolation by precipitation-in-gel did not eliminate ATPase activity from NCAM immunoprecipitates (Dzhandzhugazyan and Bock, unpublished results), an unlikely feature for a soluble ATPase.

To label the catalytic component in immunoisolated NCAM preparation, affinity modification was employed. Nagy and Shuster (*57*) have recently shown by photoaffinity labeling with azido derivatives of ATP, that synaptosomes contain a large number of ATP-binding proteins. We decided to test ATP analogues containing chemically active groups. Compared to photoaffinity reagents, analogues of this type react with a more limited number of amino acid residues localized at the nucleotide binding site (for review, see ref 49). Therefore, they supposedly modify a lower number of ATP-binding proteins but are capable of a more complete and uniform derivatization at the ATP-binding sites (*49*, *58*). One such analogue, FSBA, has successfully been employed in studies of several nucleotide-binding proteins (*49*, *59*, *60*). Using immunoblotting for the detection of FSBA (*48*) an ATP binding site in ecto-ATP diphosphohydrolase from pig pancreas has been demonstrated (*60*). FSBA was found to inhibit the NCAM-associated ATPase activity (Figure 4B,C and Figure 6A) and was selectively incorporated at an ATP-binding site of NCAM (Figure 6A,B). In this connection it may be mentioned that photoinsertion of 8-N_3-ATP and 2-N_3-ATP, but not of N_3-cAMP into NCAM-B, in association with a highly purified preparation of adenylate cyclase, has been observed by Castets et al. (*61*). The fact that we detected the affinity label in all three major NCAM isoforms, including the lipid-anchored NCAM-C (Figure 6A) indicates, that NCAM contains an ATP-binding site, and that this site is localized extracellularly.

FSBA almost completely and irreversibly inactivated the NCAM-associated ATPase activity. The degree of inactivation correlated with the intensity of labeling (Figure 6A), and the stoichiometry was consistent with a single specifically labeled site at complete enzyme inhibition (Figure 6B) indicating that the nucleotide-binding site of NCAM probably is catalytic. This assumption is also supported by the demonstration of a similar conformational response of NCAM to the binding of each of the NCAM substrates: ATP, ADP, or the substrate analogue FSBA (Figure 7 and 8)—a feature common to many ATP-hydrolyzing enzymes (*62*). Nevertheless, it cannot be excluded that FSBA modifies a hypothetical regulatory nucleotide-binding site in NCAM. Since NCAM is immunoisolated as an individual protein (*43*) and not as a subunit of a stable heterooligomeric complex, a strong blocking effect from a regulatory site implies a localisation of the catalytic site within the NCAM polypeptide rather than in a putative contaminating enzyme, indicating that NCAM is the catalytic component in the preparation. These issues may be elucidated by isolation and sequencing of specifically labeled sulfonylbenzoyladenosine-containing peptide(s) and/or site-directed mutagenesis of the putative nucleotide-binding site.

No other FSBA-modified protein bands were detected by immunoblotting of NCAM. This fact, nevertheless, does not completely rule out the possibility, that the NCAM immune complex contains a highly active, FSBA-sensitive ATPase, whose modification cannot be detected due to low ATPase protein amounts. Studying a highly active ATPase from chicken gizzard Stout and Kirley (*63*) found no indication of an association between the ATPase and any adhesion molecule, since no cross-linking between the ATPase and any other protein was found. However, no data concerning a possible expression of NCAM in the chicken gizzard membranes were presented. Furthermore, the cross-linking procedure was unable to cross-link all the contacting protomers in the active trimer, since it only generated dimers. Therefore, a possible association between NCAM and a highly active ATPase has to be excluded otherwise. It has been reported that a homologue to the chicken enzyme E-type ATPase with ca. 1000-fold higher specific activity than NCAM has been isolated from transverse tubules of rabbit skeletal muscle, and by immunoblotting with antibodies raised against a synthetic peptide corresponding to the N-terminal sequence, the enzyme has been demonstrated in rat brain. The authors suggested that it here represents the major fraction of enzyme activity obtained from digitonin-solubilized rat brain microsomes (*45*). However, this highly active ATPase has been shown to be insensitive to FSBA modification (*5*) and differs in substrate specificity from the ATPase activity of immunoisolated NCAM. Therefore, it is unlikely that this enzyme is associated with NCAM.

Recently, a membrane glycoprotein CD39 originally described as a marker of activated lymphoid cells (*9*, *64*) has been identified as an apyrase (*7*), an enzyme hydrolyzing ATP and ADP, as NCAM does. Expression of CD39 was demonstrated on activated B cells, T cells, and natural killer cells (*9*), and the ATP-hydrolyzing activity of natural killer cells could be inhibited by FSBA treatment (*59*). FSBA

incorporation into an ecto-apyrase from pancreas, which also is a member of the CD39-like family, has recently been demonstrated (*60*). Expression of CD39 has been demonstrated in other, nonlymphoid tissues indicating a broad distribution of this protein (*8*). Large central extracellular loops in human and murine CD39 and in a recently cloned chicken gizzard ecto-ATPase (*65*) are homologous and contain "putative apyrase conserved regions" found also in yeast GDPase and in a group of apyrases expressed in some plants and obligatory parasites (*6*). This indicates a possible structural-functional relationship of all these enzymes known to be involved in a rapid degradation of nucleotides. However, no considerable homology within the CD39 structure was found with the N- and C-terminal regions of the chicken gizzard ecto-ATPase (*65*). Likewise, the N-terminal sequence of a rabbit T-tubule ATPase, which was used as immunogen for production of antibodies for detection of the enzyme in rat brain microsomes (*45*), shows no homology with the N-terminal or internal sequences of CD39, for which reason the antigen recognized in brain hardly can be CD39.

Data on expression of CD39-like proteins in brain are contradictory. There are indications based on cross-immunoreactivity with apyrases, that CD39-like proteins are expressed in rat brain (*12*), but no transcripts could be detected in brain RNA hybridized with full-length CD39 cDNA (*8*). No CD39 has been demonstrated in fibroblasts by flow cytometry (*9*). Thus, this protein probably does not contribute to the ATPase activity immunoisolated from the transfected L-cells. Furthermore, the mentioned ATPases, apyrases and CD39 are integral membrane proteins not supposed to be released by PI-PLC (*63*, *65*−*67*). Thus, our data favour the notion, that the extracellular ATP-binding site of NCAM is catalytically active. Furthermore, another enzyme involved in the conversion of ATP to adenosine, 5′-nucleotidase, also termed CD73, has recently been identified as an adhesion molecule (*21*). Data indicating a relationship between adhesion phenomena and ecto-ATP hydrolysis are thus accumulating (for review, see ref 3). The functional significance of combining an enzymatic and an adhesive function in the same molecule is currently not clear. A clue may be provided by the demonstration of inhibition of spontaneous release of NCAM after occupation of the ATP-binding site by ATP, ADP, or FSBA. However, FSBA (or any slowly- or nonhydrolyzable ATP analogue) might influence NCAM conformation and/or release indirectly by modification of other ATP-binding proteins, such as protein kinases, purinergic receptors, etc., especially since NCAM has been shown to be phosphorylated by an ecto-protein kinase (*68*). In case FSBA has an indirect effect, ATP must induce the opposite effect. Thus, a stimulating effect of ATP on the proteolytic release of L-selectin, mediated *via* the purinoreceptor P_{2z} in intact cells has been demonstrated by Jamieson et al. (*69*). This effect was blocked by modification with oATP. On the other hand, the effects may be expected to be similar or identical, if substrates and affinity reagent directly affect the same site in a protein, as observed for NCAM. The fact that FSBA, ATP, and ADP, but not AMP, affected the release of NCAM similarly, indicates that these reagents directly bind to the NCAM molecule. Occupation of the ATP-binding site, without hydrolysis as observed using the nonhydrolyzable compound FSBA, induced a conformational response in NCAM leading to protection of the proteolytic cleavage site near the membrane (Figures 7 and 8). This conformational transition seems to be specific, since the site of attack by PI-PLC localized C-terminally to the proteolytic cleavage site, was unaffected by the affinity modification (Figures 7 and 8). A conformational change induced by substrate binding is a rather common phenomenon among nucleotide-hydrolyzing enzymes, and here, to our knowledge, demonstrated for the first time in an ecto-ATPase. The site of the proteolytic cleavage is mapped to a short segment between the second fibronectin type III repeat of NCAM and the membrane (*43*). Remarkably, a sequence, $A^{676}ENQQGKS^{683}$, homologous to a common ATP-binding consensus sequence G/AXXXXGKT/S in a "P-loop", also termed a Walker motif A (*62*, *70*) has previously been noticed as a potential ATP-binding site in NCAM (*71*). This conserved sequence is localized in the C-terminal part of the second fibronectin type III repeat in NCAM, and thus in the region of the spontaneous proteolytic cleavage site affected by FSBA and ATP. (The amino acid residues are numbered according to the translation of rat NCAM aligned along the mouse NCAM-A sequence, Swiss-Prot accession number P13595, residue no. 1 being the start of the signal peptide.) Another motif conserved in P-loop containing enzymes, DXXG, whose function involves conformational changes during the catalytic cycle (*62*), is also found in the same region of NCAM, $D^{626}DGG^{629}$, and in the first Ig-like domain, $D^{89}DAG^{92}$. Since NCAM is presumed to have a rod-like structure (*72*), the participation of the latter fragment in the formation of a catalytic site seems unlikely, at least for the NCAM protomer. Three sequences homologous to a Walker motif B, H/R/K-X_{5-8}-$\Phi X\Phi_2$D/E, where Φ is a hydrophobic residue (*70*, *73*), are localized in the region between the IgV domain and the membrane, these correspond to R^{651}−D^{665}, K^{539}−D^{552}, and R^{485}−D^{499}. Thus, the structural data support our finding of an extracellular catalytic site in NCAM.

Our results seem to have at least two functional consequences. First, it is known that NCAM, beside binding to itself in a so-called homophilic binding, is capable of *cis*- and *trans*-interactions with a variety of proteins, such as the neural cell adhesion molecule L1 (*74*) and the adhesion molecule on glia, AMOG (*31*). Furthermore, NCAM is reported to transduce signals *via* an association with the fibroblast growth factor receptor (*75*). Conformational changes in NCAM might influence these interactions. In particular, ATP hydrolysis at a low turnover rate, ca. 1000 min^{-1}, might induce cyclic conformational changes near the membrane with possible implications in signal transduction. For comparison, GTPases whose function requires cyclic conformational changes, are known to have turnover rates that are at least 10-fold lower than that of NCAM, ca. 100 min^{-1}, and a slow turnover is thought to be important for an effective coupling of the GTP hydrolysis to the process of signal transduction (*76*).

Another functional aspect concerns the soluble forms of NCAM. Shedding of soluble NCAM forms is a well-documented event (*44*), both *in vitro* and *in vivo*, and it is supposed to regulate cell−cell adhesion, since the shedded forms retain their functional properties and therefore compete with adhesion molecules at the cell surface (*44*, *53*). Thus, soluble NCAM has been shown to be lethal during embryogenesis (*77*). The observed effect of ATP on NCAM shedding provides a mechanism for extracellular regulation

of adhesion by modulating the release of NCAM into the extracellular space, and by changing the relative amounts of NCAM associated with the cell surface.

Connections between neurons are not fixed. Constant modulations of synaptic contacts take place during development, regeneration and in connection with memory consolidation. This persisting potential for structural remodulation is termed synaptic plasticity. Several reports suggest an involvement in NCAM in regenerative processes (*78*−*80*) and in learning (*81*, *82*). NCAM knock-out mice exhibit impaired ability to establish long-term memory for a spatial learning task (*83*), and recently the participation of NCAM in long-term potentiation, LTP, an *in vitro* model for learning and memory formation, has been demonstrated (*84*, *85*). In this system, a few high-frequency stimulations of the CA1 synapses in hippocampal slices increase the strength of these synapses for a long period. Antibodies against NCAM reduce this potentiation. On the other hand, extracellular ATP has been shown to stimulate LTP *via* enzymatic mechanisms requiring ATP hydrolysis (*86*). Based on the presented results we propose, that ATP, released during synaptic activity as a cotransmitter, by slow hydrolysis by the E-type NCAM-ATPase supports synaptic transmission and at the same time preserves NCAM from proteolysis in the active, i.e. ATP-containing synapses. According to this hypothesis inactive synapses may be eliminated by a mechanism reducing the strength of contacts, first, by extracellular proteolysis of NCAM, and subsequently by destabilisation of contacts by the released extracellular NCAM fragments competing with intact membrane-attached NCAM. This hypothesis combines two lines of evidence concerning the possible roles of ATP and NCAM in synaptic plasticity.

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