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Demonstration of (Ca^{2+}-Mg^{2+})-ATPase activity of the neural cell adhesion molecule

Karine Dzhandzhugazyan*, Elisabeth Bock

Protein Laboratory, University of Copenhagen, Panum Institute, Blegdamsvej 3C, Bldg. 6.2, DK-2200 Copenhagen N, Denmark

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In this study a possible association between (Ca^{2+}-Mg^{2+})-ATPase activity and the neural cell adhesion molecule, NCAM, was investigated. The effects of various detergents on ATPase activity were evaluated, and it was found that solubilization of rat brain microsomes with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS, released a major fraction of the (Ca^{2+}-Mg^{2+})-ATPase activity together with NCAM. Using different types of solid phase immunoadsorption it was shown that NCAM antibodies selectively isolated ATPase activity. Furthermore, agarose gel immunoelectrophoresis of solubilized brain microsomes followed by ATPase assay directly in the gel revealed ATPase activity associated with the NCAM immunoprecipitate. The NCAM-associated enzyme activity had a broad nucleoside triphosphate specificity and no strict selectivity for divalent cations, indicating that the enzyme probably is an ecto-ATPase. This raises a series of intriguing questions in relation to NCAM adhesive functions.

Neural cell adhesion molecule (NCAM); Ecto-ATPase; (Ca^{2+}-Mg^{2+})-ATPase; Immunopurification; Enzyme assay in gel

1. INTRODUCTION

Increasing evidence indicates a structural and functional connection between various cell adhesion molecules, CAMs, and membrane ATPases. Thus, E-cadherin seems to be responsible for a polarized distribution of the Na^+, K^+-ATPase in epithelia [1]. The adhesion molecule on glia (AMOG) is homologous to the β_1-subunit of the Na^+, K^+-ATPase [2], and in combination with the catalytic α-subunit AMOG is able to form functionally active Na^+, K^+-ATPase molecules [3]. The liver cell adhesion molecule, Cell-CAM, which is a member of the immunoglobulin superfamily [4], has been shown to have an amino acid sequence identical to that deduced from a cDNA sequence of an ecto-ATPase from rat hepatocytes [5]. Immunochemical cross-reactivity between Cell-CAM and this ecto-ATPase has also been demonstrated [6]. However, adhesive properties of the isolated liver ecto-ATPase have not been demonstrated, and attempts to detect any ATPase activity of purified Cell-CAM preparations have so far been unsuccessful [7]. The present study was undertaken in order to determine whether ATPase activity is associated with the neural cell adhesion molecule, NCAM, which is a member of the immunoglobulin superfamily. A procedure for solubilization of enzyme activity together with NCAM was established, and by means of various immunoisolation procedures evidence was obtained indicating that (Ca^{2+}-Mg^{2+})-dependent ATP hydrolysing activity is an intrinsic property of NCAM.

*Corresponding author. Fax: (45) (35) 36 01 16. On leave from the Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russian Federation.

2. MATERIALS AND METHODS

Microsomes were isolated from brains of 40-day-old rats in a buffer consisting of 0.32 M sucrose, 30 mM histidine, 1 mM EDTA, pH 7.5 according to [8], except that mitochondria were removed by sedimentation through a cushion of Percoll instead of ordinary differential centrifugation [9]. Total protein was determined by the Lowry procedure [10]. ATPase assay medium contained 50 mM NaCl, 20 mM NaN_3, 6 μM $MgCl_2$, 100 μM $CaCl_2$, 50 μM vanadate, 1 mM ATP (disodium salt), 30 mM histidine, pH 7.4. P_i liberated into 0.5 ml assay medium at 37°C during 30 min (unless otherwise stated) was determined as phosphomolybdate complex at 820 nm [11]. The effect of various detergents on ATPase activity was tested at a microsomal protein concentration of 0.7 mg/ml in a medium containing 0.15 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.5. After incubation for 30 min on ice, aliquots were taken for ATPase assay. Solubilization with 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, CHAPS, 5 mg/ml, was performed at a protein concentration of 0.7–1.5 mg/ml in the medium described above with the addition of 25% w/v glycerol and 1 mM phenylmethylsulfonyl fluoride. After incubation for 30 min on ice insoluble material was sedimented by centrifugation in a Beckman Airfuge at 100,000 × g for 30 min.

Polyclonal rabbit antibodies against rat NCAM were prepared as previously described [12]. Immunoadsorption of NCAM to nitrocellulose filters GS (Millipore) was performed in 0.1% CHAPS, 100 mM NaCl, 25 mM Tris, pH 7.5. Filter pieces of approximately 0.5 cm^2 were washed, preincubated with either control antibodies or anti-NCAM antibodies, blocked with 5% BSA and incubated overnight at 4°C with solubilized supernatant from microsomes at a protein concentration of 0.07 mg/ml. After incubation, the filters were washed, and immunoadsorbed ATPase activity per cm^2 filter was determined. Alternatively, NCAM was immunoisolated using either Protein A-agarose (Sigma) or Protein G- Sepharose Fast Flow (Pharmacia). The affinity gels were incubated with control antibodies or anti-NCAM antibodies

in 0.1% CHAPS, 100 mM NaCl, 25 mM HEPES and 1 mM EDTA. Supernatant from solubilized microsomes was added, and after suitable washing immunoadsorbed ATPase activity was determined. Immunoprecipitation in gel was performed according to [12] substituting Triton X-100 in the gel with 0.15% CHAPS, and with the addition of 0.1 M NaCl in both gel and buffer. A partially purified brain protein fraction with a known amount of NCAM standard was labelled with fluorescamine [13] and used as marker of electrophoretic migration. ATPase assay in agarose gel was performed as follows: after immunoelectrophoresis the washed and compressed gel was covered by 2-fold concentrated ATPase assay medium, to which 1 mM sodium acetate and 20 mg/ml lead ammonium citrate/acetate complex (Sigma) [14] were added. The gel was incubated for 3–6 h at 37°C in a humid chamber, and thereafter washed, dried and immersed into a solution of ammonium sulfide 5-fold diluted from a ready made solution from Merck (containing not less than 20% nitrogen according to the manufacturer) in order to convert the precipitated white lead phosphate to dark brown lead sulfide. Enzymatically active precipitates were visualized by ATPase reaction in gel, washed, excised, homogenized and submitted to standard ATPase assay. A solubilized brain membrane fraction containing 36 μg NCAM/ml was used as standard for determination of the amount of NCAM in immunoprecipitates.

3. RESULTS AND DISCUSSION

Solubilization and purification of NCAM is usually performed in the presence of non-ionic detergents, some of which inactivate ATPase activity [15,16]. Therefore, the enzyme activity in microsomes was determined in the presence of a number of detergents (Fig. 1). The enzymatic activity was strongly inactivated by treatment with Triton X-100 and Nonidet P-40. Less inactivating were polyoxyethylene 8- or 9-lauryl ethers and polyoxyethylene ether W-1. Mildest was digitonin; but this detergent was able to solubilize only 30% of the total activity at a concentration of 10 mg/ml, which caused a pronounced inactivation of the enzyme. Solubilization with CHAPS led to a relatively strong inactivation of ATPase activity, and no protective effect of 3 mM ATP was found (not shown). However, in the presence of 25% glycerol during solubilization the inactivating effect of CHAPS was reduced (Fig. 1). In Table I, (Ca^{2+}-Mg^{2+})-ATPase activity and total protein distribution in supernatant and pellet after solubilization with 0.5% CHAPS is shown. It can be seen that the enzyme retains 85% of it's initial activity after this treatment. The main fraction, 89%, of the total activity in the membrane-detergent suspension remained in the supernatant after centrifugation. 67% of the total protein was solubilized by this procedure. The pellet contained approximately 30% of the total protein and less than 10% of the ATPase activity. Therefore, the procedure was regarded as effective and rather selective with regard to solubilization of brain membrane (Ca^{2+}-Mg^{2+})-ATPase. The distribution of solubilized NCAM in the same fractions was analyzed by means of immunoelectrophoresis in gel (Fig. 2) and found to be similar to that of the (Ca^{2+}-Mg^{2+})-ATPase: a major fraction of NCAM was released into the supernatant after solubilization with CHAPS. The solubilized (Ca^{2+}-Mg^{2+})-ATPase activity was rather stable; it was not affected by freezing at −20°C, and enzyme activity after 24 h at ambient temperature in the various buffers used in this study was approximately 50–70% of the initial activity. Addition of 100 mM NaCl resulted in a stabilization of the en-

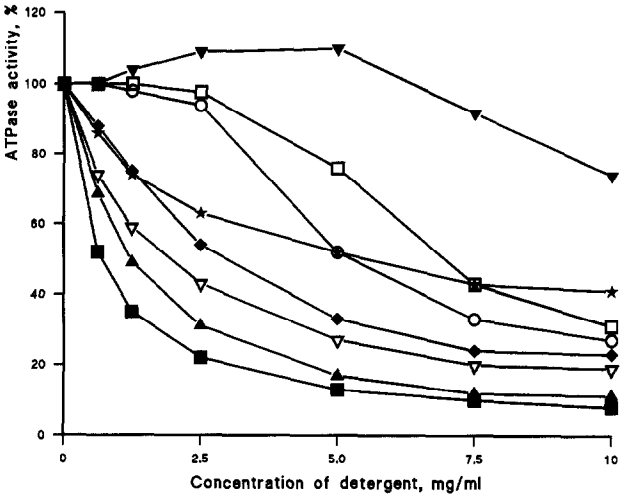

Fig. 1. Effect of detergents on (Ca^{2+}-Mg^{2+})-ATPase activity of rat brain microsomes. ■ Triton X-100; ▲ Nonidet P-40; ▽ Polyoxyethylene 8 lauryl ether; ◆ Polyoxyethylene 9 lauryl ether; * Polyoxythylene ether W-1; ▼ Digitonin; ○ CHAPS; □ CHAPS in the presence of 25% glycerol. 100% correspond to ATPase activity of microsomes without detergent treatment, 0.18 μmol P_i /mg protein/min.

Table I

Solubilization of (Ca^{2+}-Mg^{2+})-ATPase activity by CHAPS

Fraction	Total protein (mg)	Total activity (nmol P_i/min)	Specific activity (nmol P_i/mg protein/min)	Recovery of total activity (%)
Microsomes	1.82	327.6	180	100
Membrane suspension in CHAPS	1.82	280.3	154	85.6
Solubilized fraction (supernatant)	1.22	248.8	204	75.9
Pellet	0.61	30	49	9.1

Mean values of triplicate determinations of ATPase activity and protein are presented.

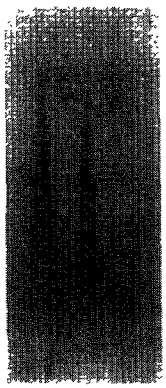

Fig. 2. Solubilization of NCAM by CHAPS. Rocket immunoelectrophoresis against NCAM antibodies of 15 μl unfractionated mixture of detergent and microsomes (1), 15 μl supernatant (2) and 15 μl pellet resuspended in original volume of solubilization buffer (3).

zyme in all buffers tested. Therefore, 100 mM NaCl was included in media employed for immunochemical procedures. The ATPase assay medium contained 50 mM NaCl, since it has been shown that NaCl has an activating effect on (Ca^{2+}-Mg^{2+})-ecto ATPase [17].

ATPase activity associated with purified NCAM was demonstrated by two immunoisolation procedures. In one procedure, ATPase activity on pieces of nitrocellulose filters with either anti-NCAM antibodies or preimmune antibodies was determined after incubation with supernatant of solubilized brain microsomes. In Fig. 3a it can be seen that NCAM antibodies selectively bound an ATPase activity. NCAM antibodies were not able to inhibit ATPase activity when incubated for 30 min at room temperature with supernatant of solubilized membranes before ATPase assay, indicating that NCAM-associated ATPase activity either is a minor fraction of the total activity or that the antibodies do not affect enzymatic activity, probably due to absence of antibodies specific to the functionally important conformational epitopes of the native enzyme [18,19]. The latter assumption was supported by the fact that ATPase activity immunoadsorbed on nitrocellulose filters was also unaffected by incubation with anti-NCAM antibodies.

Immunoisolation was also performed using either Protein A- or Protein G-agarose to which either control or anti-NCAM immunoglobulins were adsorbed. In Fig. 3b the bound ATPase activities are shown. NCAM antibodies immobilized on Protein G-agarose isolated more ATPase activity than antibodies on Protein A, and control antibodies immobilized on Protein G-agarose only bound an activity corresponding to 10% of that isolated by the specific antibodies bound to the same matrix. Enzyme activity was determined both after 1 hour and 4 hours at 37°C, see Fig. 3b. The results indicated that the enzyme was stable and that the reaction was linear in the test period. The effect of addition of 1 mM Ca^{2+} or 1 mM Mg^{2+} or both to an ATPase assay medium in which the routinely added divalent metal ions had been omitted is shown in Fig. 3c. It can be seen that in the presence of Ca^{2+} the activity was higher than in the presence of Mg^{2+}. The level of activity in medium containing both 1 mM Ca^{2+} and 1 mM Mg^{2+} showed that the effects of the two ions were not addi-

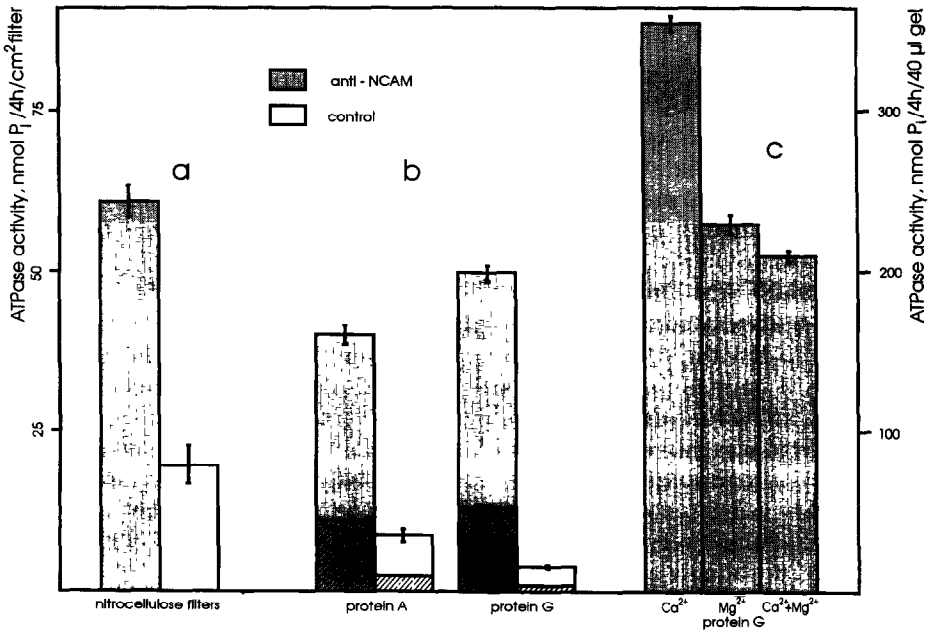

Fig. 3. Solid-phase immunoadsorption of (Ca^{2+}-Mg^{2+})-ATPase. NCAM antibodies or control antibodies were immobilized on (a) nitrocellulose filters or (b) Protein A-agarose, left, and Protein G-agarose, right. Activity of immunoadsorbed ATPase was measured after hydrolysis at 37°C during 4 h or 1 h, hatched areas; (c) effect of 1 mM Ca^{2+}, 1 mM Mg^{2+} or both together on the activity of Protein G-agarose immunoadsorbed ATPase. Mean values ± S.D. (bars) of five (a), four (b) and three (c) independent experiments are shown.

tive, indicating that the same enzyme probably is responsible for the activities observed in Ca^{2+} as well as in Mg^{2+} (see Fig. 3c). Similar characteristics have been observed for some known ecto-ATPases [15–17]. In order to determine whether the demonstrated NCAM associated ATPase activity was an intrinsic function of NCAM or caused by co-isolation of a separate protein, NCAM was also isolated by immunoprecipitation in gel. By means of this procedure it is possible to obtain very pure preparations of NCAM [20]. The procedure provides separation of e.g. NCAM and the L1 adhesion molecule which are known to be tightly associated in brain and during purification [21]. ATPase activity of NCAM immunoprecipitates was demonstrated by direct staining in the gel as described above. No acceleration of the nonenzymatic ATP hydrolysis or (Ca^{2+}-Mg^{2+})-ATPase inactivation was observed in the presence of the lead ammonium citrate/acetate complex in accordance with [14]. By application of P_i on the gel surface before ATPase assay it was found that an amount of 0.25 ng per mm^2 (dot number 4 from the left, Fig. 4a) could easily be visualized, whereas no precipitate formation was observed when pyrophosphate was applied (Fig. 4a).

Brain membranes mixed with CHAPS, and supernatant and pellet obtained after centrifugation were submitted to immunoelectrophoresis in gel and subsequent ATPase assay. P_i staining of both unfractionated detergent-membrane mixture and supernatant was observed, whereas it was negligible for the pellet fraction, see Fig. 4b, lanes 3–8. When the P_i staining was removed by treatment with 1 M HCl/50% EtOH, and protein subsequently was stained with Coomassie blue, an immunoprecipitate at a position identical to the P_i precipitate was seen, Fig. 4b, confer lanes 1 and 2 to lanes 3 and 4. If Triton X-100 was added in a final concentration of 0.25% to supernatant from CHAPS solubilized microsomes a pronounced enzyme inactivation was observed, see Fig. 4b, lane 9, indicating that the P_i staining of the NCAM immunoprecipitates was proportional to the general (Ca^{2+}-Mg^{2+})-ATPase activity of the sample, see Fig. 1. If supernatant from solubilized membranes was preincubated with anti-NCAM antibodies, no NCAM precipitate or ATPase activity could be demonstrated in the gel. In order to test the possibility that ATPase activity of the NCAM immunoprecipitate was due to unspecific retention of an ATPase during electrophoresis, human serum albumin was mixed with supernatant of CHAPS solubilized membranes and submitted to immunoelectrophoresis using rabbit antibodies against human albumin. The albumin precipitates were submitted to either P_i or protein staining and as seen in Fig. 4b, lanes 10 and 11, a slight, unspecific P_i staining of the albumin precipitate was observed. However, protein staining revealed that the amount of antigen–antibody complexes in the albumin precipitate was much higher than in the anti-NCAM precipitates (confer lanes 1 and 2 with lanes 12 and 13 in Fig. 4b). In order to separate the NCAM immunoprecipitate from unrelated ATPases and thereby reduce a possible, unspecific trapping of enzymes by the antigen-antibody complexes in the gel, supernatant from CHAPS solubilized membranes was also submitted to two-dimensional immunoelectrophoresis. In Fig. 5a and b, protein and P_i stainings of NCAM precipitates obtained by this procedure are shown. An intensive P_i staining was observed along the NCAM precipitate, indicating that ATPase activity is an intrinsic function of the NCAM protein. The specific (Ca^{2+}-Mg^{2+})-ATPase activity was determined in excised NCAM precipitates, and found to be 4.5 μmol P_i/mg NCAM protein/min with ATP and 1.4 μmol P_i/mg NCAM protein/min with ADP as substrate. No AMP hydrolysis could be demonstrated. Therefore, AMP may be the final product of this enzymatic reaction. The specific activities determined in this way must obviously be regarded as minimum values. For comparison, liver ecto-ATPase has a specific activity of 20 μmol P_i/mg protein/min [15]. The NCAM ATPase activity

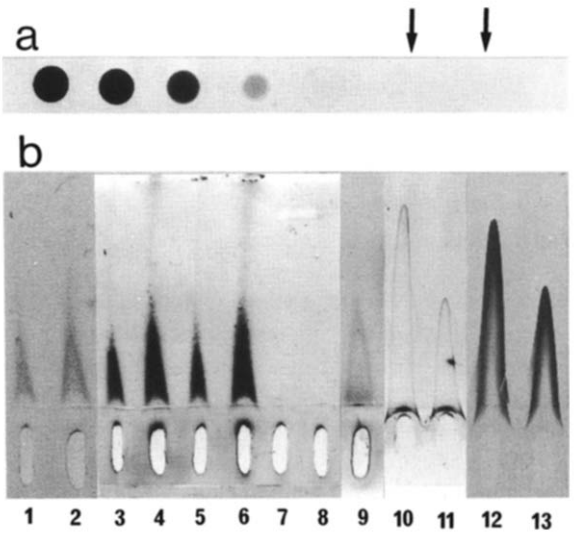

Fig. 4. Determination of ATPase activity in agarose gel. (a) Specificity of P_i staining was tested by application (from the left) of orthophosphate (10; 5; 2.5; 1.25; 0.62 ng) or pyrophosphate (30; 15 ng) on the gel surface before ATPase assay, perfomed in the presence of lead ammonium citrate/acetate complex. Pyrophosphate applications are indicated with arrows. (b) ATPase activity of NCAM immunoprecipitates was tested after electrophoresis of 10 and 20 μl of unfractionated mixture of detergent and microsomes (lanes 3, 4), supernatant (lanes 5, 6) and pellet, resuspended in original volume of buffer containing 0.5% CHAPS (lanes 7, 8). Effect of Triton X-100 was tested by application of a CHAPS solubilized fraction, to which Triton X-100 was added to a final concentration of 0.25% (lane 9). In lanes 1 and 2, Coomassie staining of NCAM immunoprecipitates was performed after destaining of the P_i-precipitates shown in lanes 3 and 4. Activity of ATPase, unspecifically bound to immunoprecipitates, was tested on albumin immunoprecipitates. 5 μg and 2.5 μg of human serum albumin were mixed with 10 μl of solubilized microsomal supernatant; after electrophoresis followed by ATPase assay the precipitates were P_i stained (lanes 10, 11); Coomassie staining of human serum albumin immunoprecipitates is shown in lanes 12 and 13.

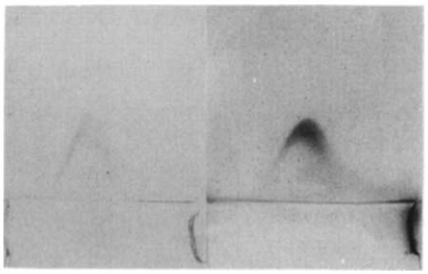

Fig. 5. ATPase activity of NCAM immunoprecipitates after crossed immunoelectrophoresis of supernatant from CHAPS-solubilized microsomes. Coomassie staining (left) and P_i staining (right).

had a broad nucleoside triphosphate specifity; using 1 mM ATP, UTP or GTP as substrates specific activities in the range of 4.4 ± 0.42 μmol P_i/mg protein/min were determined. These characteristics: a broad nucleoside triphosphate and diphosphate specificity and the absence of strict selectivity with regard to Ca^{2+} or Mg^{2+} are common features of several known ecto-ATPases [15–18].

The similarity of the solubilization pattern of NCAM and of ATPase activity, the immunoadsorption of (Ca^{2+}-Mg^{2+})-ATPase activity by means of NCAM antibodies bound to different solid supports, and finally, the co-localization of NCAM and ATPase activity in immunoprecipitates in gel, lead us to the conclusion that ATPase activity is a function of the NCAM molecule, although we cannot rule out the possibility that a tight, stoichiometric and probably functionally important coupling of NCAM with an unknown (Ca^{2+}-Mg^{2+})-ATPase exists. However, the latter seems unlikely, since previous studies have shown that NCAM isolated by immunoprecipitation in gel yields the characteristic NCAM polypeptides in uncontaminated form [20]. Furthermore, a recent report has predicted an ATP binding consensus sequence corresponding to amino acid residues 665–672 in bovine NCAM [22], and this sequence is highly homologous to an extracellular sequence on rat NCAM. In accordance with the fact that another immunoglobulin superfamily member, Cell-CAM, presumably is an ecto-ATPase, the NCAM-specific (Ca^{2+}-Mg^{2+})-ATPase activity may also be assumed to be expressed extracellularly.

Recently, adenylate cyclase activity has been suggested to be an intrinsic function of NCAM or of a tightly associated protein [22]. The enzymatic activity observed in our study can easily be distinguished from adenylate cyclase activity. Ortho-, rather than pyrophosphate is measured in the P_i-selective phosphomolybdate complex [23] and as P_i precipitate in gel (Fig. 4a). The hydrolysis of ADP, the broad nucleoside triphosphate specificity and the effective Ca^{2+} substitution for Mg^{2+} further support the assumption that under the chosen experimental conditions the NCAM associated ATPase activity, rather than adenylate cyclase activity, is responsible for the observed ATP hydrolysis.

Our results raise a number of fundamental questions. Is enzymatic activity a general property of cell adhesion molecules of the immunoglobulin superfamily? Is the activity specific for certain NCAM isoforms or NCAM expressing cell types and does the expression of the activity depend on certain posttranslational events? Most intriguently, what is the functional role of ecto-ATPase hydrolysis in relation to NCAM mediated adhesion and adhesion-triggered processes?

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