28 kDa adenosine-binding proteins of brain and other tissues

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Membranes prepared from calf brain were solubilized and chromatographed on a column containing 5'-amino-5'-deoxyadenosine covalently linked to agarose through the 5'-amino group. When the column was eluted with adenosine, a pure protein emerged with subunit molecular mass of 28 kDa. The protein was extracted from the membranes with sodium cholate, but not with 100 μ M-adenosine or 0.5 M-NaCl. A similar 28 kDa protein was isolated from the soluble fraction of calf brain. The yield of membrane-bound and soluble 28 kDa protein per gram of tissue was about the same. The 28 kDa protein was also found in membrane and soluble fractions of rabbit heart, rat liver and vascular smooth muscle from calf aorta. The yield per gram of tissue fell into the order brain > heart $\approx$ vascular smooth muscle > liver for the 28 kDa protein from the membrane fraction, and brain $\approx$ heart > vascular smooth muscle > liver for the 28 kDa protein from the soluble fraction. Polyclonal antibodies to pure 28 kDa protein from calf brain membranes cross-reacted with the 28 kDa protein from calf brain soluble fraction and with 28 kDa proteins isolated from other tissues. The 28 kDa protein from calf brain membranes was also eluted from the affinity column by AMP and 2',5'-dideoxyadenosine, but at a concentration higher than that at which adenosine eluted the protein, but *N*⁶-(*R*-phenylisopropyl)adenosine, 5'-*N*-ethylcarboxamidoadenosine, ADP, ATP, GTP, NAD⁺, cyclic AMP and inosine failed to elute the protein at concentrations up to 1 mM. The 28 kDa protein from the soluble fraction was not eluted by 3 mM-AMP or 1 mM-*N*⁶-(*R*-phenylisopropyl)adenosine, -5'-*N*-ethylcarboxamidoadenosine or -cyclic AMP. Unexpectedly, the soluble 28 kDa protein was eluted by AMP in the presence of sodium cholate. Soluble 28 kDa protein from calf brain had a *K*_D for adenosine of 12 μ M. Membrane 28 kDa protein from calf brain had a *K*_D of 14 μ M in the presence of 0.1 % sodium cholate. Amino acid compositions of the 28 kDa proteins were similar, but not identical.

INTRODUCTION

Three types of adenosine receptors have been characterized on the basis of their effects on adenylate cyclase: inhibitory (*A*₁) and stimulatory receptors (*A*₂), which occur on the cell surface, and an intracellular adenosine receptor (P-site). The adenosine-analogue affinity for the *A*₁ receptor is *N*⁶-(*R*-phenylisopropyl)-adenosine (PIA) > adenosine > 5'-*N*-ethylcarboxamidoadenosine (NECA), whereas that for the *A*₂ receptor is NECA > adenosine > PIA (Wolff *et al.*, 1981; Londos *et al.*, 1983).

The P-site can be distinguished from other adenosine receptors because it binds analogues modified in the 2'- and 3'-positions of the ribose moiety, such as 2',3'-dideoxyadenosine and binds analogues with an unaltered adenine moiety (Londos *et al.*, 1983). All adenosine receptors bind analogues modified in the 5'-position of adenosine. Apparent affinities have been determined by measuring the response of adenylate cyclase to adenosine; *A*₂ receptors and P-sites respond with half-maximum effects in the micromolar range, whereas the *A*₁ receptor responds in the nanomolar range (Wolff *et al.*, 1981). Proteins solubilized from rat brain membranes have a *K*_D value for PIA of 3.3 nM and for NECA of 6 nM and 100 nM (Nakata & Fujisawa, 1984). *A*₁ receptors are abundant in brain and adipocytes (Londos *et al.*, 1983; Garcia-Sainz & Torner, 1985). *A*₂ receptors are abundant

in liver (Londos & Wolff, 1977), fibroblasts (Rozenfurt, 1982; Ravid & Lowenstein, 1988) and vascular smooth muscle (Anand-Srivastava *et al.*, 1982). The P-site is believed to exist in all cell types (Londos *et al.*, 1983). Stiles *et al.* (1985) found that a 38 kDa protein from cerebral cortex and adipocyte membranes is covalently labelled when an ¹²⁵I-labelled *A*₁-specific receptor analogue is allowed to bind to the membranes and is then cross-linked to the protein with *N*-succinimidyl-6-(4-azido-2-nitrophenylamino)hexanoate. Klotz *et al.* (1985) found that a 35 kDa protein from rat brain and human platelet membranes is covalently labelled with the *A*₁ receptor analogue *R*-2-azido-*N*⁶-(*p*-hydroxyphenylisopropyl)adenosine.

Soluble adenosine and cyclic AMP-binding proteins with subunit molecular masses of 45–48 kDa have been isolated from various mammalian tissues (Yuh & Tao, 1974; Sugden & Corbin, 1976; Ueland & Doskeland, 1977; Ueland, 1978; Ollson, 1978) and identified as 5'-*S*-adenosylhomocysteine hydrolase (Hershfield & Kredich, 1978; Ueland & Saebo, 1979; Ueland & Helland, 1983).

In the present study we have used an affinity column in which 5'-amino-5'-deoxyadenosine is linked to agarose to isolate potential adenosine receptors. Londos & Preston (1977) found that this adenosine analogue inhibits adenylate cyclase of liver in the micromolar range. By using this affinity column, adenosine-binding

Abbreviations used: NECA, 5'-*N*-ethylcarboxamidoadenosine; PIA, *N*⁶-(*R*-phenylisopropyl)adenosine; PAGE, polyacrylamide-gel electrophoresis; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); PMSF, phenylmethanesulphonyl fluoride; DMA, dimethyl adipimidate; DMS, dimethyl sulfoxide; Sulpho-EGS, ethylene glycolbis(sulphosuccinimidyl succinate).

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proteins with molecular masses of 28 kDa were isolated in pure form from membrane and soluble fractions prepared from brain, heart, liver and vascular smooth muscle. Some properties of these proteins are reported.

EXPERIMENTAL

Materials

Calf aortas and brains were obtained from J. T. Trelegan Inc., Cambridge, MA, U.S.A. Frozen rabbit hearts and rat livers were obtained from Pel-Freez Biologicals, Rogers, AR, U.S.A. Electrophoresis and Western-blot chemicals, Affi-Gel 10 and protein-staining kit were from Bio-Rad. Hepes was obtained from Research Organics International. Phenylmethanesulphonyl fluoride (PMSF), benzoyl-L-arginine methyl ester, tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl), sodium cholate, cholic acid, 5'-amino-5'-deoxyadenosine, NECA, ATP, GTP, inosine, cyclic AMP, NAD⁺, ADP, Naphthol Blue Black (Amido Black 10B), phosphorylase *b* (rabbit muscle), bovine serum albumin, ovalbumin, carbonic anhydrase (bovine erythrocytes), lactate dehydrogenase (rabbit muscle) and trypsin inhibitor (soybeans) were purchased from Sigma Chemical Co. PIA, adenosine and adenosine deaminase were obtained from Boehringer-Mannheim. Vacuum dialysis was conducted in a Micro-ProDiCon obtained from Bio-Molecular Dynamics. 2',5'-Dideoxyadenosine and AMP were purchased from Pharmacia. Basic Fuchsin was from Aldrich Chemical Co. T.l.c. plates were from Eastman-Kodak. Goat anti-sheep IgG conjugated to horseradish peroxidase was from Organon Teknika-Cappel. Freund's adjuvant was obtained from Difco Laboratory. [2-³H]Adenosine was obtained from New England Nuclear. Riboflavin was obtained from ICN Pharmaceuticals.

The Ampholines were from LKB. Dimethyl suberimide (DMS), dimethyl adipimide (DMA) and ethylene glycolbis(sulphosuccinimidyl succinate) (Sulpho-EGS) were obtained from Pierce. Antibody to β -galactosidase-binding protein was generously given by Dr. Samuel Barondes, Department of Psychiatry, University of California at San Francisco, CA, U.S.A.

Preparation of membrane and soluble fractions

Unless otherwise indicated, all operations were performed at 4 °C.

Buffers. Buffer A: 50 mM-sodium Hepes buffer, pH 7.4, 150 mM-NaCl, 1 mM-EDTA and a freshly prepared mixture of proteinase inhibitors consisting of 0.1 mM-PMSF, benzoyl-L-arginine methyl ester (10 mg/l), TPCK (10 mg/l) and trypsin inhibitor (10 mg/l). Buffer B: 4.3% (w/v) sodium cholate, 50 mM-sodium Hepes buffer, pH 7.4, and 150 mM-NaCl. Buffer C: 0.86% sodium cholate, 50 mM-Hepes buffer, pH 7.4, and 150 mM-NaCl.

Calf brain. A calf brain was obtained from a local slaughterhouse, packed in ice, and used within 30 min after the animal was killed. The brain was homogenized in 2 vol. of ice-cold Buffer A using a Waring Blendor (four bursts of 20 s each, with waiting periods of 30 s). The homogenate was centrifuged at 169 *g* for 10 min to remove cellular debris. The pellet was resuspended in 0.6 vol. of Buffer A, using a Waring Blendor (four bursts

of 20 s each, with waiting periods of 30 s). The two supernatants were combined, filtered through gauze and centrifuged at 130 000 *g* for 45 min. The supernatant was either applied to the 5'-amino-5'-deoxyadenosine affinity column immediately or stored at -20 °C. The membranes were washed twice by resuspension in 50 ml of Buffer A by using a Polytron homogenizer (PCU-2; Brinkmann Instruments, Westbury, NY, U.K.) with a 10 mm-diameter probe (PTA 10S), employing three bursts of 20 s at half-maximum speed with waiting periods of 30 s. All homogenizations with the Polytron were performed while the suspensions were cooled in an ice bucket. The suspension was adjusted to a volume of 300 ml and homogenized with the Polytron by using one burst of 20 s. The suspension was then centrifuged at 130 000 *g* for 45 min. This washing procedure was repeated once. The membranes were then resuspended in 20 ml of Buffer A by using the Polytron with one burst of 20 s. The membranes were solubilized by adding 100 ml of Buffer B and mixing with the Polytron employing three bursts of 20 s with waiting periods of 30 s. The homogenate was centrifuged at 130 000 *g* for 45 min. The resulting supernatant was applied to the affinity column as described below.

The washed brain membranes were also extracted with 0.5 M-NaCl or 100 μ M-adenosine, instead of sodium cholate, using the Polytron with a 10 mm-diameter probe (PTA 10S) and employing three bursts of 20 s with waiting periods of 30 s. The resulting suspension was centrifuged at 130 000 *g* for 45 min. In the case of the NaCl extraction, the supernatant (80 ml) was diluted to 0.26 M-NaCl by adding 170 ml of Buffer A before its application to the 5'-amino-5'-deoxyadenosine affinity column. In the case of the extraction with adenosine, the high-speed supernatant was dialysed against 500 vol. of Buffer A at 8 °C for 12 h. The buffer was changed twice during this period. The dialysed sample was then applied to the affinity column.

Calf vascular smooth muscle. Calf aortas, 30 in all, were suspended in 500 ml of Buffer A. Strips of medial tissue were dissected and frozen in liquid N₂. The yield was 273 g. The tissue was pulverized in a stainless-steel percussion mortar cooled with liquid N₂, and homogenized in 600 ml of Buffer A by using the Polytron with a 35 mm-diameter probe (PTA 35/2M) at half-maximum speed, several times, for 20 s. The homogenate was centrifuged at 169 *g* for 10 min. The membrane and soluble fractions were then prepared essentially as described for brain.

Rabbit hearts and rat livers. The procedures used were similar to those described for brain.

Washed membranes from different tissues were assayed for lactate dehydrogenase activity (Bergmeyer & Bernt, 1974) as a soluble enzyme marker. The enzyme activity in the washed membranes from different sources was 0.2–0.9% of the activity detected in the soluble fraction.

Affinity chromatography

Preparation of 5'-amino-5'-deoxyadenosine affinity column. A portion (25 ml) of Affi-Gel 10 was washed with 100 ml of cold distilled water. The gel was added to 25 ml of 2 mM-5'-amino-5'-deoxyadenosine in 50 mM-sodium Hepes buffer, pH 8.5, and shaken gently for 1 h at room temperature. The progress of the reaction was

measured as follows: 500 μ l aliquots were taken at 10 min intervals and centrifuged for 5 min in a micro-centrifuge. The supernatant (100 μ l) was diluted to 2 ml with 0.1 M-HCl to hydrolyse the u.v.-absorbing *N*-hydroxysuccinimide that was displaced when Affi-Gel 10 reacted with the amino group of the adenosine analogue. The absorbance of 5'-amino-5'-deoxyadenosine in the supernatant was then measured at 259 nm. A constant absorbance close to zero indicated that the coupling reaction had been completed and that most of the ligand had been linked to the gel. The gel was filtered, washed with 100 mM-Tris/HCl buffer, pH 8.4, and shaken gently in this solution at room temperature for 2 h to allow for the destruction of the remaining *N*-hydroxysuccinimide ester groups by reaction with the amino group of Tris. The amount of ligand residues linked to the gel was determined by dissolving 0.1 g of the 5'-amino-5'-deoxyadenosine affinity gel in 1 ml of 1 M-HCl at 90 °C. The solution was diluted with water and the absorbance at 259 nm was determined. A millimolar absorption coefficient of 15.4 (Dawson *et al.* 1969) was used to calculate ligand bound to the gel. The gel was stored at 8 °C in a solution containing 25 mM-Tris/HCl buffer, pH 7.5, 0.04% NaN_3 and 50% (v/v) glycerol.

Affinity chromatography of soluble and membrane proteins. The following procedures were conducted at 8 °C. Affinity gel containing 2 μ mol of 5'-amino-5'-deoxyadenosine residues/ml of gel was poured in a column (gel bed: 0.8 cm diam. $\times$ 18 cm high). The column was equilibrated by washing with 250 ml of Buffer A without proteinase inhibitors. The soluble fraction was applied to the column at a flow rate of 8 ml/h. Next the column was washed with 450 ml of Buffer A at a flow rate of 25 ml/h. The column was then eluted with adenosine or adenosine analogue in Buffer A at a flow rate of 8 ml/h. Fractions (7–8 ml) were collected, dialysed, and concentrated to 0.4–1.0 ml against 90 vol. of Buffer A in a Micro-ProDiCon using membranes with a molecular-mass cut-off of 10 kDa. The same procedure was followed when detergent-solubilized membranes were applied to the column, except that Buffer C was used in place of Buffer A.

Affinity chromatography of detergent-solubilized membranes was also carried out in the absence of detergent. Furth (1980) has shown that cholate is dialysable. The sample of detergent-solubilized membrane proteins was dialysed in tubing with a 10 kDa cut-off against 60 vol. of Buffer A with two changes of buffer. The dialysis was performed at 8 °C for 20 h. The dialysed sample was applied to the affinity column as described for the soluble fraction.

The column was regenerated by washing with 5 vol. of 7 M-urea in 1 M-NaCl. It was then washed with 50 vol. of the desired buffer.

Antibodies

Pure 28 kDa protein from calf brain membranes was subjected to SDS/polyacrylamide-gel electrophoresis (SDS/PAGE). The gel was stained with Coomassie Brilliant Blue for 10 min and destained as described by Laemmli & Favre (1973). The band was cut out, and the gel slices were homogenized in 1 ml of phosphate-buffered saline (136 mM-NaCl/8 mM- Na_2HPO_4 /2.6 mM-KCl/1.4 mM- KH_2PO_4 , pH 7.4) with a Polytron at half-maximum speed. Freund's complete

adjuvant (1.5 ml) was added to the homogenate. The mixture was homogenized again. The mixture, which contained about 500 μ g of 28 kDa protein, was injected subcutaneously into a New Zealand rabbit weighing 2 kg (Vaitukaitis, 1981). The rabbit was boosted with 300 μ g and 250 μ g of the 28 kDa protein at 4 weeks and 6 weeks respectively after the first injection. The 28 kDa protein used for boosting was prepared as described above, except that Freund's incomplete adjuvant was used.

Equilibrium-dialysis binding

A sample of 28 kDa protein that had been concentrated and dialysed in a ProDiCon, as described above, was dialysed further against 500 vol. of Buffer A in the case of soluble 28 kDa protein or against Buffer A supplemented with the desired concentration of detergent in the case of membrane 28 kDa protein, using dialysis bags with a 10 kDa cut-off. The dialysis was performed at 8 °C for 6–8 h, with two changes of buffer.

Binding of adenosine or adenosine analogues to the 28 kDa protein was measured at 8 °C in dialysis cells with a capacity of 0.15 ml in each chamber, using a dialysis membrane with a 10 kDa cut-off. The solution used contained 20 mM-sodium Hepes buffer, pH 7.4, 150 mM-NaCl and sodium cholate as indicated, in a total volume of 0.1 ml. The 28 kDa protein was injected into chamber 1 at a concentration of 2 μ M. The experiment was started by placing 0.5–150 μ M-[2- ^3H]adenosine [(1–2) $\times$ 10⁸ d.p.m./ μ mol] into chamber 2. The distribution of adenosine in the two chambers was determined as a function of time. Typically, equilibrium was reached after 9–10 h. At the beginning, and after equilibrium was reached, 10 μ l aliquots were withdrawn from each chamber and counted for radioactivity in a liquid-scintillation counter.

When 28 kDa protein was prepared for binding studies, purified detergent was used. Cholic acid (Sigma) was decolorized with activated charcoal and recrystallized twice from 70% (v/v) ethanol. Alternatively, pure sodium cholate (Calbiochem) was used.

Other methods

SDS/PAGE slab gels were prepared and run as described by Laemmli & Favre (1973), with 12% (w/v) polyacrylamide for the running gel. Gels were stained with either Coomassie Brilliant Blue or silver as described by Merril *et al.* (1984). Immunoblotting of proteins was performed as described by Towbin & Gordon (1984). Soluble proteins were assayed by the method of Bradford (1976) or with Amido Black (Schaffner & Weissmann, 1973). Membrane proteins were assayed with Amido Black. Bovine serum albumin was used as standard.

Native 28 kDa protein was cross-linked with DMA as described by Seizen *et al.* (1980) or with DMS or Sulpho-EGS by the method of Baskin & Yang (1980). In the case of DMA and DMS, the reaction mixture contained 6 μ g of purified 28 kDa protein, 25 mM-cross-linker, 0.2 M-triethanolamine/HCl, pH 8.5, and, where indicated, 50 μ M-adenosine, in a total volume of 84 μ l. The mixture was incubated at room temperature for 2 h. The reaction was stopped by adding gel sample buffer for SDS/PAGE and then boiling for 5 min. The reaction mixture for Sulpho-EGS contained 6 μ g of purified 28 kDa protein, 0.2 mM-cross-linker, 50 mM-sodium phosphate buffer, pH 7.3 and, where indicated, 50 μ M-adenosine in a total volume of 84 μ l. The mixture was in-

cubated at room temperature for 30 min, and the reaction was stopped as described above. When the membrane 28 kDa protein was used in cross-linking experiments, the reaction mixture included 0.5% sodium cholate. The reaction mixtures were examined by gel electrophoresis using an 8% running gel and a 3% stacking gel. The proteins were detected by silver staining.

The amino acid composition of 28 kDa proteins was determined by subjecting samples to SDS/PAGE. This was followed by electroblotting on to poly(vinylidene difluoride) membranes as described by Matsudaira (1987). The 28 kDa protein band was cut out, extracted, hydrolysed in 6 M-HCl for 24 h using a Pico-tage workstation (Waters Associates). The resulting amino acids were converted into their phenylthiocarbonyl derivatives and analysed by h.p.l.c. as described by Ebert (1986). The determination of each amino acid was accurate to $\pm 5\%$. These analyses were performed by Dr. David Andrews, Microchemistry Service, Biological Laboratories, Harvard University, Cambridge, MA, U.S.A.

Isoelectric focusing was performed by the method of O'Farrell *et al.* (1977), except that the pure 28 kDa protein (10–15 μg) was run only in one dimension. Gels were stained for carbohydrate by the method of Segrest & Jackson (1972). Protein blotting was performed as described by Towbin & Gordon (1984). Soluble 28 kDa protein from brain (0.2 μg) was allowed to react with sheep antiserum to mouse adenosine deaminase (1:500 dilution) for 2 h using the Western-blot procedure. The secondary antibody used was an anti-sheep IgG conjugated to horseradish peroxidase. Commercial adenosine deaminase from calf intestine was used as a positive control. The antibody to adenosine deaminase from mouse cell line B-1/50 was a gift of Dr. Rodney Kellems, Baylor College of Medicine, Houston, TX, U.S.A.

Enzyme assays

Adenosine kinase was assayed with 0.1 mM-[2- ^3H]-adenosine (2×10^9 d.p.m./ μmol), 1 mM-ATP, 1 mM-MgCl₂, 50 mM-sodium acetate/acetic acid buffer, pH 5.5, 20 μg of bovine serum albumin and 30 μg of washed brain membranes or 1 μg of 28 kDa protein in a final volume of 0.1 ml. The reaction was allowed to proceed at 30 °C for 5 min and stopped by placing the tubes in a boiling-water bath for 2 min. A mixture (20 μl) containing 25 mM-inosine, 25 mM-AMP, 25 mM-ATP, and 25 mM-adenosine was added to the tubes, which were then centrifuged in a microcentrifuge. The supernatant (5 μl) was applied to cellulose thin-layer plates and AMP was separated from other nucleotides and counted for radioactivity (Ravid *et al.*, 1984).

Adenosine deaminase was assayed with 0.1 mM-[2- ^3H]-adenosine (450 d.p.m./ μmol), 50 mM-sodium Hepes buffer, pH 7.4, bovine serum albumin (0.2 mg/ml) and 20–50 μg of brain membranes or about 1 μg of 28 kDa protein in a final volume of 0.1 ml. The reaction was allowed to proceed at 30 °C for 5 min and was stopped as described for the adenosine kinase assay. The labelled inosine formed was separated and counted for radioactivity as described above.

AMPase and IMPase activities were assayed as described by Itoh *et al.* (1978) and Truong *et al.* (1988). The 28 kDa protein used for these assays was prepared from brain in the presence of 25% glycerol. Orthophosphate was determined according to Sanui (1974).

RESULTS

Isolation of a 28 kDa protein from brain membranes

Affinity chromatography of brain membrane extract was conducted as described in the Experimental section. Samples from each fraction were subjected to SDS/PAGE. Elution of the column with adenosine yielded a protein peak (Fig. 1a) which was analysed by gel electrophoresis and found to contain a pure protein with a molecular mass of 28 kDa (Fig. 1b). The yield was about 1.4 μg of 28 kDa protein/g of calf brain. The column was washed further with 5 vol. of 7 M-urea in 1 M-NaCl. No additional 28 kDa protein was eluted by this wash (results not shown). The 28 kDa protein was eluted between 160 and 400 μM -adenosine, with the peak

Fig. 1. Elution of membrane proteins from a 5'-amino-5'-deoxyadenosine affinity column by a gradient of adenosine

(a) Detergent-solubilized proteins from calf brain membrane (572 mg; 5.6 mg/ml) were applied to a 5'-amino-5'-deoxyadenosine affinity column (●). In a separate experiment the detergent was dialysed from the solubilized proteins (568 mg; 4.8 mg/ml) before the application of the sample to the affinity column (■). The column was washed with 450 ml of buffer. It was then eluted with a linear gradient (straight diagonal line with points) of 0–400 μM adenosine in buffer over 50 ml. The buffer wash and adenosine gradient were run either in the presence (●) or in the absence (■) of detergent. Fractions of 7 ml were collected, concentrated to about 0.4 ml, and protein was determined. (b) Gel electrophoresis of 12 μl of fractions from (a) (●). The gradient started with lane 1. Molecular-mass standards (kDa) used were: phosphorylase b, 97.4; bovine serum albumin, 66; ovalbumin, 45; carbonic anhydrase, 29; and trypsin inhibitor, 20.1. The gel was silver-stained. For other details, see the Experimental section.

being eluted at 220 μ M-adenosine (Fig. 1a). In a separate experiment, the cholate was dialysed from the sample of solubilized membrane proteins as described in the Experimental section and the mixture was then chromatographed as described above, except that detergent was omitted. Under these conditions, the yield of 28 kDa protein was 25% lower than before (Fig. 1a). Possibly the 28 kDa protein still contained bound detergent that was not removed by dialysis during its application to the affinity column. However, note that the sample on the column was washed with Buffer A, which lacked cholate, and was then eluted with adenosine in the absence of cholate.

Recycling of the breakthrough protein peak through a second affinity column did not result in any additional binding of 28 kDa protein to the column (results not shown). This indicates that the first affinity column was not saturated with the 28 kDa protein.

Brain membranes were also extracted with 0.5 M-NaCl or with 100 μ M-adenosine instead of cholate. The extracts were separately applied to the affinity column and eluted as described above. No 28 kDa protein was detected by SDS/PAGE of the adenosine eluate or of the urea wash from the columns loaded with either extract (results not shown). Thus, under these conditions, the 28 kDa protein is not dissociated from the membranes by adenosine or salt.

Isolation of 28 kDa protein from soluble fraction

The soluble fraction prepared from calf brain was applied to the affinity column. The column was washed and eluted as described in the Experimental section. Adenosine eluted proteins with the following molecular masses (in kDa): 58, 50, 40, 30, 28, 14 and 12 (Fig. 2). Except for the 28 kDa protein, all of these proteins were also eluted by washing the affinity column with 5–10 vol. of 1 mM-cyclic AMP. As shown below, the soluble 28 kDa protein was obtained in pure form when the column was first eluted with 1 mM-cyclic AMP, and then with a linear gradient of 0–400 μ M-adenosine. Membrane and soluble 28 kDa proteins migrated as one band when run together on gel electrophoresis, indicating identical molecular masses (results not shown).

Ligand specificity

Several adenosine analogues and purine nucleotides were tested for their ability to elute the 28 kDa protein from the affinity column. The columns were eluted with linear gradients of ligand from 0 to 1.0 mM over 50 ml. Elution profiles of calf brain *membrane* proteins from the affinity column are shown in Fig. 3(a). Protein peaks were eluted at 0.18 mM-adenosine, 0.8 mM-2',5'-dideoxyadenosine and 0.26 mM-AMP. Gel electrophoresis showed that these ligands eluted pure 28 kDa protein (results not shown). The 28 kDa membrane protein was not eluted from the column by cyclic AMP, inosine, PIA, NECA, ADP, ATP, NAD⁺ or GTP.

The *soluble* 28 kDa protein was eluted by 0.15 mM-adenosine, but not by 3 mM-AMP, 1 mM-PIA, -NECA, -NAD or -cyclic AMP. The other nucleotides listed above were not tested. The 28 kDa protein from membranes was eluted by AMP and detergent, but the 28 kDa protein from high-speed supernatant was not eluted by AMP in the absence of cholate. We therefore tested whether addition of cholate affected elution of high-speed-supernatant proteins. The high-speed super-

Fig. 2. Elution of soluble proteins from a 5'-amino-5'-deoxyadenosine affinity column by a gradient of adenosine

Fractions obtained from affinity chromatography of cytosolic proteins were subjected to gel electrophoresis. High-speed supernatant from calf brain homogenate (900 mg of protein; 3.8 mg/ml) was applied to a 5'-amino-5'-deoxyadenosine affinity column as described in the Experimental section. The wash procedure and adenosine gradient were as described in Fig. 1(a) for elution without detergent. Fractions of 8 ml were collected, concentrated to about 0.6 ml, and 12 μ l of the concentrated fraction was applied to the gel. The gradient started with lane 1. Molecular-mass standards were as given in Fig. 1. The gel was stained with Coomassie Brilliant Blue.

natant was applied to the affinity column in the presence of 3.6% sodium cholate as described for the proteins solubilized from membrane. The column was washed with Buffer C (containing 0.86% sodium cholate), and proteins were eluted with a linear gradient of AMP from 0 to 1 mM in Buffer C over 50 ml, followed by 7 M-urea. A protein peak was eluted at 0.25 mM-AMP (Fig. 3b). Gel electrophoresis showed that this peak consisted of pure 28 kDa protein. No additional proteins were eluted by urea (results not shown). This shows that the detergent prevented the binding to the affinity column of soluble proteins other than the 28 kDa protein, a result quite different from that obtained in the absence of cholate (Fig. 2). It appears that the binding of the 28 kDa protein from the high-speed supernatant to the affinity column and to adenosine is much higher than to AMP; however, in the presence of cholate this situation is changed.

When solubilized brain membranes were applied to an *N*⁶-(3-aminopropyl)adenosine-agarose column, in which the 6-amino group of adenosine is bound to the gel support, no 28 kDa protein was retained by the column (results not shown).

Tissue distribution and immunocross-reactivity of 28 kDa proteins

Cytosolic fraction and membrane proteins solubilized with 3.6% sodium cholate were prepared from rabbit hearts, rat livers and vascular smooth muscle of calf aortas. The fractions were subjected to affinity chromatography as described for brain. All tissues tested

Fig. 3. Comparison of adenosine, 2',5'-dideoxyadenosine and AMP elution profiles from affinity column

(a) Calf brain *membrane* protein that had been solubilized with sodium cholate was applied separately to three affinity columns. Each column was loaded with 310 mg of protein (3.7 mg/ml) and eluted with a linear gradient of 0–1 mM-adenosine (●) or 2',5'-dideoxyadenosine (■) or AMP (▲) in Buffer C over 50 ml. (Note that Buffer C contained 0.86% sodium cholate.) Fractions (2 ml) were collected. (b) Calf brain *high-speed supernatant* was made 3.6% with respect to cholate and applied to the affinity column. The column was eluted as described in the legend to Fig. 1(a) by using a linear gradient from 0 to 1 mM-AMP in Buffer C over 50 ml., i.e. the elution was performed in the presence of 0.86% sodium cholate.

contained a 28 kDa protein in the soluble and membrane fractions. The yields of 28 kDa protein per mg of membrane protein were higher than yields per mg of cytosol protein. Calf brain is the richest source of 28 kDa protein from membranes (Table 1).

Crude calf brain homogenate was subjected to SDS/PAGE followed by immunoblotting. Antiserum to the 28 kDa protein from calf brain membranes reacted only with a 28 kDa protein (results not shown). The antibodies were tested for cross-reactivity with identical amounts of membrane and soluble 28 kDa proteins from different tissues. Immunoblotting showed that the 28 kDa proteins from various sources all cross-react with the antibody (Fig. 4).

Binding of adenosine to 28 kDa proteins

Binding of adenosine to the 28 kDa protein from calf brain is shown in Fig. 5. The dissociation constants calculated from these data are 12 and 14 μM for the soluble and membrane proteins respectively. Scatchard plots show approx. 2 mol of adenosine bound/mol of each protein. The binding of adenosine analogues to brain membranes is inhibited by sodium cholate in a concentration-dependent manner (Nakata & Fujisawa, 1984). The critical micelle concentration of sodium cholate is 0.7% in the presence of 150 mM-NaCl (Lichtenberg *et al.*, 1983). The binding of adenosine to the 28 kDa membrane protein was strongest in the presence of 0.1–0.2% sodium cholate (Fig. 6).

Amino acid composition and molecular mass

The amino acid composition of the membrane and soluble 28 kDa proteins of calf brain is similar (Table 2). The percentages of the hydrophobic amino acids methionine and proline in the membrane protein are 5-fold and 1.6-fold higher respectively than those in the soluble protein, and the percentage of lysine is 2.8-fold higher in the soluble as compared with the membrane protein.

Attempts to determine the molecular mass of the native protein by gel filtration were unsuccessful. When

Table 1. Tissue distribution of 28 kDa proteins

Abbreviations: Memb., membrane-bound; Sol., soluble; VSM, vascular smooth muscle.

Tissue and weight	Cell fraction	Total protein in cell fraction (mg)	Protein applied to column (mg)	Yield of 28 kDa protein eluted by adenosine		
				(μg)	(μg/mg*)	(μg/g†)
One calf brain (253 g)	Membr.	884	632‡	308	0.48	1.21
	Sol.	2700	600	73	0.12	1.29
14 Rabbit hearts (103 g)	Membr.	343	206‡	28	0.13	0.27
	Sol.	3947	600	22	0.03	1.40
VSM from 30 calf aortas (136 g)	Membr.	571	408‡	47	0.11	0.34
	Sol.	1740	580	26	0.04	0.57
14 Rat livers (110 g)	Membr.	ND§	512‡	11	0.02	0.10
	Sol.	7086	1200	5	0.004	0.26

* Yield = μg of 28 kDa protein eluted/mg of protein applied to affinity column.

† Yield = μg of 28 kDa protein/g of tissue.

‡ Membrane proteins solubilized by 3.6% sodium cholate.

§ ND, not determined.

Fig. 4. Immunocross-reactivity of 28 kDa proteins from different tissues

(a) 0.5 μ g of 28 kDa proteins from different tissues were subjected to SDS/PAGE, and the gel was silver-stained. For other details, see the Experimental section. Lanes 1 and 2, calf vascular smooth muscle; lanes 3 and 4, calf brain; lanes 5 and 6, rabbit heart. Lanes 1, 3 and 5 are of membrane proteins, whereas lanes 2, 4 and 6 are of soluble proteins. (b) Western blot of a gel similar to that described in (a), except that the gel was not stained and 0.25 μ g of each 28 kDa protein from different tissues was run on the gel. For other details, see the Experimental section.

the soluble 28 kDa protein was run through either a Sphergel-TSK 3000 SW column (Altex), using 100 mM-potassium phosphate buffer, pH 6.6, with and without 300 mM-NaCl, as running buffer, or a Sorbak GF-250 column (du Pont) using 0.3 M-potassium phosphate buffer, pH 7.0, or 40 mM-sodium Hepes buffer, pH 7.0, with 0.2 M-(NH₄)₂SO₄ as running buffer, the apparent molecular mass obtained was $\leq$ 10 kDa. This shows that the protein interacted with the columns.

DMA, DMS and Sulpho-EGS were used in an attempt to cross-link the subunits of the native 28 kDa proteins from brain. When the pure protein and the cross-linking reagent were incubated in the presence or absence of 50 μ M-adenosine, a single protein band with a molecular mass of about 28 kDa was observed on SDS/PAGE. Pyruvate kinase (7 μ g) was used as a positive control; it was cross-linked by DMS to yield a major species with a

Fig. 5. Binding of adenosine to 28 kDa proteins from soluble and membrane fractions of calf brain as a function of adenosine concentration

Equilibrium dialysis was used to determine binding. For studies with the 28 kDa protein from soluble fraction of brain ($\circ$), the dialysis cells contained 20 mM-sodium Hepes buffer, pH 7.4, and 150 mM-NaCl. One side also contained the 2 μ M 28 kDa protein and the other adenosine. For studies with the 28 kDa protein from membrane fraction of brain (Δ), the conditions were the same, except that the concentration of 28 kDa was 1.8 μ M and that the cells also contained 0.1% sodium cholate. The abscissa shows adenosine concentrations at equilibrium. Each point represents an average of two determinations. For other details, see the Experimental section.

Fig. 6. Effect of cholate on binding of adenosine to 28 kDa protein from brain membranes

Binding was determined by equilibrium dialysis. The dialysis cells contained 20 mM-sodium Hepes buffer, pH 7.4, 150 mM-NaCl and sodium cholate as indicated. Initially, one side of the cells contained 20 μ M-adenosine (2×10^8 d.p.m./ μ mol) and the other 0.7 μ M-28 kDa protein. Pure 28 kDa protein isolated from brain membranes in the absence of detergent was used. For other details, see the Experimental section.

molecular mass of 155 kDa. These negative results suggest that the 28 kDa proteins either do not form oligomers or that their oligomers do not cross-link readily.

In order to test if the 28 kDa protein is composed of

Table 2. Amino acid composition of 28 kDa protein from calf brain

The proteins were hydrolysed with 6 M-HCl at 110 °C for 24 h, and single analyses were performed.

Amino acid*	Composition (mol/100 mol)*	
	Membrane	Soluble
Asx†	15.4	16.0
Glx‡	13.4	14.0
Ser	4.6	6.0
Gly	11.4	8.1
His	1.4	2.0
Arg	8.0	6.8
Thr	4.4	3.8
Ala	7.2	6.0
Pro	4.1	2.6
Tyr	1.2	1.9
Val	7.6	7.3
Met	1.0	0.2
Ile	4.2	4.7
Leu	10.6	9.4
Phe	2.7	3.6
Lys	2.7	7.6

* Cysteine and tryptophan were not determined.

† Asparagine + aspartic acid.

‡ Glutamine + glutamic acid.

subunits which are linked via disulphide bonds, samples of the 28 kDa proteins were electrophoresed under both reducing and non-reducing conditions. No significant change in mobility was observed (results not shown), indicating that the 28 kDa protein is not composed of subunits linked through disulphide bonds.

Other tests

The 28 kDa membrane protein from brain (25 µg) and ovalbumin (2 µg) were run on SDS/PAGE, and the gel was stained for carbohydrates with periodic acid/Schiff reagent as described by Segrest & Jackson (1972). Ovalbumin (2 µg), but not 28 kDa protein (25 µg), stained for carbohydrate (results not shown), indicating that the 28 kDa protein is probably not a glycoprotein or that it has a low sugar content that was not detected.

The cytosolic protein from calf brain showed four bands on isoelectric focusing with pI values of 8.30, 7.67, 7.17 and 6.80. The 28 kDa membrane protein also showed four bands on isoelectric focusing with pI values of 8.10, 7.45, 7.17 and 6.80. Lactate dehydrogenase from rabbit muscle was used as a control; it showed pI values very close to the reported values of 8.40, 8.37 and 8.10 (Susor *et al.*, 1969). The 28 kDa membrane protein from calf brain used for determining pI values was obtained in the absence of detergent (Fig. 1), since sodium cholate was found to interfere with the running of the protein in the isoelectric-focusing gels.

In order to test if the 28 kDa protein is a known protein, its properties were compared with other proteins of similar molecular mass and tissue distribution (Table 1). Bhullar & Haslam (1987) described a 27 kDa GTP-binding protein in membranes and soluble fraction of brain and other tissues. Our 28 kDa protein has a nucleoside-binding site, but it did not bind [γ -³²P]GTP. Cerra *et al.* (1985) reported soluble β -galactoside-binding

proteins with subunit molecular masses of 14.5, 18 and 29 kDa. Our 28 kDa protein did not interact with antibody to the β -galactoside-binding protein. Soluble 28 kDa protein from brain did not react with antibody to mouse adenosine deaminase. We tested for adenosine kinase, adenosine deaminase, AMPase and IMPase activities. None was detected.

It is unlikely that the 28 kDa protein is hypoxanthine:guanine phosphoribosyltransferase (molecular mass ~ 26 kDa), adenylate kinase (molecular mass ~ 21 kDa) or AMP deaminase, since the amino acid composition of the 28 kDa protein (Table 2) is quite different from those described for hypoxanthine:guanine phosphoribosyltransferase from human erythrocytes (Olsen & Milman, 1977), rabbit muscle adenylate kinase (Mahowald *et al.*, 1962) and rabbit muscle AMP deaminase (Coffee & Kofke, 1975).

DISCUSSION

The 28 kDa adenosine-binding proteins from calf brain isolated by us are probably not degradation products of larger proteins, since all buffers used included a fresh 'cocktail' of proteinase inhibitors. This conclusion is confirmed by immunoblotting of SDS/PAGE gels of crude brain extracts, which revealed a single band with a molecular mass of 28 kDa. The yields of soluble and membrane 28 kDa protein per gram of brain were about the same (Table 1). The 28 kDa adenosine-binding protein was also found in the membrane and soluble fractions of other tissues. The immunocross-reactivity suggests possible homology between the membrane and the soluble 28 kDa proteins. Although the amino acid compositions of the 28 kDa proteins are similar, there are marked differences in the case of some hydrophobic and polar amino acids, showing that these proteins are not identical.

Calf brain yields about 89 pmol of 28 kDa protein/g wet weight. If most of the soluble 28 kDa protein is in the cytosol, the concentration of the soluble 28 kDa protein is 0.11 µM, if it is assumed that cytosolic water accounts for about 40% of the wet weight. The protein binds a maximum of 2 mol of adenosine/mol. Assuming that the intracellular adenosine concentration is about 10 µM (Frick & Lowenstein, 1976), only a small fraction of the total adenosine will be bound to the 28 kDa protein.

The 28 kDa adenosine-binding protein does not have any of the activities of known adenosine-metabolizing enzymes. Moreover, we have sequenced two peptide fragments of 22 and 25 amino acids of the 28 kDa protein from calf brain membranes. Comparison with a sequence library of known proteins (Lipman & Person, 1985) including adenosine deaminase, hypoxanthine:guanine phosphoribosyltransferase and adenylate kinase showed no significant similarities. The 28 kDa protein is eluted from the 5'-amino-5'-deoxy-adenosine affinity column by adenosine, but not by inosine. This observation suggests that the 28 kDa protein is not part of the nucleoside-transport system that transports adenosine and inosine with the same affinity (Plagemann & Wohlhueter, 1983).

PIA and NECA, which are A₁ and A₂ receptor agonists respectively, did not elute the 28 kDa proteins from the affinity column. 2',5'-Dideoxyadenosine, which is a P-site agonist, eluted the 28 kDa protein from membranes, but at a concentration that was higher than that required

for adenosine. It follows that the 28 kDa protein does not possess the agonist specificity expected for A₁ or A₂ receptors. The affinity for AMP may be an artefact produced by the presence of cholate, because the soluble 28 kDa protein was eluted by AMP only when cholate was present in the eluting buffer. The inability of NECA and PIA to elute the 28 kDa proteins makes it unlikely that they are A₂ or A₁ adenosine receptors, a conclusion confirmed by their tissue distribution (Table 1). The 28 kDa protein, may, however, be a P-site, which acts as an intracellular adenosine receptor (Londos *et al.*, 1983). The dissociation constants for the membrane and soluble 28 kDa proteins of 14 and 12 μ M-adenosine respectively are in the range of intracellular adenosine concentrations found under normoxic conditions (Frick & Lowenstein, 1976) and in the range of the affinity for adenosine reported for the P-site (Wolff *et al.*, 1981). Premont *et al.* (1979) found that the catalytic component of adenylate cyclase from rat brain and the P-site do not separate on gel filtration or on a sucrose density gradient. They suggested that the P-site is located on the catalytic component of adenylate cyclase. However, there is no evidence showing whether the P-site is an integral part of the catalytic component of adenylate cyclase or is bound to it. It is also plausible that the 28 kDa adenosine-binding protein is a regulatory protein which acts like calmodulin, but its targets have not been identified.

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