5'-S-(2-Aminoethyl)-N⁶-(4-nitrobenzyl)-5'-thioadenosine (SAENTA), a novel ligand with high affinity for polypeptides associated with nucleoside transport

Partial purification of the nitrobenzylthioinosine-binding protein of pig erythrocytes by affinity chromatography

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Derivatives of N⁶-(4-aminobenzyl)adenosine (substituted at the aminobenzyl group) and 5'-linked derivatives of N⁶-(4-nitrobenzyl)adenosine (NBAdo) were evaluated as inhibitors of site-specific binding of [³H]nitrobenzylthioinosine (NBMPR) to pig erythrocyte membranes. Potent inhibitors were SAENTA [5'-S-(2-aminoethyl)-N⁶-(4-nitrobenzyl)-5'-thioadenosine] and acetyl-SAENTA (the 2-acetamidoethyl derivative of SAENTA). SAENTA was coupled to derivatized agarose-gel beads (Affi-Gel 10) to form an affinity matrix for chromatographic purification of NBMPR-binding polypeptides, which in pig erythrocytes are part of, or are associated with, the equilibrative nucleoside transporter. When pig erythrocyte membranes were solubilized with octyl glucoside (n-octyl β-D-glucopyranoside) and applied to SAENTA-Affi-Gel 10 (SAENTA-AG10), polypeptides that migrated as a broad band on SDS/PAGE with an apparent molecular mass of 58–60 kDa were selectively retained by the affinity gel. These polypeptides were identified as components of the nucleoside transporter of pig erythrocytes by reactivity with a monoclonal antibody (mAb 11C4) that recognizes the NBMPR-binding protein of pig erythrocytes. Retention of the immunoreactive polypeptides by SAENTA-AG10 was blocked by NBAdo. The immunoreactive polypeptides were released from SAENTA-AG10 by elution under denaturing conditions with 1% SDS or by elution with detergent solutions containing competitive ligands (NBAdo or NBMPR). A 72-fold enrichment of the immunoreactive polypeptides was achieved by a single passage of solubilized, protein-depleted membranes through a column of SAENTA-AG10, followed by elution with detergent solutions containing NBAdo. These results demonstrate that polypeptide components of NBMPR-sensitive nucleoside-transport systems may be partly purified by affinity chromatography using gel media bearing SAENTA groups.

INTRODUCTION

In erythrocytes, nucleoside-specific elements of the plasma membrane mediate entry and exit of nucleoside molecules in an equilibrative (facilitated diffusion) process [1]. Nitrobenzylthioinosine (NBMPR), a potent inhibitor of equilibrative transport of nucleosides in erythrocytes and many other cell types, has been a valuable probe of nucleoside transport systems at the biological and molecular levels (reviewed in [2–6]). NBMPR binds tightly to sites that are part of, or closely associated with, membrane polypeptides involved in nucleoside translocation [4]. Occupancy of the high-affinity sites by NBMPR or by related compounds blocks transporter function in erythrocytes from humans and a variety of other species [7,8]. Photoactivation of site-bound [³H]NBMPR, which results in covalent linkage of the ligand to the binding site, has enabled identification of the NBMPR-binding protein of human erythrocytes as an intrinsic

membrane glycoprotein with an apparent molecular mass of 55 kDa [9,10].

Isolation of nucleoside-transporter polypeptides from erythrocyte membranes has been hindered by their low abundances and, in human erythrocytes, by their structural similarity to glucose-transporter polypeptides. The nucleoside and glucose transporters of human erythrocytes, which co-migrate in the band-4.5 region (nomenclature of Steck [11]) of electrophoretograms during SDS/PAGE [12], have been identified as 55 kDa polypeptides by photoaffinity labelling with respectively [³H]NBMPR [10,13,14] and [³H]cytochalasin B [10,15–18]. Separation of the nucleoside- and glucose-transporter polypeptides has been achieved by using antibodies to selectively remove the latter from human erythrocytic band-4.5 polypeptides, yielding preparations in which the protein content is more than 60% nucleoside transporter [13]. An alternative approach to the preparation of nucleoside-transporter poly-

Abbreviations used: NBMPR, nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9-(β-D-ribofuranosyl)purine}; NBAdo, nitrobenzyladenosine [N⁶-(4-nitrobenzyl)adenosine]; NBTGR, nitrobenzylthioguanosine {2-amino-6-[(4-nitrobenzyl)thio]-9-(β-D-ribofuranosyl)purine}; SAENTA, 5'-S-(2-aminoethyl)-N⁶-(4-nitrobenzyl)-5'-thioadenosine; acetyl-SAENTA, 5'-S-(2-acetamidoethyl)-N⁶-(4-nitrobenzyl)-5'-thioadenosine; SAENTA-AG10, SAENTA-Affi-Gel 10; mAb, monoclonal antibody; octyl glucoside, n-octyl β-D-glucopyranoside; Tween 20, polyoxyethylene sorbitan monolaurate; IC₅₀, concentration causing 50% inhibition; PEI-cellulose, poly(ethylenimine)-cellulose; Tris 6.9 buffer, 50 mM-Tris/HCl buffer (pH 6.9, 22 °C); Tris 7.4 buffer, 50 mM-Tris/HCl buffer (pH 7.4, 22 °C).

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peptides free of glucose-transporter polypeptides is to use membranes from cells that transport nucleosides but not glucose, such as erythrocytes of adult pigs [19–22]. Nucleoside-transporter polypeptides of pig erythrocytes have been partially purified from detergent-solubilized protein-depleted membrane preparations by DEAE-cellulose chromatography [23]. Nucleoside-transporter polypeptides enriched in this way were used to raise monoclonal antibodies (mAbs) that recognize NBMPR-binding polypeptides of pig erythrocytes [24]. The latter polypeptides, which are somewhat larger than those of human erythrocytes, migrated on electrophoretograms with an apparent molecular mass of 62–64 kDa [24].

Several 6-aminopurine nucleosides with *N*⁶-(4-nitrobenzyl) substituent groups have been recognized as inhibitors of NBMPR-sensitive nucleoside-transport processes in erythrocytes and in certain cultured cell lines [25]. Considering that a general approach to isolation of nucleoside-transporter polypeptides might be found in affinity chromatography using support matrices derivatized with molecules having affinity for NBMPR-binding sites, we have assessed (i) derivatives of *N*⁶-(4-aminobenzyl)adenosine substituted at the 4-aminobenzyl position, and (ii) two 5'-substituted derivatives of *N*⁶-(4-nitrobenzyl)adenosine (NBAdo), as inhibitors of site-specific binding of [³H]NBMPR to pig erythrocyte membranes. Because potent inhibition of NBMPR binding was seen with the 5'-substituted compounds, 5'-*S*-(2-aminoethyl)-*N*⁶-(4-nitrobenzyl)-5'-thioadenosine (SAENTA) was coupled to derivatized agarose-gel beads (Affi-Gel 10). The ability of SAENTA coupled to Affi-Gel 10 (SAENTA-AG10) to retain NBMPR-binding polypeptides was evaluated by using detergent-solubilized preparations of erythrocyte membranes from adult pigs. mAb 11C4, which recognizes NBMPR-binding polypeptides of pig erythrocytes [24], was used to identify transporter polypeptides by immunoblotting from gel electrophoretograms. SAENTA-AG10 selectively retained immunoreactive polypeptides, which could subsequently be released from the affinity matrix by elution with detergent solutions containing free NBAdo or NBMPR. This procedure, which depends on a specific interaction of NBMPR-binding sites with matrix-coupled SAENTA molecules, may be useful for isolation of polypeptides associated with NBMPR-sensitive nucleoside transporters from other, more complex, cell types.

EXPERIMENTAL

Materials

Blood from adult domestic pigs (Gainers, Edmonton, Alberta, Canada) was collected into 0.2 vol. of anticoagulant solution (90 mM-sodium citrate/16 mM-citric acid/16 mM-monosodium phosphate/2 mM-adenine/12 mM-inosine) and immediately processed by conventional methods for recovery of erythrocytes. NBMPR and NBTGR were prepared as described previously [26]. [³H]NBMPR (23 Ci/mmol), obtained from Moravsek Biochemicals, Brea, CA, U.S.A., was purified by using a Spheri-10 RP18 100 mm × 4.6 mm column (Brownlee Labs, Santa Clara, CA, U.S.A.), eluted with a methanol/water gradient. [¹²⁵I]-labelled goat anti-mouse IgG was purchased from du Pont Canada (Mississauga, Ontario, Canada).

Other research materials and their suppliers were as follows: phenylmethanesulphonyl fluoride, Dowex (Cl⁻ form) anion-exchange resin, NitroBlue Tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, Tween 20 and CHAPS (Sigma Chemical Co., St. Louis, MO, U.S.A.); Affi-Gel 10 and alkaline phosphatase conjugated to goat anti-mouse IgG (Bio-Rad, Mississauga, Ontario, Canada); centrifugal ultrafilters (Analychem, Markham, Ontario, Canada); Centricon 30 microconcentrators

(Amicon, Danvers, MA, U.S.A.); Nonidet P40 (Particle Data Laboratories, Elmhurst, IL, U.S.A.); Sephadex G-50 and molecular-mass standards for gel electrophoresis (Pharmacia, Dorval, Quebec, Canada); Protosol and Econofluor (New England Nuclear, Lachine, Quebec, Canada); PEI-cellulose (UV₂₅₄) thin-layer plates (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); polyvinylidene difluoride transfer (Immobilon) membranes (Millipore Ltd., Mississauga, Ontario, Canada); octyl glucoside (Boehringer Mannheim, Dorval, Quebec, Canada); and GF/C microfibre filters and DEAE-cellulose (Whatman, Clifton, NJ, U.S.A.).

Preparation of adenosine derivatives

The chemical syntheses of the compounds listed in Table 1 will be made available on request to C.E.C. Briefly, NBAdo was prepared by *N*¹-alkylation of adenosine with 4-nitrobenzyl bromide in dimethylformamide, and the product was subjected to Dimroth rearrangement to yield the *N*⁶-isomer as previously described [27]. Catalytic hydrogenation (Pd/C) of the latter product gave *N*⁶-(4-aminobenzyl)adenosine. The 'transient protection' methodology of Jones [28], which consisted of trimethylsilylation followed by acylation, carbamoylation, or sulphonylation with the respective acid chloride and desilylation with aqueous NH₃, provided the 'X-series' of compounds listed in Table 1.

Acetylation and *O*-deacetylation of crude 5'-*S*-(2-aminoethyl)-thioadenosine followed by alkylation and rearrangement gave 5'-*S*-(2-acetamidoethyl)-*N*⁶-(4-nitrobenzyl)-5'-thioadenosine. Analogous alkylation and rearrangement of 5'-*S*-(2-hydroxyethyl)thioadenosine and treatment of the 5'-*S*-(2-hydroxyethyl)-*N*⁶-(4-nitrobenzyl)-5'-thioadenosine with diethylazodicarboxylate/triphenylphosphine and then with phthalimide gave 5'-*S*-(2-phthalimidoethyl)-*N*⁶-(4-nitrobenzyl)-5'-thioadenosine. This compound was deprotected by heating with hydrazine in ethanol to give the phthaloylhydrazide salt of 5'-*S*-(2-aminoethyl)-*N*⁶-(4-nitrobenzyl)-5'-thioadenosine (SAENTA).

Coupling of SAENTA to Affi-Gel 10

The SAENTA phthaloylhydrazide (phthalazin-1,4-dione) salt (0.16 mmol) was dissolved in 50% (v/v) methanol (20 ml), 3 drops of acetic acid were added, and the solution was applied to a column (4 ml) of Dowex (Cl⁻ form) anion-exchange resin (prewashed with 0.1 M-NaCl and equilibrated with 50% methanol). The eluate (0.5 ml/min) was collected, the column was washed with 50% methanol (25 ml), and the two fractions were combined and stored at -20 °C. The concentration of SAENTA hydrochloride (hereafter referred to as SAENTA) was determined spectrophotometrically (ϵ_{272} 2.69 × 10⁴ litre · mol⁻¹ · cm⁻¹).

Propan-2-ol, dimethyl sulphoxide and triethylamine were dried over anhydrous MgSO₄ overnight before use. SAENTA (as the hydrochloride salt in 50% methanol) was dried by rotary evaporation, and the residue, after two washes with propan-2-ol, was dissolved in propan-2-ol/dimethyl sulphoxide (4:1, v/v) to which was added triethylamine in 4-fold molar excess over SAENTA. The reactant solutions (3–5 ml) were added to Affi-Gel 10 (1 ml) and the mixtures were incubated at 22 °C for 1 h, after which the SAENTA-AG10 beads were washed twice with water and incubated (4 h, 22 °C) with 5 vol. of 0.2 M-Hepes, pH 8.0. The SAENTA-AG10 beads were then washed extensively with 0.9% NaCl and stored at 4 °C in 0.9% NaCl containing 0.02% NaN₃. 'Control' Affi-Gel 10 (Control-AG10) beads were prepared by omitting SAENTA from reaction mixtures.

For determination of the amount of SAENTA coupled to Affi-Gel 10, [³H]SAENTA was included in reaction mixtures, and radioactivity associated with SAENTA-AG10 beads was de-

terminated after incubation (16 h) in 5 ml of 3% Protosol in Econofluor. [G-³H]SAENTA was prepared by catalysed isotope exchange in ³H₂O (Dr. L. Gati and Associates Laboratories, Edmonton, Alberta, Canada), and aqueous solutions of the product (700 μ Ci) were repeatedly evaporated to dryness (40 °C) before experimental use. [³H]SAENTA was dissolved in 1 ml of aq. 50% methanol, and 20 μ l portions were purified by t.l.c. using PEI-cellulose/UV₂₅₄ thin-layer plates developed in 0.9% NaCl solution. After development, the chromatogram region containing [³H]SAENTA was eluted with water to recover the product.

Preparation of unsealed ghosts, protein-depleted membranes and octyl glucoside-solubilized membranes

Unsealed ghosts were prepared at 4 °C by a previously described procedure [29], modified by the inclusion of 0.1 mM-phenylmethanesulphonyl fluoride, and stored at -70 °C or used for preparation of protein-depleted membranes [14]. For solubilization with octyl glucoside, protein-depleted membranes (1 mg/ml) were incubated (4 °C, 20 min) in 50 mM-Tris/HCl buffer, pH 6.9, at 22 °C, hereafter designated as 'Tris 6.9 buffer', with 1% octyl glucoside, and residues were removed by centrifugation (100 000 g, 1 h, 4 °C). Protein was determined by the Lowry method [30,31] or, where indicated, by the bicinchoninic acid method [32], with BSA as the standard.

Site-specific binding of [³H]NBMPR

Binding to unsealed ghosts was determined by a filtration assay [33]. Briefly, assay mixtures (final volume, 1.2 ml; two samples per condition) contained 2 nM-[³H]NBMPR, graded concentrations of test compound and unsealed ghosts (10 μ g of protein/ml) in 5 mM-sodium phosphate buffer, pH 7.4, at 22 °C, and, for determination of non-specific binding, 5 μ M non-radioactive NBMPR was present. After a 20 min incubation at 22 °C, membrane material from 1 ml portions of assay mixtures was collected by vacuum filtration on Whatman GF/C microfibre filters, which were then rapidly washed three times with 2 ml portions of ice-cold 5 mM-sodium phosphate buffer. The filters were placed in scintillation vials and assayed for radioactivity after a 16 h incubation in 8 ml of Triton X-100-based scintillation fluid [34]. Protein was determined by the bicinchoninic acid method [32].

Binding of NBMPR to protein-depleted membranes under equilibrium conditions was determined by incubating membranes (22 °C, 20 min) in solutions (200 μ l) containing Tris 6.9 buffer and 100 nM-[³H]NBMPR, with the addition of 20 μ M-NBTGR for determination of non-specific binding. The membranes were harvested by centrifugation (16 000 g, 5 min, 8 °C) of two 80 μ l portions, washed three times with ice-cold Tris 6.9 buffer, and solubilized in 0.5 ml of 5% Triton X-100. The solubilized samples were assayed for radioactivity and protein content as described above.

Binding of NBMPR under equilibrium conditions to octyl glucoside-solubilized polypeptides was determined by incubating the polypeptide preparations (22 °C, 20 min) in solutions (200 μ l) containing Tris 6.9 buffer with 100 nM-[³H]NBMPR, the appropriate concentration of octyl glucoside, and, for determination of non-specific binding, 20 μ M-NBTGR. The reaction mixtures were cooled (4 °C), and two 80 μ l portions were applied to 1 ml Sephadex G-50 columns and centrifuged (200 g, 2 min, 4 °C). The reaction mixtures and the excluded fractions of the applied liquid samples (void volumes) were assayed for radioactivity and protein content as above. The columns, which had been previously equilibrated with Tris 6.9 buffer containing the appropriate concentration of octyl glucoside, were centrifuged (200 g, 2 min) just before use.

Photolabelling with [³H]NBMPR

Protein-depleted membranes with site-bound [³H]NBMPR were photolabelled by exposure to u.v. light from a water-cooled 450 W mercury arc lamp (Canrad-Hanovia, Newark, NJ, U.S.A.), as in a previously described procedure [35]. The photolabelled membranes were resuspended in 50 mM-Tris/HCl buffer, pH 7.4 at 22 °C, hereafter designated as 'Tris 7.4 buffer', and stored -70 °C. Protein content was determined using the bicinchoninic acid procedure [32].

Polypeptides that had been enriched by passage over SAENTA-AG10 beads (see below) were photolabelled as follows. Reaction mixtures (total volume 200 μ l) consisted of polypeptides (6.2 μ g), 100 nM-[³H]NBMPR, 10 mM-dithiothreitol, 50 mM-NaCl, 200 μ M-EDTA, 0.25% octyl glucoside and 0.25% CHAPS in Tris 7.4 buffer; final concentrations are specified. The mixtures were kept in 5 mm $\times$ 20 mm plastic centrifuge tubes (Beckman no. 344718; Beckman Instruments, Palo Alto, CA, U.S.A.) on ice for 90 min and exposed to u.v. light from the source specified above for 1 min at a distance of 4 cm from the quartz coolant jacket. Non-specific photolabelling was determined by u.v. irradiation of parallel reaction mixtures that also contained 10 μ M non-radioactive NBMPR.

Enrichment of NBMPR-binding polypeptides with SAENTA-AG10 beads

Protein-depleted membranes (1 mg/ml) were solubilized by incubation (4 °C, 20 min) with 1% octyl glucoside in Tris 7.4 buffer, and the mixtures were centrifuged (100 000 g, 4 °C, 50 min) to remove residues. Unless otherwise noted, the supernatants were diluted 4-fold to yield solubilized membranes in 'loading buffer', which consisted of 50 mM-NaCl, 200 μ M-EDTA, 0.25% octyl glucoside and 0.25% CHAPS in Tris 7.4 buffer; final concentrations are specified. The solubilized membrane preparations were applied (8 °C, 0.2 ml/min) to columns of SAENTA-AG10 beads previously equilibrated with loading buffer. In some experiments, columns of Control-AG10 beads were used to assess non-specific adsorption of polypeptides present in membrane preparations. The columns were washed (8 °C, 1 ml/min) with 10 vol. of loading buffer without additives and then were eluted (8 °C, 0.5 ml/min) with 10 vol. of loading buffer containing 150 μ M-NBA do or 30 μ M-NBMPR. The columns were stripped of the remaining adsorbed material by elution with 2 vol. of Tris 7.4 buffer containing 1% SDS. Eluate fractions were concentrated (Centricon 30 microconcentrator), and polypeptides in the concentrates were subjected to SDS/PAGE and probed by immunoblotting with mAb 11C4, using the procedures described below. In some experiments, site-specific binding of [³H]NBMPR by polypeptides in eluate fractions was measured, after washing to remove bound NBA do by 10-15 cycles of Centricon concentration and dilution with loading buffer.

Octyl glucoside-solubilized membranes were subjected to sequential DEAE-cellulose and SAENTA-AG10 chromatography as follows. Detergent-solubilized membrane preparations in 120 mM-NaCl solution were applied (0.5 ml/min) to DEAE-cellulose columns previously equilibrated with 1.5-2 vol. of Tris 7.4 buffer containing 1% octyl glucoside and 120 mM-NaCl. The 'flowthrough' solutions, which contained band-4.5 polypeptides, were immediately applied to SAENTA-AG10 columns for enrichment of NBMPR-binding polypeptides as described above.

SDS/PAGE and immunoblotting

Various treated membrane preparations and solubilized polypeptides were subjected to SDS/PAGE, using 1 mm-thick 12% acrylamide slab gels [36]. Molecular-mass markers (BSA,

66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; trypsin inhibitor 20 kDa; and lactalbumin, 14 kDa) and proteins present in samples were detected with Coomassie Blue. For analysis of photolabelled polypeptides, radioactivity was measured in a liquid-scintillation system after extraction of 2 mm slices of the gel lanes into a solution of 3 % Protosol in Econofluor [37].

For immunoblotting, polypeptides (including prestained molecular-mass markers), initially separated by SDS/PAGE, were electrophoretically transferred to Immobilon sheets using a transfer buffer that consisted of 192 mM-glycine and 20 % or 15 % (v/v) methanol [38] in 25 mM-Tris/HCl buffer (pH 8.3, 22 °C). Transfer was accomplished at 35 V for 12–16 h. Immuno-reactive polypeptides were determined as described previously [24], using alkaline-phosphatase-conjugated goat anti-mouse IgG (heavy + light chain), as in the method of Blake *et al.* [39], except for the substitution of 0.1 M-Tris/HCl buffer, pH 9.3, for veronal acetate. In some experiments, ^{125}I -labelled goat anti-mouse IgG was used as the second antibody to determine the degree of enrichment of NBMPR-binding polypeptides, relative to protein-depleted membranes. For the latter, a standard curve was constructed by analysis of the radioactivity associated with immunoreactive polypeptides after electrophoretic transfer and immunoblotting of different quantities of protein-depleted membranes.

RESULTS

Several N^6 -(4-nitrobenzyl) derivatives of 6-aminopurine nucleosides exhibit high affinity for the NBMPR-binding sites on cultured S49 mouse lymphoma cells [25]. In the current work, the interaction of NBAdo with the NBMPR-binding sites of pig erythrocytes was assessed by measuring inhibition of site-specific binding of [^3H]NBMPR to unsealed ghosts. The K_d value (mean $\pm$ s.d.) for site-bound NBMPR on pig erythrocyte ghosts has been reported as 1.6 ± 0.4 nM [33], and in the experiments of Fig. 1, binding of 2 nM-[^3H]NBMPR was assessed in the presence of graded concentrations of NBAdo. An IC_{50} value (concn. causing 50 % inhibition) of 36 nM was obtained for NBAdo inhibition of NBMPR binding. These results suggested that NBAdo would be a good candidate for attachment to a support matrix in the development of affinity media for purification of NBMPR-binding polypeptides.

In the experiments of Table 1, derivatives of N^6 -(4-aminobenzyl)adenosine with substituents on the 4-aminobenzyl group and 5'-linked derivatives of NBAdo (see Fig. 2 for structures) were assessed as inhibitors of NBMPR binding. IC_{50} values for the compounds derivatized at the 4-aminobenzyl position ranged from 0.21 to 140 μM . SAENTA and its derivative, acetyl-SAENTA, were potent inhibitors of NBMPR binding, with IC_{50} values of 330 and 76 nM respectively. The degree of inhibition seen with acetyl-SAENTA suggested that derivatization of SAENTA at the primary amino group, with linker groups attached to macromolecular matrices, might be possible without substantial loss of binding activity.

SAENTA was coupled to Affi-Gel 10 beads, an agarose gel support derivatized with 10-atom linker arms that end with a reactive group. The efficiency of the coupling reaction over a range of SAENTA concentrations (0.5–3.0 mM) is illustrated in Fig. 3. In the studies reported below, SAENTA-AG10 was produced under conditions that yielded about 0.8 μmol of SAENTA/ml of Affi-Gel 10.

Isolation of NBMPR-binding polypeptides by site-specific retention on SAENTA-AG10 beads required detergent conditions that would solubilize the polypeptides without also

Fig. 1. Inhibition of site-specific binding of NBMPR to pig erythrocyte ghosts by NBAdo

Binding of [^3H]NBMPR to ghosts was measured with a filtration assay, as described in the Experimental section, with correction for non-specific binding determined by performing the assay in the presence of 5 μM -non radioactive NBMPR. Replicate mixtures contained ghosts, 2 nM-[^3H]NBMPR, graded concentrations of NBAdo and, in mixtures used for non-specific binding, 5 μM non-radioactive NBMPR. Values for [^3H]NBMPR specifically bound in the presence of NBAdo are expressed as percentages of that bound in the absence of NBAdo (8.56×10^3 c.p.m./filter). In the experiment presented here, the concentration of NBAdo that inhibited site-specific binding of NBMPR by 50 % (IC_{50}) was 36 nM, and the mean ($\pm$ s.d.) for three separate experiments was 36 ± 1 nM.

Fig. 2. Structures of N^6 -substituted adenosine nucleosides

Structures of the X and Y substituents are given in Table 1.

denaturing the high-affinity binding sites. Fig. 4 presents results of experiments that assessed the abilities of graded concentrations of octyl glucoside to solubilize NBMPR-binding polypeptides in protein-depleted membranes. For assessment of solubilization, membranes that had been covalently photolabelled with [^3H]NBMPR were incubated with octyl glucoside solutions, and the distribution of radioactivity between the particulate and soluble fractions was determined after high-speed centrifugation. About 75–80 % of the ^3H -labelled material was recovered in the soluble fraction after incubation with Tris 6.9 buffer containing octyl glucoside concentrations of 0.75 % or greater, indicating extensive, but not complete, solubilization of the photolabelled polypeptides.

In a separate set of experiments (also presented in Fig. 4), reversible binding of [^3H]NBMPR to protein-depleted membranes from pig erythrocytes was assessed in Tris 6.9 buffer containing graded concentrations (0.25–1 %) of octyl glucoside. The latter solutions were prepared by diluting preparations of solubilized membranes in Tris 6.9 buffer containing 1 % octyl glucoside. Although inhibited by 50 % in 0.5 % octyl glucoside

Fig. 3. Efficiency of coupling SAENTA to Affi-Gel 10

The coupling reaction and analysis of the ^3H content of the product (SAENTA-AG10) were carried out as described in the Experimental section. Each reaction mixture contained 0.25 ml of Affi-Gel 10, [^3H]SAENTA (1155 c.p.m./μmol) and non-radioactive SAENTA at the concentrations indicated up to the limit of solubility (about 3 mM). Each value (●) is the mean of two separate determinations. The theoretical coupling efficiency refers to the predicted fractional occupancy by SAENTA of the coupling sites available on the Affi-Gel 10 beads and was calculated from information provided by the manufacturer.

Table 1. Inhibition of NBMPR binding to pig erythrocyte ghosts by N^6 -substituted adenine nucleosides

Site-specific binding of [^3H]NBMPR was measured in the presence of graded concentrations of test compounds as described in Fig. 1. IC_{50} values were determined from plots of the percentage of site-bound NBMPR (in the presence of test compound) versus the concentration of the test compound. Structures of the parent compounds are shown in Fig. 2.

Substituent (structure)	IC_{50} value
X = $-\text{CO}-\text{C}(\text{CH}_3)_3$	140 μM
$-\text{CO}-[\text{CH}_2]_3-\text{CH}_3$	64 μM
$-\text{CO}-\text{CH}_3$	59 μM
$-\text{CO}-\text{N}(\text{CH}_3)_2$	41 μM
$-\text{CO}-[\text{CH}_2]_4-\text{CH}_3$	21 μM
$-\text{CO}-[\text{CH}_2]_5-\text{CH}_3$	12 μM
$-\text{SO}_2-\text{C}_6\text{H}_4-\text{CH}_3$	9 μM
$-\text{CO}-\text{O}-\text{CH}_2-\text{CH}_3$	3 μM
$-\text{CO}-[\text{CH}_2]_6-\text{CH}_3$	3 μM
$-\text{CO}-\text{N}-(\text{C}_6\text{H}_5)_2$	1 μM
$-\text{SO}_2-\text{CH}_3$	940 nM
$-\text{CO}-\text{C}_6\text{H}_5$	210 nM
Y = $-\text{NH}_3^+\text{Cl}^-$	330 nM
$-\text{NH}-\text{CO}-\text{CH}_3$	76 nM

and completely absent in 1% octyl glucoside solutions, binding activity was restored to levels comparable with those of protein-depleted membranes when the concentration of octyl glucoside was reduced to 0.25%. When the solubilized membrane prepar-

Fig. 4. Solubilization of NBMPR-binding polypeptides with octyl glucoside: restoration of binding activity by dilution

Two sets of experiments are presented. Protocol A: solubilization of photolabelled polypeptides with octyl glucoside. Protein-depleted membranes, photolabelled with [^3H]NBMPR by photoactivation, were incubated (22 °C, 20 min) in Tris 7.4 buffer containing graded concentrations of octyl glucoside (1 mg of protein/ml; final volume 300 μl). Duplicate 50 μl portions were assayed for ^3H content before and after centrifugation (120000 g, 1 h). The ^3H contents of supernatants and pellets were used to calculate percentages (●) of [^3H]NBMPR-labelled material solubilized. Protocol B: reversible binding to solubilized polypeptides. Presented are measurements of site-specific binding of 100 nM [^3H]NBMPR to protein-depleted membranes in the absence of octyl glucoside (control) and to solubilized membrane polypeptides in the presence of graded concentrations of octyl glucoside. The latter solutions were prepared by solubilizing protein-depleted erythrocyte membranes in Tris 7.4 buffer containing 1% octyl glucoside, followed by dilution with Tris 7.4 buffer. Values represent [^3H]NBMPR bound to octyl glucoside-treated membranes expressed as percentages (○) of that bound to control protein-depleted membranes (30.3 pmol/mg of protein).

ations were diluted to an octyl glucoside concentration of 0.25% in the presence of other detergents, also at 0.25%, binding activity was reduced by > 75% by Triton X-100, sodium cholate and sodium deoxycholate, but was unaffected by CHAPS (results not shown). In subsequent studies, membranes were solubilized with Tris-buffered solutions containing 1% octyl glucoside and then, to restore binding activity, the preparations were diluted with Tris-buffered solutions to achieve final octyl glucoside and CHAPS concentrations of 0.25%.

In the experiment of Fig. 5, solubilized membrane preparations in loading buffer were applied to SAENTA-AG10 and Control-AG10 beads to determine if NBMPR-binding polypeptides were retained by the SAENTA-AG10 beads. Passage of solubilized membranes through the SAENTA-AG10 column reduced the capacity of the solubilized preparation to reversibly bind [^3H]NBMPR by 55%, whereas passage through the Control-AG10 column had no such effect. When proteinaceous material retained by the columns was recovered by elution with 1% SDS (Fig. 5a, lane 3) and examined by SDS/PAGE, an unidentified component that migrated slowly on the electrophoretograms was observed after staining with Coomassie Blue, and there was no Coomassie Blue-stained material in the band-4.5 region (45–65 kDa). However, when the electrophoretograms were analysed by immunoblotting with mAb 11C4, immunoreactive polypeptides (50–58 kDa) were seen to be present in the SDS eluate from the SAENTA-AG10 column (Fig. 5b, lane 3).

Fig. 5. Binding of immunoreactive polypeptides to SAENTA-AG10 beads

Protein-depleted membranes were solubilized and applied (4.5 mg of protein) to columns containing SAENTA-AG10 beads or Control-AG10 beads as described in the Experimental section. The columns were eluted with 10 vol. of loading buffer and then 2 vol. of 1% SDS in Tris 7.4 buffer. Portions (protein quantities are given in parentheses) of the variously treated mixtures were subjected to SDS/PAGE, and proteins were detected by staining with Coomassie Blue (a) or by immunoblotting with mAb 11C4 after electrophoretic transfer to Immobilon sheets (b). Lane 1, protein-depleted membranes (5.0 μ g); lane 2, loading buffer eluate from SAENTA-AG10 column (0.5 μ g); lane 3, SDS eluate from SAENTA-AG10 column (1.0 μ g); lane 4, loading buffer eluate from Control-AG10 column (0.32 μ g); lane 5, SDS eluate from Control-AG10 column (0.88 μ g); lane 6, prestained molecular-mass markers. The NBMPR-binding activities (pmol/mg of protein) were as follows: the mixture applied to the columns, 99.6; the flow-through from the SAENTA-AG10 column, 45; the flow-through from the Control-AG10 column, 89. The positions of immunoreactive polypeptides (lanes 1 and 3) are indicated by arrows.

In contrast, there was no evidence of immunoreactive polypeptides in the SDS eluate from the Control-AG10 column (Fig. 5b, lane 5) or after washing the columns with loading buffer (Fig. 5b, lanes 2 and 4).

The specificity of the interaction of NBMPR-binding polypeptides with SAENTA-AG10 beads was examined in the experiments of Fig. 6, which showed that retention of the immunoreactive polypeptides did not occur in the presence of NBAdo. Solubilized membrane preparations in loading buffer that contained 375 μ M-NBAdo or lacked NBAdo (control) were applied to SAENTA-AG10 columns, and the retained and unretained fractions were analysed for the presence of NBMPR-binding polypeptides by electrophoresis and immunoblotting with mAb 11C4. After passage of the solubilized membrane preparations through the SAENTA-AG10 column in the presence of NBAdo, the immunoreactive material (50–58 kDa) was

Fig. 6. Inhibition of binding of immunoreactive polypeptides to SAENTA-AG10 beads by NBAdo

Protein-depleted membranes were solubilized in Tris 7.4 buffer containing 1% octyl glucoside and then diluted as described in the Experimental section with loading buffer (control) or with loading buffer containing 375 μ M-NBAdo. The solubilized membrane preparations were then applied (6.9 mg of protein) to SAENTA-AG10 columns that had been previously equilibrated with loading buffer without (control) or with 375 μ M-NBAdo. The columns were eluted with 10 vol. of loading buffer and then with 2 vol. of 1% SDS in Tris 7.4 buffer. Portions (protein quantities are given in parentheses) of the variously treated mixtures were subjected to SDS/PAGE, and NBMPR-binding polypeptides were detected by immunoblotting with mAb 11C4 after electrophoretic transfer to Immobilon sheets. Lane 1, protein-depleted membranes (5.5 μ g); lane 2, flow-through after application of NBAdo-treated membranes to the NBAdo-treated column (1.0 μ g); lane 3, loading buffer eluate from NBAdo-treated column (0.5 μ g); lane 4, SDS eluate from NBAdo-treated column (0.96 μ g); lane 5, SDS eluate from control column with SDS (1.05 μ g); lane 6, prestained molecular-mass markers. The positions of immunoreactive polypeptides (lanes 1, 2 and 5) are indicated by arrows.

detected in the unretained fraction (lane 2), but was not evident in the retained fraction (lane 3), indicating that NBAdo blocked binding of the immunoreactive polypeptides to SAENTA-AG10. In contrast, after passage of the solubilized membrane preparations through the SAENTA-AG10 column in the absence of NBAdo (control), the immunoreactive material was seen in the retained fraction (lane 5). These results are consistent with the notion that retention of NBMPR-binding polypeptides by SAENTA-AG10 beads resulted from specific interactions between the SAENTA groups of the affinity matrix and the NBMPR-binding sites on nucleoside-transporter polypeptides.

Since NBAdo blocked retention of the immunoreactive polypeptides by the SAENTA-AG10 beads, it was used as a competing ligand to selectively recover NBMPR-binding polypeptides from the beads. In the experiment of Fig. 7, a solubilized membrane preparation was applied to a SAENTA-AG10 column, which was eluted successively with loading buffer, loading buffer with 150 μ M-NBAdo, and loading buffer with 1% SDS. Analysis of eluate fractions by electrophoresis and immunoblotting with mAb 11C4 demonstrated that the NBAdo-containing eluant released most of the immunoreactive material from the column, leaving little, if any, material on the column (compare lanes 4 and 5, Fig. 7b); the same eluant without NBAdo did not release immunoreactive material from the column (lane 3, Fig. 7b). In the experiment of Fig. 7, the immunoreactive material migrated somewhat more slowly (58–65 kDa) than in the experiments of Figs. 5 and 6. In a separate experiment (results not shown), after passage of solubilized membrane preparations through SAENTA-AG10 columns, elution with loading buffer containing 30 μ M-NBMPR also released the immunoreactive material.

Fig. 7. Elution of immunoreactive polypeptides from SAENTA-AG10 beads with loading buffer containing NBAdo

Protein-depleted membranes were solubilized in Tris 7.4 buffer containing 1% octyl glucoside and then diluted as described in the Experimental section with loading buffer. The solubilized membrane preparations were applied (4.05 mg of protein) to a SAENTA-AG10 column, and the column was eluted successively with 10 vol. of loading buffer, 10 vol. of loading buffer containing 150 μ M-NBAdo and 2 vol. of 1% SDS in Tris 7.4 buffer. Portions (protein quantities are given in parentheses) of the variously treated mixtures were subjected to SDS/PAGE, and proteins were detected by staining with Coomassie Blue (a) or by immunoblotting with mAb 11C4 after electrophoretic transfer to Immobilon sheets (b). Lane 1, protein-depleted membranes (5.05 μ g); lane 2, flow-through after application of solubilized membranes to column (5.45 μ g); lane 3, loading buffer eluate (0.3 μ g); lane 4, NBAdo eluate (1.0 μ g); lane 5, SDS eluate (0.5 μ g); lane 6, prestained molecular-mass markers. The NBMPR-binding activities (pmol/mg of protein) of the mixture applied to the column and of the flow-through were 88.4 and 39.2 respectively. The positions of immunoreactive polypeptides (lanes 1, 2, and 4) are indicated by arrows.

The release by NBAdo solutions of the immunoreactive polypeptides from SAENTA-AG10 beads was assessed over a range of NBAdo concentrations (results not shown). After a solubilized membrane preparation was passed through a SAENTA-AG10 column, elution with solutions of loading buffer containing NBAdo at concentrations between 1.5 μ M and 1.5 mM released immunoreactive material retained by the column. Most of that material was recovered in eluate containing 1.5 μ M-NBAdo, and little more was released by solutions containing higher NBAdo concentrations.

Throughout this work the presence of NBMPR-binding polypeptides in the SAENTA-retained material was determined by

Fig. 8. Photoaffinity labelling of polypeptides isolated by SAENTA-AG10 chromatography

Protein-depleted membranes were solubilized in Tris 7.4 buffer containing 1% octyl glucoside, the resulting solution was diluted with loading buffer as described in the Experimental section and applied (5.2 mg of protein) to a SAENTA-AG10 column, and the column was eluted with loading buffer containing 30 μ M-NBMPR. The polypeptide fraction that was eluted with the NBMPR solution was washed with loading buffer (using ten cycles of concentration by ultrafiltration and dilution to reduce the content of NBMPR) and then photolabelled with 100 nM-[3 H]NBMPR in the presence (○) or absence (●) of 10 μ M-NBMPR. The photolabelled material (6.2 μ g of protein) was subjected to SDS/PAGE, proteins were detected by staining with Coomassie Blue, and 2 mm slices of the gel were assayed for their 3 H content. The positions of molecular-mass standards on the electrophoretograms are indicated.

SDS/PAGE followed by immunoblotting with mAb 11C4, an immune probe specific for the NBMPR-binding polypeptides of pig erythrocytes [24]. In the experiment of Fig. 8, the identity of the polypeptide fraction enriched by SAENTA-AG10 affinity chromatography was confirmed by the demonstration of site-specific photolabelling with [3 H]NBMPR. Material from solubilized membranes that was retained by a SAENTA-AG10 column and recovered by elution with loading buffer containing NBMPR was photolabelled with [3 H]NBMPR, after extensive washing to remove site-bound non-radioactive NBMPR. A control sample was photolabelled in the presence of excess non-radioactive NBMPR. The 3 H-labelled material migrated on electrophoretograms with an apparent molecular mass (58 kDa) similar to that found for the immunoreactive polypeptides.

In the present study solubilized membrane preparations were applied to, and eluted from, SAENTA-AG10 columns at 8 $^{\circ}$ C. When the affinity chromatography was conducted at 37 $^{\circ}$ C (results not shown), the immunoreactive material was retained by the SAENTA-AG10 beads and could not be eluted with NBAdo. The immunoreactive material was recovered by elution with loading buffer containing 1% SDS. These observations suggest that the NBMPR-binding polypeptides were bound more tightly to the affinity matrix at 37 $^{\circ}$ C than at 8 $^{\circ}$ C.

Table 2 summarizes the enrichment of NBMPR-binding polypeptides from protein-depleted membranes of pig erythrocytes achieved by passing solubilized membrane preparations through SAENTA-AG10 columns and eluting the columns with NBAdo solutions. Enrichment was quantified by measuring the increase in specific binding activity (pmol of NBMPR/mg of protein) at a single high concentration (100 nM)

Table 2. Enrichment of NBMPR-binding polypeptides with SAENTA-AG10 beads

Protocol A: One-step enrichment from protein-depleted membranes. Solubilized membrane preparations in loading buffer were applied to a SAENTA-AG10 column and eluted with 150 μ M-NBAdo. Reversible binding of [3 H]NBMPR and quantification of NBMPR-binding polypeptides by immunoblotting with 125 I-labelled goat anti-mouse IgG were determined as described in the Experimental section. Presented below are averaged values from two experiments. Protocol B: Two-step enrichment from protein-depleted membranes. A solubilized membrane preparation in loading buffer was applied to a DEAE-cellulose column and polypeptides recovered therefrom were applied to a SAENTA-AG10 column, which was then eluted with loading buffer containing 150 μ M-NBAdo. The relative quantities of NBMPR-binding polypeptides in protein-depleted membranes and the SAENTA eluate were determined by immunoblotting with 125 I-labelled goat anti-mouse IgG.

Parameter	Protein-depleted membranes	SAENTA-enriched material	Yield (%)	Enrichment*
Protocol A				
Protein (mg)	13.75	0.023	0.2	
Total NBMPR-binding capacity (pmol)	560	20.25	3.6	
NBMPR-binding activity (pmol/mg of protein)	40.7	880.4		21.6
125 I-IgG binding (c.p.m./ μ g of protein)	1191	85 405		71.8
Protocol B				
125 I-IgG binding (c.p.m./ μ g of protein)	1478	274 267		186

* Relative to protein-depleted membranes.

of [3 H]NBMPR. Since NBAdo is a tight-binding ligand, the enriched material was subjected to several cycles of concentration (by ultrafiltration) and dilution to remove bound NBAdo before analysis of reversible binding of [3 H]NBMPR. The 'one-step' procedure (Protocol A) achieved a 22-fold increase in NBMPR-binding activity. Enrichment was also quantified by measuring the increase in immunoreactive polypeptides using mAb 11C4 and 125 I-labelled second antibody. With Protocol A, a 72-fold increase in immunoreactive material was achieved. The disproportionate increase in immunoreactive polypeptides relative to NBMPR-binding activity suggested the presence of tightly bound NBAdo and/or the presence of denatured NBMPR-binding polypeptides in the enriched material.

Partial purification of NBMPR-binding polypeptides of pig erythrocytes by passage through DEAE-cellulose columns has been previously demonstrated [23]. Sequential passage of solubilized protein-depleted membranes through a DEAE-cellulose column and a SAENTA-AG10 column achieved a 186-fold enrichment of immunoreactive polypeptides (Protocol B, Table 2).

DISCUSSION

The potent inhibition by NBAdo of the equilibrative nucleoside transporter of S49 lymphoma cells is a consequence of NBAdo interaction with the NBMPR-binding sites [25]. In this work, we tested (i) two 5'-linked derivatives of NBAdo and (ii) derivatives of *N*⁶-(4-aminobenzyl)adenosine with various substituents on the 4-aminobenzyl group as inhibitors of NBMPR binding to membranes of pig erythrocytes. An aim was to determine if substituent groups at either of these positions would allow transport-inhibitory molecules, such as NBAdo, to interact with NBMPR-binding sites. Substituent groups at these positions on site-interactive molecules might serve as linkage points for attachment to support matrices in the development of affinity media for isolation of polypeptides associated with NBMPR-sensitive transport of nucleosides. IC₅₀ values for inhibition of reversible binding of [3 H]NBMPR to pig erythrocyte membranes by the 4-aminobenzyl derivatives were as low as 210 nM, indicating some potential of the derivatized groups as linker sites.

In the preparation of SAENTA and acetyl-SAENTA, derivatization at the 5'-position of NBAdo was examined. Potent

inhibition (IC₅₀ 76 nM) of NBMPR binding was seen with acetyl-SAENTA, indicating structural tolerance at the 5'-position of NBAdo for interaction with the NBMPR-binding sites. SAENTA, the reactive parent compound, was a less potent inhibitor (IC₅₀ 330 nM) of NBMPR binding than acetyl-SAENTA, perhaps because the charged species does not interact with the NBMPR-binding sites. In earlier studies that assessed the effects of ionization of S⁶-substituted thiopurine ribonucleosides [40] and of permeants [41,42] on interaction with nucleoside transporters, molecules with charged substituents were not active as inhibitors or permeants.

The NBMPR-binding polypeptides of adult pig erythrocytes have been partially purified by DEAE-cellulose chromatography [23], and mAbs (3E3, 11C4) that bind to these polypeptides have been obtained using the DEAE-cellulose fractions as antigen [24]. In this work, mAb 11C4 was used to identify NBMPR-binding polypeptides in the retained and unretained fractions after application of solubilized membrane preparations to columns containing SAENTA-AG10 beads. In addition, NBMPR-binding activity in membrane polypeptide fractions that were retained or not retained by SAENTA-AG10 beads was monitored by measuring reversible binding of [3 H]NBMPR. A single passage of solubilized membranes through the SAENTA-AG10 column reduced NBMPR-binding activity by about 50 %, and multiple passages (results not shown) reduced binding activity further, to < 10 % of that present before exposure to the SAENTA-AG10 beads. Polypeptides recognized by mAb 11C4 were retained by SAENTA-AG10 beads and could be released therefrom by elution with solutions containing NBAdo or NBMPR. Retention of the immunoreactive polypeptides by SAENTA-AG10 beads is attributed to site-specific binding to SAENTA groups, since the polypeptides (i) were retained by SAENTA-AG10 beads, but not by Control-AG10 beads, (ii) did not bind when NBAdo was present in the loading buffer, and (iii) were released when the column was eluted with loading buffer containing NBAdo. The immunoreactive polypeptides, after extensive washing to remove bound NBMPR, were photo-labelled with [3 H]NBMPR, confirming their identity as NBMPR-binding polypeptides. In this work, the immunoreactive polypeptides migrated with an apparent molecular mass of 58–60 kDa, which is lower than that found previously (62–64 kDa) for the NBMPR-binding polypeptides of pig erythrocytes

[18,23,24]. We do not have an explanation for these differences.

A major difficulty encountered in the development of procedures for enriching nucleoside-transporter polypeptides with SAENTA-AG10 beads was the loss of binding activity in the presence of detergent. The concentrations of octyl glucoside required to solubilize NBMPR-binding polypeptides from pig erythrocyte membranes completely inhibited site-specific binding of [³H]NBMPR, and, although binding activity could be restored by reducing the concentration of octyl glucoside from 1% to 0.25%, a time-dependent loss of binding activity (results not shown) occurred even at low concentrations of octyl glucoside. Preparations with the highest binding activity were obtained when the chromatographic steps were conducted immediately after solubilization of membranes. Time-dependent denaturation in the presence of detergent has been shown for glucose-transporter polypeptides from human erythrocytes [43].

The NBMPR-binding polypeptides retained by SAENTA-AG10 beads at 37 °C were not released by competitive elution with high concentrations of NBAdo, suggesting that the interaction of NBMPR-binding polypeptides with SAENTA was tighter at 37 °C than at 8 °C. A similar, temperature-dependent increase in affinity of band-3 polypeptides for an affinity matrix has been reported [44]. In the latter study, detergent-solubilized membrane preparations from human erythrocytes were applied to an affinity matrix that had been derivatized with an inhibitor of the anion transporter.

When enrichment of NBMPR-binding polypeptides from protein-depleted membranes was estimated by measuring NBMPR-binding activity, a 22-fold increase was achieved by a single passage of solubilized membranes through a SAENTA-AG10 column with release therefrom by elution with an NBAdo-containing solution. Measurement of binding activity (pmol/mg of protein) evidently underestimated the extent of enrichment, since there was a disproportionate 72-fold increase in the material recognized by mAb 11C4. The enrichment of immunoreactive polypeptides from protein-depleted membranes was increased to 186-fold by prior passage of the solubilized membrane preparations through a DEAE-cellulose column, probably because of removal of band-3 polypeptides. The latter procedure represents a significant improvement over the 60-fold purification obtained by Kwong *et al.* [23] using conventional gradient-elution ion-exchange chromatography. The major contaminant of the preparation of Kwong *et al.* [23] was a low-molecular-mass species (43 kDa), whereas in the present study the major contaminant was of high molecular mass (see Fig. 5a, lane 3).

Kwong *et al.* [23] have concluded, from analysis of binding of NBMPR to highly purified human NT polypeptides, that the nucleoside transporter contains one NBMPR-binding site per 55 kDa polypeptide chain. Assuming a 1:1 stoichiometry for NBMPR binding to the 55 kDa polypeptide, purification of the latter to homogeneity from protein-depleted membranes from pig erythrocytes would require a 400–450-fold enrichment. If so, the preparation obtained by sequential DEAE-cellulose and SAENTA-AG10 chromatography in the present study was about 50% pure.

In summary, we have developed a novel reactive ligand (SAENTA) for the NBMPR-binding site of the equilibrative nucleoside transporter of erythrocytes. When coupled to Affi-Gel 10 through the primary amino group of SAENTA, the affinity of the latter group for the NBMPR-binding site was the basis for retention by the derivatized gel of NBMPR-binding polypeptides present in octyl glucoside-solubilized preparations of membranes from pig erythrocytes. The NBMPR-binding polypeptides were released from the affinity gel by elution with loading buffer containing a competing ligand, NBAdo. SAENTA, when coupled to an appropriate affinity support, provides a new approach for

isolation of polypeptides associated with 'NBMPR-sensitive' equilibrative transporters of nucleosides in erythrocytes and other mammalian cell types.

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