Biochemical Pharmacology, Vol. 52, pp. 1375–1385, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00465-0

Characterization of a Novel Adenosine Binding Protein Sensitive to Cyclic AMP in Rat Brain Cytosolic and Particulate Fractions

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ABSTRACT. A novel binding site for the adenosine receptor agonist 5′-N-ethylcarboxamidoadenosine (NECA), which was enriched in rat forebrain, was characterized in cytosolic and particulate preparations. The site showed a pharmacological profile different from other [^{3}H]NECA binding proteins and was named adenotin 2. [^{3}H]NECA was bound in the presence of 100 μM 2-chloroadenosine with a K_d of 45.4 nM and a B_{max} of 4711 fmol/mg in the cytosol and a K_d of 72.4 nM and a B_{max} of 4844 fmol/mg in the crude membrane fraction. The presence of two different binding sites on adenotin 2 for [^{3}H]NECA was shown in kinetic experiments. This protein showed identical pharmacological profiles in both subcellular preparations. [^{3}H]NECA was displaced by purine analogues with a rank order of potency of NECA > 3′5′ cyclic AMP (cAMP) > 5′-deoxy-5′-chloroadenosine > S-adenosylhomocysteine ≈ 5′-deoxy-5′-methylthioadenosine (MeSA) > adenosine ≈ adenine. cAMP inhibited [^{3}H]NECA binding allosterically, whereas adenine and MeSA acted competitively. Inhibitors and activators of protein kinases such as N-(2-aminoethyl)-5-isoquinolinesulfonamide, Sp-adenosine cyclic monophosphothioate and (8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2, 7b, 11a-triazadibenzo-(a,g)cycloocta(cde)-trinden-1-one (K 252a) interacted with [^{3}H]NECA binding to adenotin 2 in nanomolar concentrations. Adenosine-5′-O-(3-thiotriphosphate) (100 μM) increased the affinity of [^{3}H]NECA to a K_d of 9 nM and diminished the affinity of cAMP. The pharmacological characteristics of this novel binding site for [^{3}H]NECA resemble those of the inhibition of phosphorylation processes by adenosine and its derivatives in heart and smooth muscle but are distinct from known adenosine receptors, adenosine binding proteins and protein kinases. BIOCHEM PHARMACOL 52;9:1375–1385, 1996. *Copyright © 1996 Elsevier Science Inc.*

KEY WORDS. adenosine binding protein; adenosine receptor; cAMP; NECA; radioligand binding; rat brain

The characterization of adenosine receptors with the radioligand [^{3}H]NECA† has been complicated by the lack of selectivity of this agonist. Similarly to NECA, binding sites of unknown function for the A_1-selective antagonist DPCPX and the A_2-selective agonist CGS 21680 have also been described [1–3]. The lack of selectivity of NECA, however, has facilitated the detection of different adenosine binding proteins. NECA activates A_1, A_{2a}, A_{2b} and A_3 receptors [4–7]. In addition to adenosine receptors, mammalian tissues contain several NECA binding proteins that can be discriminated from each other and from adenosine receptors by their characteristic pharmacological profiles. A high-affinity adenosine binding protein, which has been named A_x, also binds adenine nucleotides and inosine with high affinity [8–10]. The low-affinity adenosine binding protein adenotin 1 can be distinguished from adenosine receptors by its lack of affinity for N^6-substituted adenosine derivatives and xanthines [11, 12]. Adenotin 1 has been detected in human platelets [11, 12], human placenta [13], bovine and rat brain [8, 14], PC 12 cells [15] and mouse P815 mastocytoma cells [16]. This protein shows amino acid sequence homologies with heat shock proteins, a calcium-binding protein and a protein kinase [12, 17–21]. Its physiological significance remains to be elucidated. The existence of another adenosine binding protein, which is

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† *Abbreviations:* ATP[S], adenosine-5′-O-(3-thiotriphosphate); cAMP, 3′5′-cyclic AMP; CGS 21680, 2-[p-(carboxyethyl)-phenethylamino]-5′-N-ethylcarboxamidoadenosine; CHAPS, 3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate; 2ClA, 2-chloroadenosine; 5′ClA, 5′-deoxy-5′-chloroadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; H 7, 1-(5-isoquinolinesulfonyl)-2-methylpyrazine; H 9, N-(2-aminoethyl)-5-isoquinoline-sulfonamide; HeNECA, 2-hexynyl-5′-N-ethylcarboxamidoadenosine; IBMX, 3-isobutyl-1-methylxanthine; K 252a, (8R*, 9S*, 11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H, 8H, 11H-2, 7b, 11a-triazadibenzo(a,g)cycloocta(cde) trinden-1-one; MeSA, 5′-deoxy-5′-methylthioadenosine; NBTG, S-(4-nitrobenzyl)-6-thioguanosine; NBTI, S-(4-nitrobenzyl)-6-thioinosine; NECA, 5′-N-ethylcarboxamidoadenosine; NECG, 5′-N-ethylcarboxamidoguanosine; NECI, 5′-N-ethylcarboxamidoinosine; PIA, N^6-phenylisopropyladenosine; RpcAMPS, Rp-adenosine cyclic monophosphothioate; R 59949, 3-(2-(4-(bis-{4-fluorophenyl}methylene)-1-piperidinyl)-ethyl)-2,3-dihydro-2-thioxo-4(1H)quinazolinone (diacylglycerol kinase inhibitor II); Ro 20-1724, 4-[(butoxy-4-methoxyphenyl)-methyl]-2-imidazolidinone; SAH, S-adenosylhomocysteine; SpcAMPS, Sp-adenosine cyclic monophosphothioate; XAC, xanthine amine congener.

Received 8 March 1996; accepted 5 June 1996.

only detectable in the presence of 2ClA, has been demonstrated in human platelets [22]. As with adenotin 1, this site displays virtually no affinity toward N^6-substituted adenosine analogues and adenosine receptor antagonists. This protein, which differs from adenotin 1, has been named adenotin 2. The present work was done to characterize adenotin 2 further with respect to its localization in rat tissues, its interaction with purine derivatives and its relation to adenosine receptors and other adenosine binding proteins and protein kinases.

MATERIALS AND METHODS

Materials

[^{3}H]NECA (15–25 Ci/mmol) was obtained from NEN-Du Pont (Bad Homburg, Germany). Adenine, adenosine, adenosine-2′3′-dialdehyde, ATP[S], AMP, bovine serum albumin, 8-bromoadenosine, 2ClA, 5′ClA, 2′3′-cyclic AMP, 3′5′-cyclic AMP, 6-chloropurine riboside, guanosine, 6-mercaptopurine riboside, MeSA, NBTG, NBTI, polyethyleneimine, R-PIA, S-adenosylhomocysteine, and theophylline were purchased from Sigma (Deisenhofen, Germany). Protein kinase inhibitors K 252a, KT 5720, KT 5823, KT 5926 and R 59949 were supplied by Calbiochem (Bad Soden, Germany). The following compounds came from RBI/Biotrend (Cologne, Germany): CGS 21680, DPCPX, genistein, H 7, H 9, IBMX, N^6-methyladenosine, NECA, Ro 20-1724, RpcAMPS, SpcAMPS, tamoxifen and XAC. Adenosine deaminase (from calf intestine; 200 U/mg), CHAPS, and NAD were obtained from Boehringer (Mannheim, Germany). Eritadenine was kindly supplied by Dr. Takeyama, Tanabe Seiyaku (Saitama, Japan). NECI was a kind gift from Prof. Ray Olsson (Dept. of Internal Medicine, University of South Florida, Tampa). HeNECA was a generous gift from Dr. G. Cristalli (University of Camerino, Italy). NECG was kindly provided by Byk Gulden (Konstanz, Germany). Deoxycoformycin was a gift from Gödecke AG (Freiburg, Germany). GF/B glass fiber filters were purchased from Whatman (Maidstone, England). All other chemicals were from standard sources and of the highest purity available.

Preparation of Cytosolic and Membrane Fractions from Rat Tissues

Forebrain, cerebellum, liver, kidney, spleen, skeletal muscle, heart, lung and fat tissue from male Wistar rats (8–12 weeks old) were homogenized in 9 volumes of ice-cold 0.32 M sucrose with a Polytron (setting 6). For the preparation of fat tissue cytosolic and membrane fractions, the upper phase, which contained fat, was discarded. The lower phase was aspirated and filtered over nylon gauze (mesh, 200 μm). The homogenate was centrifuged at 100,000 *g* (37,000 rpm) for 60 min at 4°C in a Beckman Ti 60 rotor. The supernatant was used as the cytosolic fraction. The cytosol was frozen in liquid nitrogen and kept at −70°C. The pellet was washed twice by resuspension in water and centrifuged at 100,000 *g* for 60 min at 4°C. The final pellet was resuspended in 50 mM Tris-HCl, pH 7.4, frozen in liquid nitrogen and stored at −70°C. Protein content was measured according to Peterson [23] by using bovine serum albumin as reference protein.

Radioligand Binding

Binding assays of [^{3}H]NECA (final concentration: 20 nM, if not indicated otherwise) were performed in a total volume of 250 μL in 50 mM Tris-HCl buffer, pH 7.4, containing 0.02% (w/v) CHAPS. All incubations were routinely performed on ice. For the determination of thermodynamic parameters, incubations were done 3 hr at 25°C, 4 hr at 20°C, 8 hr at 10°C and 18 hr at 0°C, according to previous time-course experiments. The samples contained 75–100 μg of cytosolic or membrane protein, 0.5 U/mL adenosine deaminase and 100 μM 2ClA. Nonspecific binding was determined in the presence of 100 μM 2ClA and 100 μM MeSA. When adenosine was used as a competitor for [^{3}H]NECA binding, membranes and cytosol (1 mg/mL) were preincubated with adenosine deaminase (1 U/mL) for 30 min at 22°C to degrade endogenous adenosine. After addition of deoxycoformycin (final concentration: 10 μM), membranes and cytosol were used immediately for competition experiments with adenosine as described above but without adenosine deaminase. Incubations were stopped under steady-state conditions (0°C: cytosol, 5 hr incubation; membranes, 18 hr incubation) by filtration of 200 μL aliquots through GF/B glass fiber filters, followed by two 4-mL washes with ice-cold 50 mM Tris-HCl buffer, pH 7.4. The filters had been impregnated with 0.3% (w/v) polyethyleneimine for filtration of cytosolic samples.

Data Analysis

Equilibrium binding data were analyzed by nonlinear curve fitting by using the SCTFIT program [24]. Kinetic data were fitted as described by Lohse *et al.* [4] by using the SigmaPlot program. All experiments were fitted to a one-site model when fitting to a two-site model did not improve the fit significantly ($P < 0.05$). K_d and K_i values were calculated from 3–10 independent experiments and are given as geometric means with 95% confidence limits. B_{max} values are arithmetic means ± SEM. Standard free energy was calculated as $\Delta G° = -RT\ln K_A$ (T = 298.15 K, R = 8.314 J/mol/K), standard enthalpy $\Delta H°$ was calculated from van't Hoff plots ($\ln K_A$ vs. 1/T; slope = $-\Delta H°/R$) and standard entropy $\Delta S°$ as $(\Delta H° - \Delta G°)/T$.

RESULTS

Localization of Adenotin 2 in Rat Tissues

A novel binding site for adenosine, which has been named adenotin 2, has been characterized by direct radioligand binding studies with the nonselective agonist [^{3}H]NECA. Total binding was measured in the presence of 100 μM

2ClA, which suppressed radioligand binding to adenosine receptors and to the nonreceptor adenosine binding proteins adenotin 1 and A_x. Nonspecific binding was defined in the presence of 100 μM 2ClA and 100 μM MeSA. The concentration of adenotin 2 in cytosolic and particulate fractions from nine different rat tissues was examined by measurement of specific [^{3}H]NECA binding to this site. In cytosolic fractions, highest levels of adenotin 2 were detected in forebrain and fat (835 ± 36 and 652 ± 70 fmol/mg protein, respectively; Fig. 1). The concentration of adenotin 2 in cytosolic preparations from these tissues was approximately three to four times higher than that in all other tissues investigated, which displayed levels of 150–200 fmol specific [^{3}H]NECA binding to adenotin 2. In particulate fractions, rat forebrain showed the highest levels of radioligand binding to adenotin 2 (716 ± 36 fmol/mg), followed by cerebellum (319 ± 21 fmol/mg) and lung (294 ± 13 fmol/mg) (Fig. 1). Because the highest concentrations of adenotin 2 were detected in forebrain in both cytosolic and particulate fractions, this binding site was characterized in detail in these preparations.

Association and Dissociation of [^{3}H]NECA

Kinetic experiments were performed in the presence of 20 nM of the radioligand, and dissociation was induced by adding unlabeled cAMP (10 μM) or MeSA (100 μM) to the incubations. Association of [^{3}H]NECA to cytosolic adenotin 2 reached equilibrium after 5 hr, whereas maximum levels of radioligand binding to adenotin 2 in the crude membrane preparation were measured after approximately 12 hr. Nonlinear curve fitting revealed that the time course of association in both preparations followed a biphasic rather than a monophasic time course ($P < 0.05$; Fig. 2). Cyclic AMP dissociated [^{3}H]NECA completely from adenotin 2 in the cytosol, whereas approximately 30% of the radioligand bound to the particulate fraction was not dissociated by this nucleotide (Fig. 2). The lower efficacy of cAMP in this fraction was not due to degradation because the stable analogue SpcAMPS also induced an incomplete dissociation of [^{3}H]NECA from membrane-associated adenotin 2 (data not shown). MeSA, however, induced a complete displacement of [^{3}H]NECA from both compartments. Dissociation by cAMP in the cytosol showed a monophasic time course. In contrast, MeSA induced a biphasic dissociation of [^{3}H]NECA both in the cytosol and in membranes. In contrast to the cytosolic preparation, curve fitting for the dissociation by cAMP in the crude membrane fraction revealed that the process was biphasic rather than monophasic (Fig. 2, lower panel). Due to the very rapid dissociation time courses, kinetic K_d values could only be calculated for the membrane preparation when the disso-

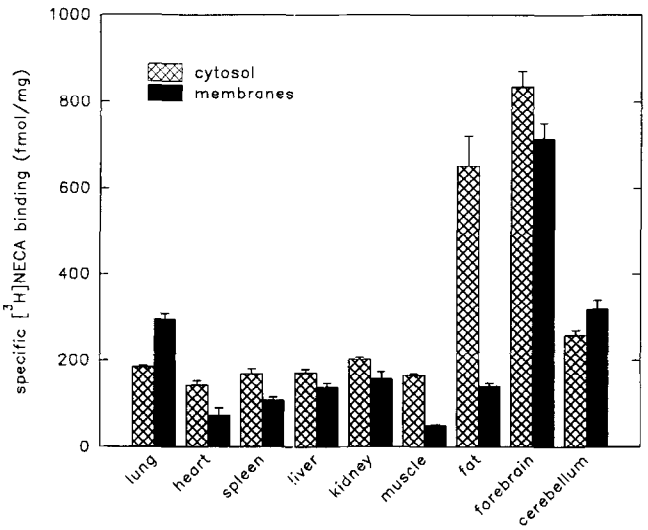

FIG. 1. Localization of adenotin 2 in rat tissues. [^{3}H]NECA (20 nM) was incubated with 75 μg of protein from cytosolic (crosshatched bars) or particulate fractions (solid bars) of rat tissues in a total volume of 250 μL on ice for 5 hr (cytosol) or 18 hr (particulate fractions). Total binding was defined as the binding in the presence of 100 μM 2ClA, and nonspecific binding was determined by the addition of 100 μM MeSA. Results ± SEM from three independent experiments are shown.

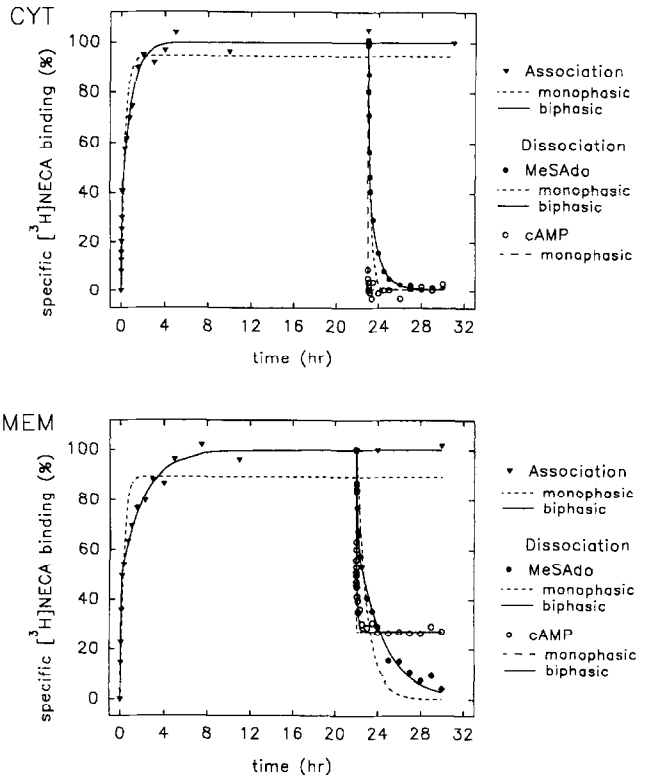

FIG. 2. Association (triangle) and dissociation of [^{3}H]NECA to adenotin 2 in rat forebrain. Radioligand binding was determined after incubation with 20 nM of the radioligand in the presence of 100 μM 2ClA for increasing time intervals on ice. Top: Cytosolic fraction; bottom: membrane fraction. After equilibrium was attained, dissociation of the radioligand was induced by the addition of 10 μM cAMP (open circle) or 100 μM MeSA (solid circle). Association and dissociation curves were calculated for monophasic (dashed lines) or biphasic models (solid lines). The data in the figure show the specific binding to adenotin 2 from one out of four independent experiments.

TABLE 1. Kinetics of radioligand binding to adenotin 2

Association				Dissociation				Affinity			
Cytosol				cAMP							
Monophasic	k_{obs}	0.0601	min^{-1}	Monophasic	k_{-1}	31.04	min^{-1}				
Biphasic	k_{obsA}	0.299	min^{-1}	MeSA							
	k_{obsB}	0.0172	min^{-1}	Monophasic	k_{-1}	0.0857	min^{-1}				
				Biphasic	k_{-1A}	0.563	min^{-1}				
					k_{-1B}	0.0215	min^{-1}				
Membranes				cAMP							
Monophasic	k_{obs}	0.0673	min^{-1}	Monophasic	k_{-1}	1.528	min^{-1}				
Biphasic	k_{obsA}	0.302	min^{-1}	Biphasic	k_{-1A}	6.70	min^{-1}				
	k_{obsB}	0.0068	min^{-1}		k_{-1B}	0.0254	min^{-1}				
				MeSA							
				Monophasic	k_{-1}	0.0128	min^{-1}	k_{+1}	0.00273	$nM^{-1} \times min^{-1}$	K_d	4.69 nM
				Biphasic	k_{-1A}	0.161	min^{-1}	k_{+1A}	0.00705	$nM^{-1} \times min^{-1}$	K_{dA}	22.8 nM
					k_{-1B}	0.0057	min^{-1}	k_{+1B}	0.0565×10^{-3}	$nM^{-1} \times min^{-1}$	K_{dB}	100.4 nM

Twenty nmol/L of [^{3}H]NECA were incubated with 75 μg protein from cytosolic or particulate fractions for increasing time intervals on ice. Association curves were fitted to a monophasic or biphasic model. Dissociation was initiated after an association of 23 hr (cytosol) or 22 hr (membranes) by the addition of 10 μM cAMP or 100 μM MeSA. K_{+1} values could be calculated only for [^{3}H]NECA binding to membranes when dissociation was induced by MeSA. Data are mean values from four independent experiments.

ciation was induced by MeSA. Two sites with affinities for [^{3}H]NECA of 22.8 nM and 100.4 nM were detected (Table 1).

Pharmacological Characterization of Adenotin 2

Cytosolic and membrane-associated adenotin 2 were characterized in more detail in saturation and competition studies with [^{3}H]NECA. [^{3}H]NECA binding to both preparations was saturable in a single component, with K_d values of 45.4 (33.0–62.4) nM and 72.4 (60.3–87.1) nM for the cytosolic and for the crude membrane preparation, respectively (Fig. 3). Pseudo Hill coefficients (cytosol: 1.00 ± 0.02; membranes: 0.98 ± 0.01) did not indicate heterogeneity of binding sites. Maximum binding capacities of 4711 ± 427 fmol/mg (cytosol) and 4844 ± 209 fmol/mg (membranes) were determined. Thus, in contrast to kinetic experiments, saturation of adenotin 2 under equilibrium conditions did not reveal the presence of two binding sites for [^{3}H]NECA. The K_d value calculated from saturation experiments for adenotin 2 in the particulate fraction was intermediate between the kinetic K_d values, and it seems

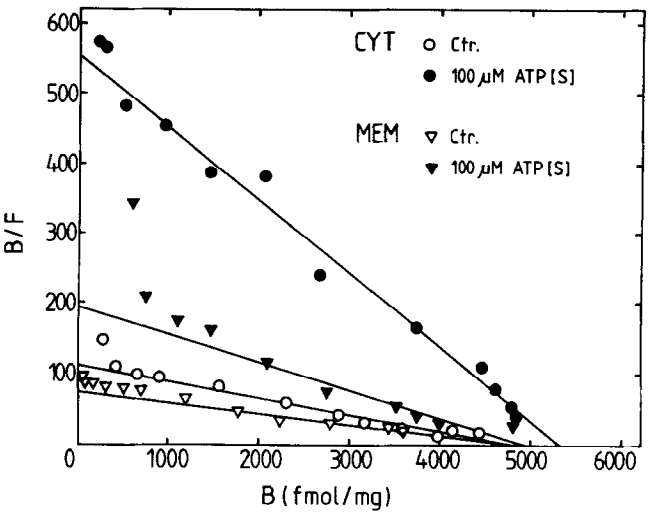

FIG. 3. Saturation of adenotin 2 with [^{3}H]NECA. Seventy-five micrograms of protein from the cytosol (circles) or membrane fraction (triangles) were incubated with increasing concentrations of [^{3}H]NECA in the absence (open symbols) or presence (closed symbols) of 100 μM ATP[S]. Total binding was measured in the presence of 100 μM 2ClA, and nonspecific binding was defined by the addition of 100 μM MeSA. Data shown are from one representative experiment of 5–10 independent experiments. Nonlinear curve fitting gave K_d values of 45.4 (95% confidence limits: 33.0–62.4) nM in the cytosol and 72.4 (60.3–87.1) nM in the membrane fraction. Maximum binding capacities (±SEM) were 4711 ± 427 fmol/mg protein and 4844 ± 209 fmol/mg, respectively; 100 μM ATP[S] significantly increased the affinity of [^{3}H]NECA in the cytosol to 9.00 (8.05–10.1) nM ($P < 0.005$ vs. control) and in the particulate fraction ($P < 0.0001$) to 24.1 (20.5–28.4) nM. B_{max} values were not changed significantly in the presence of ATP[S]; 5551 ± 296 fmol/mg adenotin 2 were measured in the cytosol, and 4489 ±282 fmol/mg adenotin 2 were found in the membranes.

unlikely that two sites that differ in their affinity by a factor of 5 could be detected in saturation experiments. Moreover, saturation experiments with a wider range of radioligand concentrations did not yield biphasic Scatchard plots (data not shown).

Competition experiments with [^{3}H]NECA revealed identical pharmacological profiles for the soluble cytosolic site and for the membrane-associated adenotin 2 (Table 2). All inhibition curves were monophasic. All compounds that displaced [^{3}H]NECA led to a complete inhibition of radioligand binding at adenotin 2 (Fig. 4). Adenosine derivatives substituted in the 5′ position, such as NECA, cAMP, 5′ClA, SAH and MeSA, were the most potent displacers of [^{3}H]NECA, with K_i values in the nanomolar or low micromolar range (Table 2). Adenine (K_i 5.91 μM for the membrane-associated site; K_i 8.38 μM for the cytosolic site) was almost as potent as MeSA or SAH. The affinity of adenotin 2 for adenosine was tested after degradation of endogenous adenosine present in the membranes and in the cytosol by adenosine deaminase and subsequent inhibition of this enzyme in the presence of 10 μM deoxycoformycin. Adenosine showed an affinity of 7.06 μM for membrane-associated and 6.29 μM for cytosolic adenotin 2. N^6-methyladenosine showed intermediate potency. Other N^6-substituted adenosine receptor agonists were not bound, and substitution of the N^6-amino group by a sulfhydryl group or by chlorine abolished the affinity of these adenosine derivatives. The inosine derivative NECI was not bound. In contrast with adenosine receptors, adenotin 2 did not bind xanthine antagonists such as XAC or DPCPX or the A_{2a}-selective agonists HeNECA or CGS 21680. Guanosine, NECG, and GMP did not inhibit [^{3}H]NECA binding to adenotin 2. The nucleoside transport inhibitors NBTG and NBTI were also inactive. Because the affinity of adenotin 2 for cAMP and SAH might indicate a relationship of this site with phosphodiesterases or SAH hydrolase, the respective abilities to displace [^{3}H]NECA of inhibitors of these enzymes were investigated. However, neither Ro 20-1724 nor IBMX, eritadenine nor adenosine-2′3′-dialdehyde competed for radioligand binding to adenotin 2. NAD, AMP and 2′3′-cAMP were also ineffective.

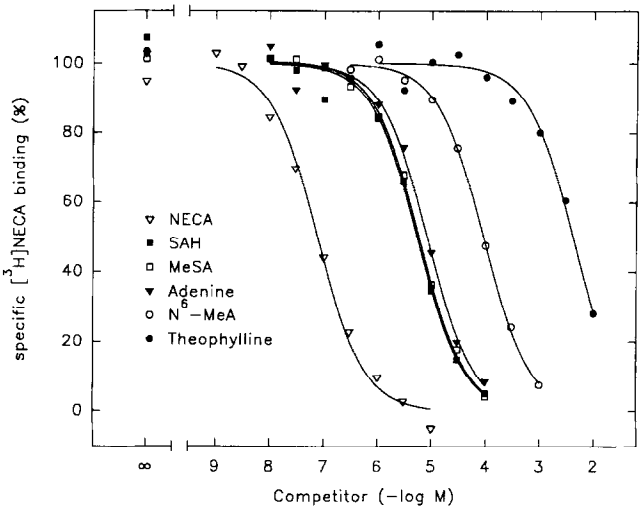

FIG. 4. Inhibition of [^{3}H]NECA binding to membrane-bound adenotin 2 by purine derivatives. Increasing concentrations of NECA (open triangle), SAH (solid square), MeSA (open square), adenine (solid triangle), N^6-methyladenosine (open circle) or theophylline (solid circle) were incubated with 75 μg membrane protein and 20 nM [^{3}H]NECA for 18 hr on ice. Total binding was the binding in the presence of 100 μM 2ClA, and nonspecific binding was determined by addition of 100 μM MeSA. The figure shows the inhibition of specific radioligand binding to adenotin 2 from one typical experiment out of three or four independent experiments.

TABLE 2. Pharmacological profile of adenotin 2 in particulate and cytosolic fractions of rat forebrain

Ligand	K_i (nM) Membranes	K_i (nM) Cytosol
NECA	57	54
3′-5′-cAMP	196	162
5′ClA	3,350	2,390
SAH	4,020	3,850
MeSA	4,800	5,900
Adenine	5,910	8,380
Adenosine	7,060	6,290
N^6-Methyladenosine	56,200	51,800
Theophylline	6,400,000	7,200,000
HeNECA, NECG, GMP, NBTG, NBTI, NECI, 8-bromoadenosine, 6-SH-purine riboside, 6-Cl-purine riboside, CGS 21 680, DPCPX, XAC, eritadenine, ado-2′-3′-dialdehyde	>100,000	>100,000
Guanosine, Ro 20-1724, IBMX, NAD, AMP, 2′-3′-cAMP	>1,000,000	>1,000,000

Seventy-five micrograms of the tissue preparation were incubated with 20 nM [^{3}H]NECA and increasing concentrations of unlabeled compounds for 5 hr (cytosol) or 18 hr (membranes) on ice. Total binding was measured in the presence of 100 μM 2ClA, while nonspecific binding was defined in the presence of 100 μM 2ClA and 100 μM MeSA. Curves were fitted to a one-site model, since fitting to two sites did not improve the quality of the fit. Data are the means of at least three independent experiments.

Thermodynamic parameters for four ligands of adenotin 2 were calculated from equilibrium binding experiments, with rat brain membrane fractions at 0–25°C. [^{3}H]NECA, cAMP, MeSA and adenine were all more potent at lower temperatures (Table 3), pointing to a mainly enthalpy-driven mechanism of ligand binding to adenotin 2. Negative values for equilibrium standard enthalpy ΔH° were found for all four compounds (Table 4), ranging from −36.22 kJ/mol for adenine to −19.06 kJ/mol for [^{3}H]NECA. Equilibrium standard entropy ΔS° was positive for [^{3}H]NECA (66.84 J/mol/K) and cAMP (20.58 J/mol/K), whereas this term was negative for adenine (−33.08 J/mol/K) and MeSA (−14.64 J/mol/K) (Table 4). The scatter plot of standard enthalpy ΔH° versus standard entropy ΔS° (Fig. 5) illustrates that the binding of adenine and MeSA was merely enthalpy driven, whereas both enthalpy and entropy contributed to the binding of cAMP and [^{3}H]NECA. The

TABLE 3. Affinities of adenotin 2 in rat brain membranes for [^{3}H]NECA, cAMP, MeSA and adenine at 0, 10, 20 and 25°C

	Temperature			
	0°C (273°K)	10°C (283°K)	20°C (293°K)	25°C (298°K)
[^{3}H]NECA, K_d (nM)	72.4 (60.3–87.1)	80.6 (60.9–107)	125 (119–131)	141 (114–175)
cAMP, K_i (nM)	196 (142–270)	378 (295–486)	476 (376–601)	619 (407–943)
MeSA, K_i (nM)	4,800 (4,200–5,500)	9,990 (9,160–10,900)	13,400 (12,300–14,700)	16,000 (15,300–16,600)
Adenine, K_i (nM)	5,910 (4,570–7,650)	11,700 (9,570–14,300)	17,100 (14,800–19,600)	24,200 (19,700–29,600)

Samples were incubated for 3 hr (25°C), 4 hr (20°C), 8 hr (10°C) or 18 hr (0°C). K_d and K_i values were calculated from 3–10 independent experiments. Data are geometric means with 95% confidence limits.

relation between the entropic term ($-T\Delta S°$) and standard enthalpy ($\Delta H°$) is described by the equation $-T\Delta S° = -1.77\Delta H° - 54.41$ (for T = 298.15 K; r = 0.98).

The effects of cAMP and MeSA were investigated in more detail with respect to an allosteric or competitive interaction with [^{3}H]NECA binding to adenotin 2. Membrane-associated adenotin 2 was incubated with 2 or 50 nM [^{3}H]NECA, and IC_{50} and K_i values were determined for MeSA and cAMP at these two radioligand concentrations (Table 5). For a competitive mechanism, higher IC_{50} values and identical K_i values were expected for the inhibition of binding of the higher [^{3}H]NECA concentration, and identical IC_{50} values and a lower K_i were expected for an allosteric effect on radioligand binding to adenotin 2. IC_{50} values for cAMP were identical at 2 and 50 nM [^{3}H]NECA (450 nM and 394 nM, respectively) (Table 5), and a significantly lower K_i value was determined at 50 nM [^{3}H]-NECA, pointing to an allosteric mechanism of inhibition by cAMP. In contrast, higher concentrations of MeSA were necessary to displace the radioligand when incubations were performed in the presence of the higher [^{3}H]-NECA concentration. The IC_{50} value of MeSA increased from 5.99 μM at 2 nM [^{3}H]NECA to 9.08 μM at 50 nM [^{3}H]NECA, and the K_i values obtained for this compound were identical (5.78 μM and 4.79 μM). These data support the assumption that cAMP interacts with the radioligand binding to adenotin 2 by an allosteric mechanism, whereas [^{3}H]NECA and MeSA directly compete for the same binding site. A competitive mechanism was also found for adenine (data not shown).

TABLE 4. Thermodynamic parameters for the displacement of [^{3}H]NECA binding from adenotin 2 in rat brain membranes

Ligand	Δ G° (kJ/mol)	Δ H° (kJ/mol)	Δ S° (J/mol/K)
[^{3}H]NECA	−38.99	−19.06	+66.84
cAMP	−35.31	−29.18	+20.58
Adenine	−26.35	−36.22	−33.08
MeSA	−27.17	−31.53	−14.64

Incubations were done at 25°C for 3 hr, 20°C for 4 hr, 10°C for 8 hr and 0°C for 18 hr. Equilibrium standard free energy (ΔG°), standard enthalpy (ΔH°) and standard entropy (ΔS°) were calculated from 3–10 independent experiments at each temperature for each ligand.

Interactions of Adenotin 2 with Protein Kinase Ligands

Because adenotin 2 showed a considerable affinity for cAMP but not for inhibitors for phosphodiesterases and SAH hydrolase, we investigated a possible relationship of adenotin 2 with cyclic nucleotide-dependent protein ki-

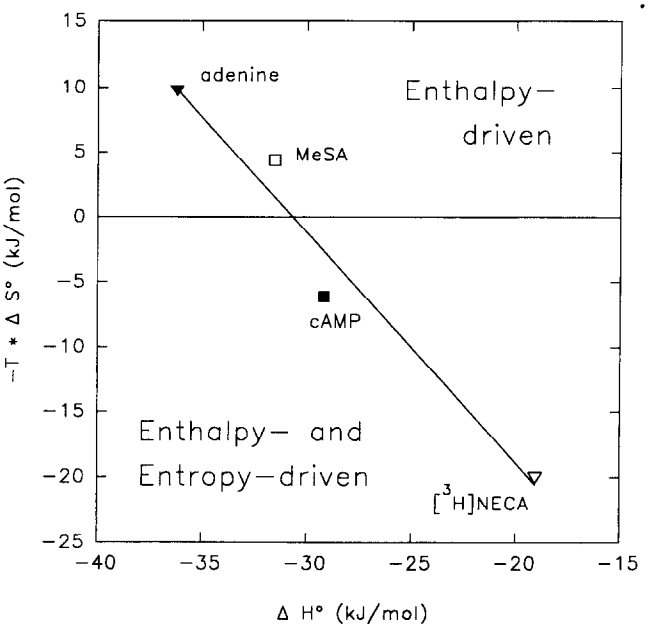

FIG. 5. Scatter plot of $-T\Delta S°$ versus $\Delta H°$ for the binding of [^{3}H]NECA (open triangle), cAMP (solid square), MeSA (open square) and adenine (solid triangle) to adenotin 2 in rat brain membranes. Individual thermodynamic parameters were calculated from equilibrium binding experiments and are given in Table 3. The line in the figure gives the linear regression for the correlation between standard enthalpy, $\Delta H°$, and the entropic term, $-T\Delta S°$ (T = 298 K), which is expressed by the equation $-T\Delta S° = -1.77\Delta H° - 54.41$ (r = 0.98).

TABLE 5. Inhibition of [^{3}H]NECA binding to adenotin 2 in rat brain membranes by cAMP and MeSA by an allosteric or a competitive interaction

	2 nM [^{3}H]NECA		50 nM [^{3}H]NECA	
Ligand	**IC_{50} (nM)**	**K_i (nM)**	**IC_{50} (nM)**	**K_i (nM)**
cAMP	450	434 (324–582)	394	208* (187–231)
MeSA	5990	5780 (4410–7570)	9080	4790 (4120–5560)

* $P < 0.005$ vs. 2 nM [^{3}H]NECA.

Particulate fractions (75 μg protein) were incubated with 2 or 50 nM [^{3}H]NECA and increasing concentrations of unlabeled ligands for 18 hr on ice. IC_{50} and K_i values are means from four to five experiments.

nases and other kinases. Because all kinases require ATP for their enzymatic activities, we tested the influence of the metabolically stable ATP analogue ATP[S] on [^{3}H]NECA binding to adenotin 2. ATP[S] was added to incubations of membrane-associated adenotin 2 in the presence of three different radioligand concentrations (2, 20 and 200 nM [^{3}H]NECA). At all three concentrations of [^{3}H]NECA, the nucleotide showed a biphasic effect on radioligand binding (Fig. 6). The effects of ATP[S] were not due to changes in nonspecific binding because this nucleotide did not influence binding in the presence of 100 μM 2ClA and 100 μM MeSA (data not shown). In concentrations up to 30 μM, ATP[S] enhanced specific radioligand binding to adenotin 2. This effect was most prominent at the lowest concentration of [^{3}H]NECA. Radioligand binding at 10 μM ATP[S] amounted to approximately 280% of control binding in the absence of the nucleotide when the incubation was performed in the presence of 2 nM [^{3}H]NECA. Maximum stimulation at 200 nM [^{3}H]NECA was measured at 30 μM ATP[S] and was approximately 130% of control binding. In higher concentrations of ATP[S], radioligand binding to adenotin 2 was decreased (Fig. 6). At 1 mM ATP[S], the binding of 200 nM [^{3}H]NECA had returned to control levels; in the presence of 20 and 2 nM of the radioligand, the binding was still higher than in the absence of ATP[S].

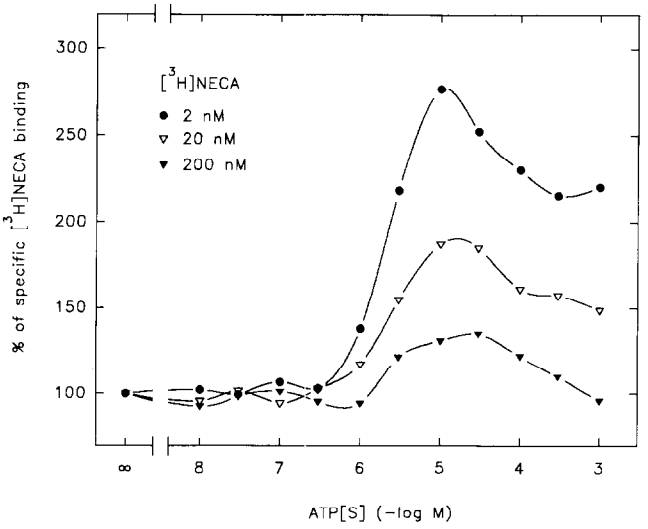

FIG. 6. Influence of ATP[S] on [^{3}H]NECA binding to adenotin 2. Seventy-five micrograms of the particulate fraction from rat brain was incubated with either 2, 20 or 200 nM [^{3}H]NECA and increasing concentrations of ATP[S] on ice for 18 hr. Total binding was defined in the presence of 100 μM 2ClA, and nonspecific binding was the binding in the presence of 100 μM 2ClA and 100 μM MeSA. The figure shows the specific binding to adenotin 2, the binding in the absence of ATP[S] being used as reference (100%).

These effects of ATP[S] might be due to an increase in the affinity of adenotin 2 for [^{3}H]NECA or to an increase in the maximum binding capacity. Therefore, we performed saturation experiments in the presence of 100 μM ATP[S] and compared these results with K_d and B_{max} values under control conditions. ATP[S] did not change the maximum binding capacity of adenotin 2 in either the cytosolic or the membrane fraction of rat brain; however, in both fractions, the affinity for [^{3}H]NECA was enhanced significantly (Fig. 3). In cytosol, the affinity increased fivefold from 45.4 nM to 9.00 nM ($P < 0.005$). A threefold higher affinity for the radioligand was determined in the particulate fraction in the presence of ATP[S] (K_d control: 72.4 nM; 100 μM ATP[S]: 24.1 nM; $P < 0.0001$). Also in the presence of ATP[S], pseudo Hill coefficients (cytosol: 0.99 ± 0.01; membranes: 1.02 ± 0.01) pointed to a single binding site.

The interaction of adenotin 2 with activators and inhibitors of protein kinases was investigated in more detail in the absence and presence of 100 μM ATP[S] (Table 6). Cyclic AMP and H 9 were the most potent competitors and inhibited radioligand binding to both the cytosolic and the particulate fraction in a monophasic manner in the absence of ATP[S]. In the presence of 100 μM ATP[S], however, different effects were observed in membranes and in the cytosol (Fig. 7). Cyclic AMP in concentrations up to 1 μM stimulated [^{3}H]NECA binding to adenotin 2 to 150% of control binding, and higher concentrations of cAMP reduced radioligand binding. In contrast, the competition curve of cAMP in membranes was shifted to the right in the presence of ATP[S], and the affinity of cAMP was decreased approximately 15-fold. Similar to cAMP, the affinity of the metabolically stable activator of protein kinase A, SpcAMPS, was diminished significantly by ATP[S] (Table 6). The K_i value of SpcAMPS was shifted from 249 nM in membranes and in the cytosol to 803 nM and 549 nM, respectively. RpcAMPS (up to 10 μM), the stable inhibitor of protein kinase A, had no effect on [^{3}H]NECA binding in either preparation in the absence or presence of ATP[S]. In concentrations higher than 10 μM, RpcAMPS increased [^{3}H]NECA binding to membrane-associated adenotin 2 in the presence of ATP[S].

The majority of other inhibitors act competitively at the ATP binding site of kinases. K_i values in the high nanomolar or low micromolar range were determined for the

TABLE 6. Interactions of ligands of protein kinases with adenotin 2 in rat brain

	K_i (nM)			
	Membranes		Cytosol	
Ligand	Control	ATP[S] 100 μM	Control	ATP[S] 100 μM
cAMP	196	3,320*	162	increase
SpcAMPS	249	803*	249	549*
RpcAMPS	>10,000	increase	>10,000	>10,000
H 7	2,890	1,010*	2,350	1,780
H 9	130	87	214	87
K 252 a	469	341	326	173*
KT 5720	>10,000	>10,000	>10,000	>10,000
KT 5823	>10,000	>10,000	>10,000	>10,000
KT 5926	>10,000	>10,000	>10,000	>10,000
R 59 949	>100,000	>100,000	>100,000	>100,000
Genistein	794,000	660,000	149,000†	235,000‡
Tamoxifen	increase	increase	increase	increase

* Significantly different from control †54.2%; ‡54.7%.

[^{3}H]NECA (20 nM) binding to adenotin 2 in rat brain cytosolic or particulate fractions was studied in the presence of activators or inhibitors of protein kinases and in the absence or presence of 100 μM ATP[S]. The samples were incubated for 5 hr (cytosol) or 18 hr (membranes) on ice. The K_i values given in the table are geometric means from three or four independent experiments.

nonselective inhibitors H 7 and K 252a, which were in part significantly lower in the presence of 100 μM ATP[S] (Table 6). The isoquinolinesulfonamide H 9, which is relatively selective for cyclic nucleotide-dependent kinases, was the most potent ligand to inhibit [^{3}H]NECA binding within the group of kinase inhibitors or activators. Other kinase inhibitors (KT 5720, KT 5823, KT 5926, R 59949) had no effect on radioligand binding to adenotin 2. Genistein inhibited only 54% of specific [^{3}H]NECA binding in the cytosol. Tamoxifen in concentrations of 100 μM and higher increased radioligand binding in membranes and in the cytosol.

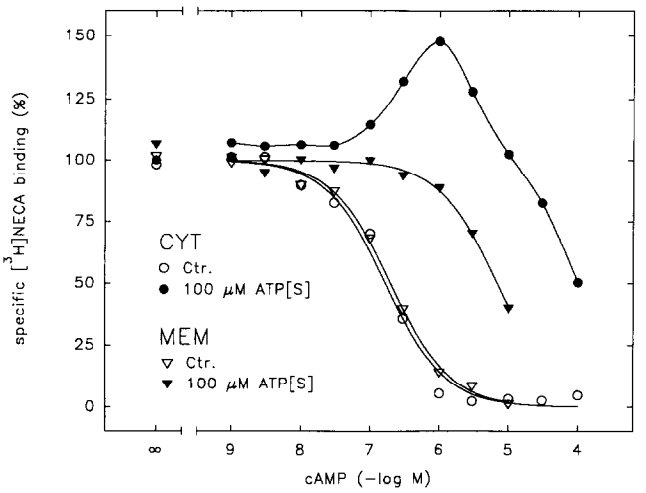

FIG. 7. Influence of cAMP on [^{3}H]NECA binding to adenotin 2. Rat brain cytosolic (circles) or membrane (triangles) fractions (75 μg) were incubated with 20 nM [^{3}H]NECA and increasing concentrations of cAMP in the absence (open symbols) or in the presence (closed symbols) of 100 μM ATP[S] on ice (cytosol: 5 hr; membranes: 18 hr). The figure shows specific radioligand binding from one out of three to four independent experiments.

DISCUSSION

Although most physiological functions of adenosine are attributed to its effects at membrane-bound receptors, intracellular cytoplasmic sites are also relevant, e.g. enzymes such as adenosine kinase or SAH hydrolase. Adenosine stimulates histamine release from mast cells by an as-yet unidentified intracellular adenosine-sensitive mechanism [25, 26]. Adenosine induces constriction of arterioles subsequent to induction of histamine release by interaction with an intracellular site after uptake of adenosine and possibly degradation to inosine [27]. Therefore, because adenosine exerts relevant actions through intracellular sites, cytosolic adenosine binding proteins are of potential physiological and pharmacological interest. The present study describes a novel binding site for adenosine derivatives, which has been named adenotin 2. All tissues investigated contained adenotin 2 in the cytosol and in the particulate fraction (Fig. 1). Because the highest levels of adenotin 2 were found in forebrain, the detailed characterization of this site was done with this material.

Kinetic experiments revealed that [^{3}H]NECA binding to adenotin 2 was a biphasic rather than a monophasic process (Fig. 2, Table 1), suggesting the presence of two different binding sites for the radioligand. In contrast, saturation experiments with [^{3}H]NECA, which yielded monophasic Scatchard plots and pseudo Hill coefficients close to unity, indicated only one class of binding sites. The K_d value from saturation experiments of the membrane-associated site was intermediate between the two kinetic K_d values, which have only a fivefold difference. It seems unlikely that two such

relatively similar affinities could be discriminated by saturation or competition studies. Because K_d values determined from kinetic studies are calculated as the ratio $k_{-1}:k_{+1}$, it is theoretically possible to detect and differentiate two binding sites with identical or very similar K_ds with this method; this is impossible in equilibrium binding experiments. All purine analogues that inhibited radioligand binding to adenotin 2 were equally potent at the membrane-associated and at the soluble cytosolic sites (Table 2). All derivatives showed monophasic displacement curves (Fig. 4), which may be due to the relatively similar or identical affinities of these compounds for the two [^{3}H]NECA binding sites and the limited possibility to discriminate such binding sites in equilibrium binding studies.

Thermodynamic experiments indicated an enthalpy- and entropy-driven mechanism of binding of cAMP and [^{3}H]NECA, whereas binding of adenine and MeSA was merely enthalpy driven. Adenotin 2 ligands were investigated in more detail with respect to a competitive or an allosteric mechanism of inhibition of radioligand binding. Competition experiments at two different [^{3}H]NECA concentrations revealed an allosteric interaction of cAMP with ligand binding to adenotin 2, whereas MeSA showed a competitive mechanism of inhibition. These results confirm that the interactions of MeSA and cAMP with adenotin 2 proceed via distinct mechanisms, which is supported by the different kinetics of [^{3}H]NECA dissociation (Fig. 2). We conclude that cAMP, by an allosteric interaction with adenotin 2, induces a rapid release of the radioligand, whereas MeSA induces a slower dissociation by direct competition at the [^{3}H]NECA binding site.

The biphasic kinetics of [^{3}H]NECA binding, the incomplete dissociation of [^{3}H]NECA from adenotin 2 in the particulate fraction by cAMP and SpcAMPS and the different mechanisms of cAMP and MeSA to inhibit radioligand binding might also be interpreted as the presence of two different proteins. In addition, minor differences between the membrane-associated and the cytosolic forms of adenotin 2 could be attributed to different proportions of the two similar [^{3}H]NECA binding proteins in membranes and cytosol. Evidence for the presence of different proteins came from kinetic experiments but was not supported by saturation and competition data. This hypothesis was ultimately rejected because all competition experiments showed a complete monophasic displacement of the radioligand, also by cAMP, in the particulate fraction and in the cytosol. A part of these sites may become refractory to dissociation by cAMP after prolonged incubation with the radioligand by an unidentified mechanism. The relatively slight differences between membrane-associated and cytosolic adenotin 2 are attributable to the differences in localization because membrane anchoring may affect the conformation of proteins.

The affinity of adenotin 2 for NECA and its tritiated analogue is lower than the affinity of this compound at A_1 receptors [4] and at A_{2a} receptors [28] but higher than at A_{2b} receptors [6], at A_3 receptors [7] or at the low-affinity adenosine binding protein adenotin 1 in human platelets [11] or bovine striatum [8]. For another adenosine binding protein from bovine striatum or bovine cerebral cortex, affinities between 17 nM and 109 nM have been determined [8, 9]. Based on the results of competition experiments, we can exclude identity of adenotin 2 with any known adenosine receptor subtype or the low-affinity adenosine binding protein adenotin 1. Rather, the relatively high affinities of cAMP, SAH and adenine might suggest some relation with SAH hydrolase [29], phosphodiesterases or protein kinases. Eritadenine and adenosine-2′3′-dialdehyde, which act on SAH hydrolase in nanomolar concentrations [29–32], were completely inactive in concentrations up to 100 μM. Moreover, SAH hydrolase binds some purine derivatives, such as 8-bromoadenosine and 6-chloropurine riboside [29], with an affinity of approximately 100 nM, which did not alter [^{3}H]NECA binding to adenotin 2. We conclude that adenotin 2 is not identical to SAH hydrolase. The binding of cAMP by adenotin 2 might also hint at a cyclic nucleotide phosphodiesterase activity. However, the lack of affinities of inhibitors of various phosphodiesterases does not support this notion. The affinity of theophylline at adenotin 2 was 7.2 mM (Table 2), whereas IC_{50} values for inhibition of phosphodiesterase subtypes in human platelets [33, 34] and human lung [35] never exceeded 500 μM. The lack of affinity of two other potent and nonselective inhibitors, IBMX and Ro 20-1724, also argues against identity of adenotin 2 with a cAMP phosphodiesterase [35–37]. Adenotin 2 can also be distinguished from several other adenosine binding proteins. The high-affinity adenosine binding protein A_x binds inosine derivatives and adenine nucleotides with high affinity [8, 9], whereas adenotin 2 displays no affinity to inosine analogues (Table 2). [^{3}H]NECA binding to adenotin 2 is displaced by cAMP but not by AMP. Ravid *et al.* [38] and Bembenek [39] purified adenosine binding proteins of unknown functions from calf brain, which, however, lack an apparent affinity for NECA or adenine.

The observation that cAMP interacted with adenotin 2 hints at a relation of this protein with a cyclic nucleotide-dependent protein kinase. Adenosine and some 5′-substituted adenosine analogues such as 5′ClA or NECA inhibit the phosphorylation of phosphatidylinositol, myosin light chain [40, 41] and troponin I [42]. Although the sensitivity of these effects to adenosine receptor antagonists has not been investigated, the insensitivity of the phosphorylation process to GTP may indicate that the actions of adenosine and 5′ClA were not mediated by adenosine receptors [40].

The metabolically stable ATP[S] in low concentrations enhanced [^{3}H]NECA binding to adenotin 2 (Fig. 6). Because this nucleotide did not compete with the radioligand, we assume that ATP[S] regulates [^{3}H]NECA binding allosterically. ATP[S] increased the affinity of [^{3}H]NECA to the cytosolic and to membrane-associated adenotin 2 (Fig.

3). In contrast, ATP[S] decreased the affinity of cAMP in membranes (Fig. 7). In cytosol, ATP[S] exerted a biphasic effect. The impairment of the inhibitory effect of cAMP in the presence of ATP[S] might be interpreted as a competition between these two adenine nucleotides. Alternatively, ATP[S] might regulate the effectiveness of cAMP allosterically. The stimulation rather than inhibition of [^{3}H]NECA binding by cAMP in the cytosol in the presence of ATP[S] argues against a direct competition between cAMP and ATP[S], at least in low concentrations of cAMP. The metabolically stable cAMP derivatives SpcAMPS and RpcAMPS interact with protein kinase A as a stimulator or an inhibitor of this enzyme, respectively [43], and both modify radioligand binding to adenotin 2 under different conditions (Table 6). Various other inhibitors of protein kinase A, which act at the ATP binding site of the enzyme, affected the interaction between [^{3}H]NECA (Table 6). H 9, which is selective for cyclic nucleotide-dependent protein kinases [44], and the nonselective inhibitor K 252a [45] were the most potent compounds. However, the very selective inhibitors of protein kinase A, KT 5720 [46], and of protein kinase G, KT 5723 [46], did not influence radioligand binding to adenotin 2. Therefore, on the basis of the results with cAMP and ATP analogues, we cannot unequivocally identify adenotin 2 as one of the known protein kinases. The exact relationship between phosphorylation processes and adenotin 2 remains to be clarified. The identity of pharmacological profiles of the inhibition of some phosphorylation processes by adenosine and its derivatives, e.g. phosphorylation of phosphatidylinositol, myosin light chain or troponin I [41–43], suggests a role for adenotin 2 in the regulation of the phosphorylation state of a number of physiologically relevant cellular constituents. Additional experimental evidence, such as the purification of adenotin 2 and the study of possible enzymatic activities, are necessary to understand the nature of this protein.

This study was supported by the European Commission through the BIOMED I concerted action ADEURO.

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