Solubilized Adenosine Receptors in the Brain: Regulation by Guanine Nucleotides

Abstract. *Adenosine receptors associated with a reduction of adenylate cyclase and labeled by tritium-labeled cyclohexyladenosine can be solubilized from brain membranes with sodium cholate. Regulation of receptor binding by guanine nucleotides is retained in the soluble state. Influences of cations observed in membrane preparations of adenosine receptors are no longer detected with the solubilized receptors. The apparent retention of a complex of receptors and guanosine triphosphate binding but not cation binding protein in the soluble state may permit a molecular analysis of receptor regulation.*

In addition to its roles in nucleic acid, adenosine triphosphate (ATP), and general intermediary metabolism, adenosine modulates cellular function in the brain and peripheral tissues through receptors which regulate adenylate cyclase activity (*1*). In the brain adenosine inhibits neuronal firing (*2*), prevents the release of neurotransmitters such as acetylcholine, dopamine, and γ-aminobutyric acid (GABA) (*3*) and thus may function as a neuromodulator or neurotransmitter. The behavioral stimulant effects of caffeine may be mediated by a blockade of adenosine receptors (*4, 5*). At least two distinct adenosine receptors have been described: those associated with a reduction of adenylate cyclase are referred to as A_1 receptors; A_2 receptors mediate augmentations of adenylate cyclase (*6*). Adenosine A_1 receptors can be labeled in brain membranes with ^{3}H-labeled N^6-cyclohexyladenosine (^{3}H-CHA), phenylisopropyladenosine (^{3}H-PIA) and, under some circumstances, 2-chloroadenosine (*7, 8*). In bovine, rat, and rabbit brain, the xanthine adenosine antagonist, ^{3}H-labeled 1,3-diethyl-8-phenylxanthine (^{3}H-DPX), binds to adenosine A_1 receptors, but in guinea pig brain membranes ^{3}H-DPX may label A_2 receptors (*7*).

Many hormone and neurotransmitter receptors, including adenosine receptors (*7, 9*) associated with adenylate cyclase, are regulated by guanine nucleotides, which selectively decrease the affinity of agonists but not antagonists (*10*). These effects are thought to involve a guanosine triphosphate (GTP) binding protein that has guanosine triphosphatase activity and is separate from the receptor recognition protein (*10, 11*). If the receptor-GTP binding protein complex could be maintained intact in solubilized membrane fractions, then molecular interactions of the proteins could be studied more efficiently. We report successful solubilization of adenosine A_1 receptors from bovine brain membranes. Regulation of receptor binding by GTP is retained in the soluble state, but influences of divalent and monovalent cations on the receptor occur in membrane-bound, but not soluble, receptors.

Binding of ^{3}H-CHA to adenosine A_1 receptors is retained by solubilized bovine brain membranes. In typical experiments with 0.5 n*M* ^{3}H-CHA (11.5 Ci/mmole), total binding is about 550 count/min, and nonspecific binding measured in the presence of 10 μ*M* PIA is about 50 count/min, which is similar to measurements from intact brain membranes. Analysis by Scatchard plots of saturation curves (Fig. 1) indicates a single component of ^{3}H-CHA binding to soluble preparations with a dissociation constant (K_d) of 0.44 n*M* and maximal number of binding sites (B_{max}) of 340 fmole per milligram of protein. The K_d value in the soluble state is the same as in the membranes, but the B_{max} value is only about 40 percent of that obtained in parallel experiments with membranes (data not shown). The ^{3}H-CHA binding sites have essentially the same substrate specificity in the solubilized and the membrane-bound states, indicating an association with adenosine A_1 receptors (Table 1). The most potent of these adenosine derivatives, L-PIA, is about 10 times more potent than CHA and almost 100 times as potent as 2-chloroadenosine. The most potent of the methylxanthines, DPX, is somewhat more active than 8-phenyltheophylline and substantially more potent than caffeine and theophylline.

The reduction of ^{3}H-CHA binding by guanine nucleotides is similar in soluble and membrane-bound receptors. At concentrations of 1 to 3 μ*M*, GTP, its nonmetabolized analog 5′-guanylylimidodiphosphate [Gpp(NH)p], and guanosine diphosphate (GDP) inhibit binding to both soluble and particulate receptors by

Table 1. Drug specificity of membrane-bound and solubilized ^{3}H-labeled N^6-cyclohexyladenosine (^{3}H-CHA) binding. To determine concentrations to inhibit specific binding by 50 percent (IC_{50}), five to eight concentrations of each displacer were examined in triplicate. For the membrane-bound assay, the tissue was prepared as described in the legend to Fig. 1 and tested as described in (*7*), with ^{3}H-CHA (1.5 n*M*). Soluble extracts were tested as described in the legend to Fig. 1. The results are the means of three independent determinations which varied by less than 20 percent.

Substance	IC_{50} (n*M*)	
	Membrane bound	Soluble
Nucleosides		
Agonists		
N^6-(L-Phenylisopropyl)adenosine	0.3	0.5
N^6-Cyclohexyladenosine	3	3
2-Chloroadenosine	30	20
N^6-(D-Phenylisopropyl)adenosine	10	7
2-(*p*-Methoxyphenyl)adenosine	3,000	2,000
8-Bromoadenosine	$>10^5$	$>10^5$
Antagonists		
5′-Deoxy-5′-methylthioadenosine	700	400
Purine bases		
1,3-Diethyl-8-phenylxanthine	130	160
8-Phenyltheophylline	250	230
3-Isobutyl-1-methylxanthine	8,000	1,200
Theophylline	25,000	30,000
Caffeine	150,000	130,000
Adenine	$>10^5$	$>10^5$

Table 2. Nucleotide regulation of ^{3}H-CHA binding to solubilized adenosine receptors. Data are the percentage of control specific binding for specific ^{3}H-CHA binding determined in the presence of varying concentrations of nucleotides. The values are the means of three or more determinations, each in triplicate, which varied by less than 15 percent. Binding tests for intact membranes were carried out with membrane preparation as described in the legend to Fig. 1 and assayed as before (*7*). Tests of the soluble receptors were carried out as described in the legend to Fig. 1. App(NH)p, 5′-adenylylimidodiphosphate.

Nucleotide	Binding (percent of control) at molar concentrations			
	10^{-7}	10^{-6}	10^{-5}	10^{-4}
Membranes				
Gpp(NH)p	90	68	45	38
GTP	80	53	36	34
GDP	82	52	37	32
GMP	100	98	94	92
App(NH)p	90	85	95	96
ATP	105	106	100	98
ADP	100	98	96	93
Soluble extract				
Gpp(NH)p	93	60	44	28
GTP	92	82	30	9
GDP	94	62	25	16
GMP	100	104	110	113
App(NH)p	96	96	95	99
ATP	97	97	90	86
ADP	102	100	92	81

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about 50 percent. By contrast, guanosine monophosphate (GMP) and the adenine nucleotides are essentially inactive (Table 2).

Several neurotransmitter receptors are regulated by divalent cations, with interaction of the cation and nucleotide effects. This suggests that the cations might influence the GTP binding protein (*12*). In the membrane state divalent cations augment ^{3}H-CHA binding, with manganese being the most potent and magnesium and calcium similar in activity (*9*) (Table 3). If the divalent cation effects involve the GTP binding protein, influences on soluble adenosine receptors would be expected. However, no increase in ^{3}H-CHA binding is observed over a wide range of manganese, magnesium, and calcium concentrations (Table 3). In fact, at a concentration of 1 m*M*, manganese and magnesium reduce binding, as they do at this concentration at other neurotransmitter receptors (*12*). Moreover, in contrast to their influence on membrane receptors, manganese and magnesium (1 μ*M* to 1 m*M*) do not alter the reduction in ^{3}H-CHA binding to soluble receptors elicited by 10 μ*M* GTP (data not shown).

Table 3. Cation regulation of ^{3}H-CHA binding to solubilized adenosine receptors. Data are the percentage of control specific ^{3}H-CHA binding determined in the presence of varying concentrations of ions. Values are as described in the legend to Table 2.

Ion	Binding (percent of control) at molar concentrations				
	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}
$MnCl_2$	129	130	93		
$MgCl_2$	109	139	152		
$CaCl_2$	108	139	152		
LiCl			95	93	86
NaCl			100	82	72
KCl			96	102	91
$MnCl_2$	98	73	43		
$MgCl_2$	101	99	79		
$CaCl_2$	98	100	98		
LiCl		98	98	102	109
NaCl		97	96	98	105
KCl		100	99	100	105

Some receptors are also regulated selectively by sodium ions. At physiological concentrations sodium reduces the binding of agonists, but not antagonists, to opiate, α-adrenergic, and histamine H_1 receptors; lithium and potassium cause substantially less reduction in binding (*13*). Binding of ^{3}H-CHA to membrane-bound adenosine A_1 receptors is also reduced by sodium. There is about a 30 percent reduction at sodium concentrations of 10 to 100 m*M* (Table 3). Lithium is less potent, and potassium does not reduce receptor binding. As with the divalent cation effect, the influence of sodium on receptor binding is no longer observed when receptors are solubilized.

The regulation of soluble adenosine receptors by guanine nucleotides indicates that the complex between the receptor and the GTP binding protein remains intact throughout the solubilization procedure. This should permit a molecular analysis of the forces that determine the interactions between these proteins. For instance, it may prove feasible to dissociate the two proteins and reunite them.

In contrast, when β-adrenergic (*14*), α-adrenergic (*15*), and histamine H_1 (*16*) receptors are solubilized in the absence of agonist, GTP regulation of ligand binding is lost, although ionic effects may be retained for α-adrenergic (*15*) and histamine H_1 (*16*) receptors. The GTP binding protein interacts strongly with divalent cations and consists of several subunits (*17*). If distinct subunits of the GTP binding protein complex were to bind cations and GTP, then perhaps the solubilized adenosine receptor would retain the GTP but not the cation binding subunits; the reverse might hold for adrenergic receptors. Levitski and Tolkovsky's (*18*) evidence that adenosine receptors are more tightly linked than β-adrenergic receptors to the GTP binding protein might also explain the different behavior of the two solubilized receptors.

Influences of guanine nucleotides have been reported for solubilized opiate receptors (*19*). If opiate receptors are solubilized, the differentiation by guanine nucleotides of agonist and antagonist receptor interactions is lost. Such an effect cannot yet be ascertained for adenosine receptors because of our inability to label soluble receptors with a ^{3}H-labeled antagonist.

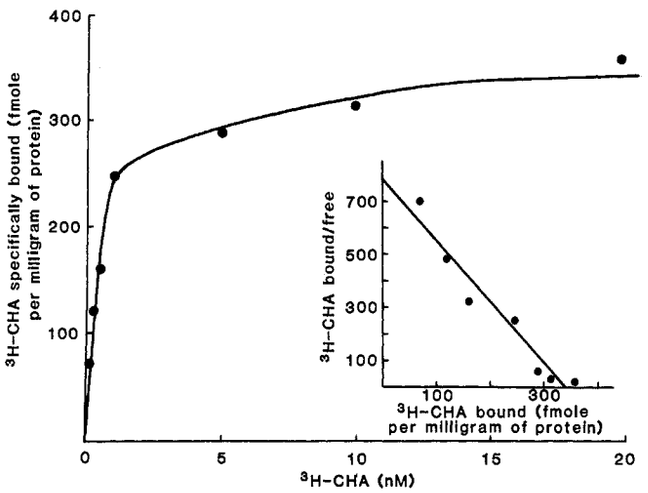

Fig. 1. Saturation analysis of ^{3}H-CHA binding to solubilized bovine brain membranes. Bovine forebrain was disrupted for 30 seconds with a Polytron (setting 5) in 5 volumes (5 ml/g, wet weight) of 50 m*M* tris-HCl buffer (*p*H 7.7) within 2 hours of decapitation. The homogenate was centrifuged at 50,000*g* for 10 minutes, the pellet resuspended in the same buffer, centrifuged, and resuspended in tris containing adenosine deaminase (Sigma, type III, 2 I.U./ml) to destroy endogenous adenosine. After 30 minutes of incubation at 37°C, membranes were centrifuged and resuspended in 5 volumes of tris containing 500 m*M* sodium ethylenediaminetetraacetic acid (EDTA). After 40 minutes of incubation with EDTA at 25°C to chelate tightly bound divalent cations, the homogenate was centrifuged, resuspended in tris, incubated for 10 minutes at 25°C four times with resuspension and recentrifugation, and then final pellets were stored at −70°C until used. This membrane preparation was used to examine ^{3}H-CHA binding to bovine brain membranes. For solubilization, pellets were thawed and homogenized in a glass homogenizer with a Teflon pestle in 5 volumes of tris containing 1 percent cholic acid. The suspension was maintained on ice for 30 minutes, diluted with 200 ml of tris, and centrifuged at 75,000*g* for 90 minutes. The supernatant was used for binding assays. Routine binding assays included 1.3 ml (0.3 mg of protein) of soluble extract, 0.05 ml of ^{3}H-CHA (final concentrations as shown), and 0.15 ml of tris buffer or L-phenylisopropyladenosine (final concentration, 1 μ*M*). The mixture was incubated for 120 minutes at 25°C, then maintained at 0°C, whereupon 0.4 ml of bovine gamma globulin (10 mg/ml) and 0.8 ml of 30 percent polyethylene glycol (molecular weight, ~ 6000; Sigma) were added. Five minutes after the addition of polyethylene glycol, the mixture was filtered on GF/B filters under a vacuum and washed twice with 5 ml of 8.5 percent polyethylene glycol. Filters were placed in vials containing 10 ml of Formula 947 (New England Nuclear), shaken for 60 minutes, and radioactivity was measured by liquid scintillation spectrometry. Specific binding is defined as total binding minus nonspecific binding measured in the presence of 1 μ*M* of L-PIA. The figure shows the direct saturation of the soluble extract by increasing concentrations of ^{3}H-CHA. The results are the means of triplicate determinations. The inset is a Scatchard plot of the data from which the K_d and B_{max} were determined. The experiment was replicated three times.

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Impulse-Coded and Analog Signaling in Single Mechanoreceptor Neurons

Abstract. *Although most sensory neurons convey temporally coded impulses to the central nervous system, certain nonspiking receptors use only graded afferent signals. Each of three large nerve fibers from the lobster oval organ, a mechanoreceptor subserving ventilation, carry both impulses and graded potentials. Thus, both impulse frequency and receptor potential amplitude are available for information transfer.*

In a variety of sense organs, adequate stimuli impinging upon the sensory receptor terminals are transduced into a common type of membrane response, the receptor potential, the characteristics of which have been extensively documented (*1*). It is a graded, nonpropagated potential limited in its electrotonic spread by the passive cable properties of the cell membrane. Usually an electrically responsive region separate from the transduction site converts this analog signal into a frequency-modulated train of nerve impulses, which, in turn, relay the sensory information to the central nervous system.

However, studies on the thoracicocoxal muscle receptor organ (the T-C MRO) and other stretch receptors at the base of the walking legs (*2, 3*), swimmerets, and uropods (*4*) in decapod Crustacea, and on certain invertebrate photoreceptors (*5, 6*) have shown that some sensory neurons lack the usual spike-initiating zone and electrically excitable membrane, but have sufficiently large length constants to permit the electrotonic spread of the receptor potential itself right into the ganglionic neuropil. This "slow" analog signal evidently causes graded release of chemical transmitter at identified synaptic zones, thereby evoking corresponding postsynaptic potentials (*7*) and consequent impulse activity in associated motoneurons (*8*). Several other examples of such graded, nonspiking transmission have recently been found, in both sensory and interneuronal systems (*3, 9*). The experiments reported here show that certain sensory nerve fibers have both large length constants and active spike-supporting membranes and are capable of carrying both the analog and impulse signals over long distances. This raises the possibility that single afferent fibers can use both methods of transmission for conveying sensory information.

The oval organ (*10*) lies within the ventilatory appendage (the second maxilla) of all decapod crustaceans so far studied, and its position and anatomy suggest that it is a proprioceptor capable of monitoring the beat of the gill bailer (the scaphognathite). It consists of a rich arborization of dendrites arising from three afferent fibers of large diameter, supported by a conical array of connective tissue strands which span the region of maximum flexion and extension near the base of the gill bailer. The cell bodies of the three sensory neurons lie centrally in the subesophageal ganglion, adjacent to the motoneurons innervating the ventilatory muscles, as has recently been revealed by cobalt backfills (*11*).

The nerve trunk of an isolated preparation from the lobster (*12*) (Fig. 1) was removed from its sheath and securely pinned to a Sylgard platform with cactus spines, so that controlled stretching of the connective tissue strands of the oval organ caused no movement of the nerve fibers beneath the recording electrodes. The three sensory fibers were readily distinguishable—both from other nerve fibers in the same trunk and from one another—by their large diameter and relative positions; they were labeled X, Y, and Z in descending order of size (*13*). All other fibers were discarded except two motor axons going to a muscle intrinsic to the gill bailer; these two axons (MN1 and MN2 in Fig. 1), whose diameters were similar to those of Y and Z, served as controls.

Intracellular recordings from the individual fibers, made 3 to 8 mm from the oval organ, showed that each of the three sensory fibers, but not the two motor axons, responded to a ramp-and-hold pull stimulus in a characteristic way. Each response comprised, in differing proportions, two distinct components: overshooting action potentials and an underlying graded potential (Fig. 1a). The responses of fiber X had the fewest spikes (one to six) and the largest graded potentials, yielding depolarizations as much as 35 mV from the resting membrane potential in the unstretched state. Fiber Y responded with a sustained, slowly adapting discharge superimposed upon a smaller graded potential. Fiber Z showed the smallest graded potentials but an intermediate degree of spike adaptation.

Addition of tetrodotoxin (TTX) to the saline bathing a preparation undergoing standard pulls at 1-minute intervals produced first a progressive diminution and finally the complete disappearance of all spikes, but left the graded potentials unchanged (Fig. 1b). Thus, while the overshooting action potentials evidently depend on fast sodium channels (as in the majority of known nerve cells), the graded potentials are independent of

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