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PHYLOGENETIC DISTRIBUTION OF [^{3}H]CYCLOHEXYLADENOSINE BINDING SITES IN NERVOUS TISSUE

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SUMMARY. The specific binding of the A_1 adenosine receptor ligand, [^{3}H]CHA, was investigated in membrane fractions prepared from brains of eleven vertebrate species and ganglia of four invertebrate species. Substantial amounts of specific [^{3}H]CHA binding sites were demonstrated in brain membranes of all vertebrate species examined; however, [^{3}H]CHA binding sites were not detectable in nervous tissue of the invertebrate species studied. The densities of [^{3}H]CHA binding sites in vertebrate brains increase in higher vertebrates. Moreover, the pharmacological characteristics of the site labeled by [^{3}H]CHA in two divergent classes of vertebrates were similar. The broad phylogenetic distribution of A_1 adenosine receptors in primitive as well as advanced vertebrate species suggests a fundamental role for adenosine in neuronal modulation.

Adenosine appears to play an important neuromodulatory role in the mammalian central nervous system (CNS), where mechanisms for its synthesis, release, degradation, high-affinity uptake and both pre- and postsynaptic action have been characterized (1,2). The neurophysiologic actions of adenosine are largely inhibitory and appear to involve both presynaptic and postsynaptic sites of action (3,4). It is now well established that many tissues contain at least two classes of membrane associated adenosine receptors which are coupled to adenylate cyclase. The A_1 adenosine receptor has a high affinity for adenosine

Abbreviations: CHA, cyclohexyladenosine; R-PIA, R-phenylisopropyladenosine; S-PIA, S-phenylisopropyladenosine; NECA, N-ethylcarboxamidoadenosine; 2-ClAdo, 2-Chloroadenosine.

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(nM) and is negatively coupled to adenylate cyclase, whereas the A_2 adenosine receptor has a lower affinity for adenosine (μM) and is positively coupled to adenylate cyclase (5,6). Although the precise mechanism by which adenosine depresses neuronal activity is presently unknown, in the rat hippocampus it appears to exert its effects via an activation of A_1 receptors (7).

Recently it has been possible to label A_1 adenosine receptors in brain membranes using radioactive adenosine analogues such as [^{3}H]CHA (8). The binding of this adenosine analogues has been investigated in brain membranes from mouse, rat, guinea pig and cow (9). In all cases, the data and profiles of inhibition of binding with adenosine analogues are consistent with [^{3}H]CHA binding to an A_1 adenosine receptor. An evaluation of the phylogenetic distribution of A_1 adenosine receptors should provide insight into the relative importance of adenosine as a modulator of neuronal activity throughout the animal kingdom. We now report that specific [^{3}H]CHA binding sites are present in brain membranes of a wide range of vertebrate species but were not detectable in nervous tissue of the invertebrate species examined.

METHODS

Materials. The [adenine-2,8-^{3}H]CHA (13.5 Ci/mmole) was obtained from New England Nuclear Corporation (Boston, Mass.); (-)-N^6(R-phenylisopropyl)-adenosine (R-PIA) and (+)-N^6(S-phenylisopropyl)-adenosine(S-PIA) were obtained from Research Biochemicals Incorporated (Wayland, Mass.); 5'(N-ethyl)-carboxamido-adenosine (NECA) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); and 2-chloroadenosine (2-ClAdo) was obtained from Sigma Chemical Company (St. Louis, MO). Unless otherwise stated, other materials were from standard commercial sources. The squid Moroteuthis robusta and Taonius pavo were taken with a 10 ft Isaacs-Kidd midwater trawl, and the hagfish Eptatretus deani, the ray, Raja rhina, and the bony fishes Sebastes alutus (Pacific perch), Sebastolobus altivelis and Sebastolobus alascanus were taken by otter trawl at their typical depths of abundance on two cruises of the R/V Wecoma off the coast of Oregon. Nervous tissues were dissected and frozen in liquid nitrogen at sea until transportation to the laboratory where they were stored at -80°C until used. Dungeness crabs (Cancer magister) were collected locally in Yaquina Bay and maintained in running sea water for up to 1 week prior to use. Aplysia californica were purchased from Pacific Biomarine (Venice, CA). Frozen brains of guinea pig (Cavia porcellus), chicken (Gallus domesticus) and cow (Bos taurus) were purchased from Pel-Freez Biologicals (Rogers, AR). Male Sprague-Dawley rats (Rattus rattus) and Swiss-Webster mice (Mus musculus) were purchased from Simonsen Labs. (Gilroy, CA), and bull frogs (Rana catesbeiana) were purchased from Carolina Biological Supply.

Methods. Vertebrate brains or invertebrate ganglia were dissected free from non-nervous tissue and immediately frozen on dry ice or in liquid nitrogen prior to storage at -80°C. On the day of the experiment frozen brain or ganglia

were thawed and then homogenized in 10-100 volumes of 50 mM Tris-HCl buffer (pH 7.7) with a Brinkmann Polytron (setting 6, 20 sec). The resulting homogenates were centrifuged at 48,000 x g for 10 min at 4°C and the supernatants discarded. The pellets were washed by resuspension in identical volumes of Tris buffer using a Polytron and recentrifuged at 48,000 x g for 10 min. The membranes were then resuspended as above in an appropriate volume of Tris buffer and incubated with adenosine deaminase (2.0 IU/ml) (Sigma, Type VI) at 22°C for 30 min. The homogenates were then recentrifuged at 48,000 x g for 10 min and the resulting pellets resuspended in an appropriate volume of Tris buffer containing 5 mM $MgSO_4$. Aliquots of the homogenate were then used immediately in receptor binding assays. The specific binding of [^{3}H]CHA to neuronal membranes was determined using a previously described (8,10) rapid filtration assay with minor modifications. Aliquots of the membrane preparations (100-900 µl depending on the species) were incubated in triplicate with 50 µl of [^{3}H]CHA and 50 µl of buffer or competing drugs as indicated in a total incubation volume of 1 ml. Nonspecific binding was determined in parallel triplicate assay tubes with 2 µM R-PIA. Incubations were terminated by vacuum filtration through Whatman GF/B glass-fiber filters using a Brandel M-24R Cell Harvester (Brandel Instruments, Gaithersburg, MD) followed by four x 4 ml washes of ice-cold Tris buffer. Filter-bound radioactivity was measured by liquid scintillation spectrometry at 43% efficiency following overnight extraction at room temperature. Saturation binding data were analyzed by Scatchard analysis using concentrations of [^{3}H]CHA ranging from 0.25 to 12 nM. In competition experiments the IC_{50} values were derived from logit-log plots of competition data, and the corresponding dissociation constants (K_I) calculated as described by Cheng and Prusoff (11). The protein content of membrane preparations was determined by the method of Lowry et al. (12) following solubilization of the samples in 0.5 N NaOH.

RESULTS

A significant amount of specific [^{3}H]CHA binding was detected in brain membranes of all vertebrate species examined. In each species [^{3}H]CHA bound saturably and with high affinity as previously reported for several mammalian species. Scatchard analysis of saturation data indicated a single class of specific [^{3}H]CHA binding sites in all vertebrate brains investigated. As shown in Table 1, there appeared to be an evolutionary trend for increasing Bmax values in phylogenetically more advanced species. Thus, the density of A_1 adenosine receptors as labeled by [^{3}H]CHA ranged from 3 fmol/mg protein in hagfish brain membranes to 542 fmol/mg protein in bovine cortical membranes. In contrast to the substantial levels of specific [^{3}H]CHA binding in vertebrate brain membranes, there were no detectable binding sites for [^{3}H]CHA in nervous tissue of any of the invertebrate species examined (Table 1).

In addition to the evolutionary trend with regard to [^{3}H]CHA binding sites in vertebrate brains, it appeared that primitive vertebrates such as the hagfish (Class Agnatha) and the skate (Class Chondrichthyes) had affinity constants (K_D =

Table 1. Phylogenetic distribution of A_1 adenosine receptors. Tissues were prepared and assayed as described in text. The apparent K_D values and densities (B_{max}) ± S.E. were derived by linear regression of Scatchard plots of saturation isotherms. The [^{3}H]CHA binding isotherms were replicated 3 times for each species. Values for rat (Rattus rattus) and cow (Bos taurus) were obtained from cortex, while for all other vertebrate species whole brain was used.

	K_D (nM)	B_{max} (fmol bound/mg protein)
PHYLUM MOLLUSCA		
Class Decapoda		
Moroteuthis robusta	-	< 2
Taonius pavo	-	< 2
Class Gastropoda		
Aplysia californica	-	< 2
PHYLUM ARTHROPODA		
Subphylum Crustacea		
Class Malacostraca		
Cancer magister	-	< 2
PHYLUM CHORDATA		
Subphylum Vertebrata		
Class Agnatha		
Eptatretus deani	9.08 ± 2.30	3.0 ± 0.4
Class Chondrichthyes		
Raja Rhina	7.02 ± 1.45	30.5 ± 13.6
Class Osteichthyes		
Sebastes alutus	1.11 ± 0.011	175.4 ± 2.1
Sebastolobus alascanus	1.67 ± 0.07	212.5 ± 5.8
Sebastolobus altivelis	2.35 ± 0.09	186.8 ± 1.3
Class Amphibia		
Rana catesbeiana	2.38 ± 0.34	42.1 ± 3.0
Class Aves		
Gallus domesticus	3.19 ± 0.09	356.9 ± 7.0
Class Mammalia		
Cavia porcellus	3.85 ± 0.07	580.4 ± 10.5
Mus musculus	1.63 ± 0.08	317.6 ± 13.7
Rattus rattus	1.24 ± 0.08	335.3 ± 9.4
Bos taurus	0.45 ± 0.02	542.2 ± 3.0

9.08 and 7.02 nM respectively) which were 14 to 18-fold higher than that of cow brain membranes (K_D = 0.45 nM). Other mammalian species and the other classes of vertebrates displayed intermediate K_D values ranging from 1.11 to 3.85 nM. Representative saturation isotherms for a lower vertebrate, Sebastolobus altivelis (Class Osteichthyes), and an advanced vertebrate, Bos taurus (Class Mammalia), are depicted in Figures 1 and 2. To determine whether the pharmacological characteristics of the [^{3}H]CHA binding sites from a lower vertebrate such as the fish were similar to those of the mammalian receptor (cow), we compared the potencies of 4 selected adenosine analogues as inhibitors of specific [^{3}H]CHA binding in the two species. In both species the drug potencies as inhibitors of [^{3}H]CHA binding reflected labeling of A_1 adenosine receptors. Thus, in both species the absolute affinities for R-PIA, 2-ClAdo, NECA and S-PIA were in the

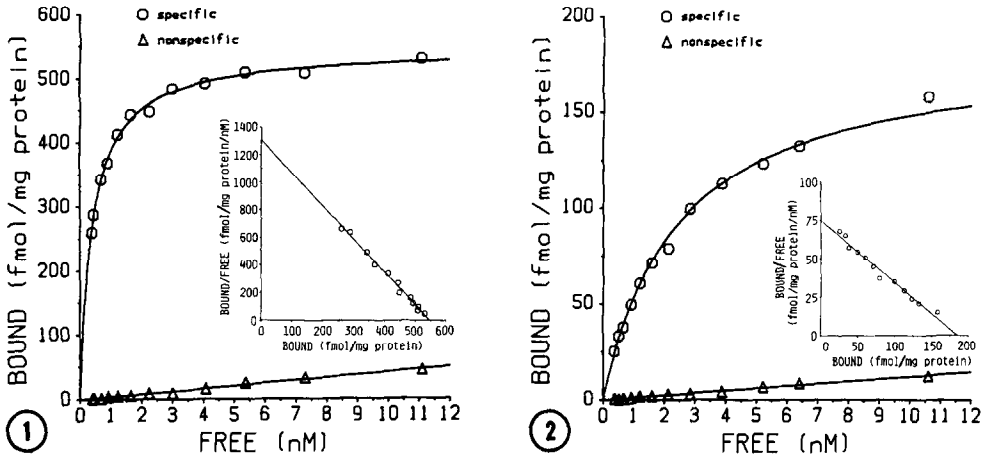

Fig. 1. Equilibrium saturation binding of [^{3}H]CHA to cow brain membranes. Membranes were incubated with 12 concentrations of [^{3}H]CHA ranging from 0.27 to 11.2 nM. Specific binding is defined as the total binding minus the amount bound in the presence of 2μM R-PIA. Values are from a single experiment which was replicated three times. The inset is a Scatchard replot of saturation binding data. In this representative experiment the K_d value for [^{3}H]CHA was 0.42 nM, and the B_{max} value was 546.4 fmol/mg protein. The r^2 value for the regression of the Scatchard plot is 0.984.

Fig. 2. Equilibrium saturation binding of [^{3}H]CHA to fish (S. altivelis) brain membranes. Membranes were incubated with 12 concentrations of [^{3}H]CHA ranging from 0.25 to 10.6 nM. Specific binding is defined as the total binding minus the amount bound in the presence of 2μM R-PIA. Values are from a single experiment which was replicated three times. The inset is a Scatchard replot of saturation binding data. In this representative experiment the K_d value for [^{3}H]CHA was 2.47 nM, and the B_{max} value was 185.0 fmol/mg protein. The r^2 value for the regression of the Scatchard plot is 0.971.

nanomolar range which is consistent with the previously reported potencies of these adenosine analogues as inhibitors of adenylate cyclase in brain membranes (13). In addition, in membranes derived from both fish and cow brain, R-PIA was more potent than NECA which is characteristic of A_1 but not A_2 receptors (14) (Table 2 and Fig. 3). The R-diastereomer of PIA was 8.3 times more potent than

Table 2. Relative potencies of adenosoine analogs as inhibitors of [^{3}H]CHA binding. IC_{50} values were determined using 1-2 nM [^{3}H]CHA and six concentrations of each analogue as shown in Fig. 3. K_i values were calculated using the Cheng-Prusoff equation (11). Values presented are means ± standard error of the mean of four separate experiments for each analogue in both species.

Compound	K_i (nM)	
	Fish	Cow
R-PIA	1.17 ± 0.28	0.12 ± 0.05
2-ClAdo	2.94 ± 0.62	4.33 ± 1.41
NECA	5.78 ± 2.67	5.74 ± 2.51
S-PIA	9.76 ± 1.01	1.87 ± 1.07

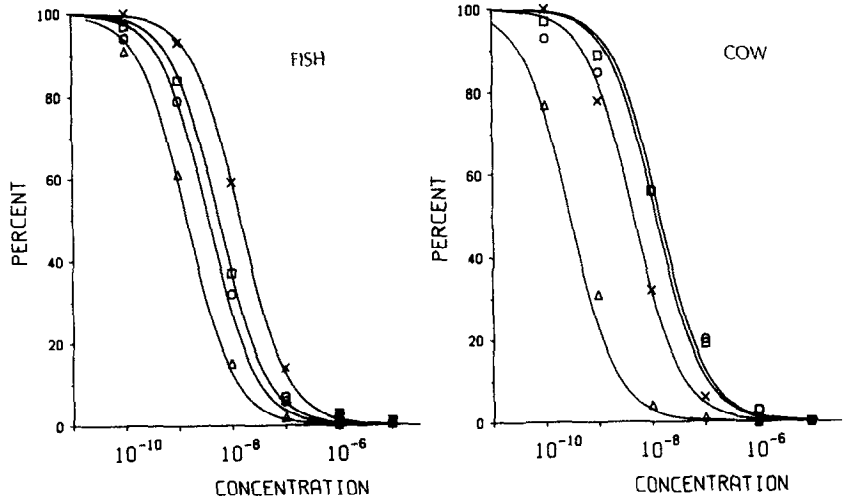

Fig. 3. Inhibition of specific [^{3}H]CHA binding in fish and cow brain membranes by adenosine analogues. R-PIA (Δ), 2-ClAdo(O), NECA (□), and S-PIA (X). Six concentrations of each analogue were incubated with aliquots of membrane homogenates and [^{3}H]CHA (1.2 nM) for 120 min at 25°C. Each point represents the mean percent inhibition of triplicate samples determined in a single experiment. These experiments were repeated three times with similar results. The curves were drawn by computer assuming, ideal, noncooperative binding described by the equation [^{3}H]CHA Bound = $B_{max}F/[F+K_D(1+I/K_i)]$, where F is the concentration of free [^{3}H]CHA, K_D is the dissociation constant for [^{3}H]CHA in a given species, I is the concentration of competing analogue, and K_i is the dissociation constant for competing analogue. The K_i values were determined using the IC_{50}'s derived from logit-log plots of data as described (11). The K_i values for each adenosine analogue in both the fish brain (Sebastolobus altivelis) and cow brain (Bos taurus) are given in Table 2.

S-PIA in fish brain membranes and 15.6 times more potent in cow brain membranes (Table 2). The magnitude of the stereoselective inhibition of [^{3}H]CHA binding by R- and S-PIA observed in cow brain membranes is in agreement with previous reports in calf brain (15) and is characteristic of A_1 adenosine receptors. The rank order potencies for the adenosine analogues were similar in both species; however, the isomers of PIA exhibited higher affinities for the cow brain binding site compared to that of fish brain.

DISCUSSION

The results of the present study indicate that the adenosine receptor agonist [^{3}H]CHA binds with a high affinity to a single population of specific sites in brain membranes of six classes of vertebrates. A detailed comparison of the pharmacological characteristics of specific [^{3}H]CHA binding in fish and cow brain membranes revealed that adenosine analogues displayed nanomolar affinities for the

[^{3}H]CHA recognition site with stereoselectivity for the isomers of PIA. These characteristics are consistent with the labeling of A_1 adenosine receptors in both a primitive and phylogenetically more advanced vertebrate nervous system. The presence of substantial amounts of specific [^{3}H]CHA binding sites in the hagfish, the most primitive vertebrate species, suggests a functional role for adenosine throughout the course of vertebrate evolution. In contrast, our inability to detect specific binding sites for [^{3}H]CHA in nervous tissue from 4 invertebrate species argues against the physiological relevance of adenosine as a neuromodulator in these species. Alternatively, it is conceivable that the absence of [^{3}H]CHA binding sites in invertebrate nervous tissue is related to their adenosine receptors possessing unique pharmacological characteristics.

Our results on the phylogenetic distribution of A_1 adenosine receptors are in accordance with previous reports concerning the evolutionary appearance of opiate receptors (16) and benzodiazepine receptors (17). In both cases invertebrate nervous tissue was found to be devoid of receptors, while, with the exception of benzodiazepine receptors in hagfish brain, all vertebrate brains investigated possessed substantial amounts of opiate and benzodiazepine receptor binding. These findings suggest that the evolutionary appearance of A_1 adenosine, opiate and benzodiazepine receptors was associated with the development of the vertebrate nervous system. The late evolutionary development of A_1 adenosine receptors implies that the physiologic function of these recognition sites is more specialized than that of neurotransmitter receptors such as muscarinic cholinergic receptors. Considerable evidence indicates that muscarinic receptors have a broader phylogenetic distribution including invertebrates such as molluscs, annelids and arthropods (18,20). Hence, the delayed phylogenetic development of A_1 adenosine receptors suggests that their appearance may be related to adenosine's neuromodulation of an increasingly complex nervous system.

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