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Comparison of the binding properties of A_1 adenosine receptors in brain membranes of two congeneric marine fishes living at different depths

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Summary. The binding properties of A_1 adenosine receptors in brain membranes were compared in two congeneric marine teleost fishes which differ in their depths of distribution. Adenosine receptors were labeled using the A_1 selective radioligand [^{3}H]cyclohexyladenosine ([^{3}H]CHA). The A_1 receptor agonist [^{3}H]CHA bound saturably, reversibly and with high affinity to brain membranes prepared from *Sebastolobus altivelis* and *S. alascanus*; however, the mean K_d values differed significantly (Figs. 1–3, Table 1). Saturation data fit to a one site model indicated that the A_1 receptor in *S. alascanus* exhibited a higher affinity ($K_d = 1.49$ n*M*) for [^{3}H]CHA whereas A_1 receptors in *S. altivelis* exhibited a significantly lower affinity ($K_d = 3.1$ n*M*). Moreover, *S. altivelis*, but not *S. alascanus*, parameter estimates for [^{3}H]CHA binding to two sites of receptor were obtained (Fig. 3, Table 1). The mean dissociation constant values for the high and low affinity sites for [^{3}H]CHA in *S. altivelis* were 0.43 n*M* and 16.3 n*M*, respectively. In equilibrium competition experiments the adenosine analogs R-phenylisopropyladenosine (R-PIA), N-ethylcarboxamidoadenosine (NECA) and S-phenylisopropyladenosine (S-PIA) all displayed higher affinities for A_1 receptors in *S. alascanus* as compared to *S. altivelis* brain membranes (Table 2, Fig. 6). The specific binding of [^{3}H]CHA was significantly increased by 0.1 and 1.0 m*M* $MgCl_2$ in both fishes; however, the sensitivity (95–131% increase) of *S. altivelis* to this effect was significantly greater than that of *S. alascanus* (48–91% increase) (Fig. 5). The results of kinetic, equilibrium saturation and equilibrium competition experiments all suggest that A_1 adenosine receptors of *S. altivelis* and *S. alascanus* brain membranes differ with respect to their affinities for selected adenosine agonists.

Abbreviations: CHA cyclohexyladenosine; *R-PIA* R-phenylisopropyladenosine; *S-PIA* S-phenylisopropyladenosine; *NECA* N-ethylcarboxamidoadenosine; *NEM* N-ethylmaleimide; *2-ClAdo* 2-chloroadenosine; *GTP* guanosine triphosphate; *N protein* guanine nucleotide binding protein; n_H Hill slope

Introduction

High hydrostatic pressure is an important component of the marine environment which may disrupt the biochemical functions of organisms colonizing the deep ocean (Somero et al. 1983). Comparisons of cytoplasmic proteins of deep- and shallow-living fishes have identified some of the loci and types of adaptations required for the deep-sea environment (e.g., Somero et al. 1983, 1987; Siebenaller 1986). Few studies (e.g., Moon 1975; Pfeiler 1978), however, have examined membrane-associated proteins, although the disruptive effects of pressure on membranes are potentially of great consequence (Chong and Cossins 1983; Macdonald 1984). We have undertaken a comparison of the A_1 adenosine receptor in brain membranes of two congeneric teleost fishes which experience similar habitat temperatures, but differ in their depths of distribution. These two species have been used as a model system with which to identify and study pressure-related adaptations (e.g., Siebenaller 1978, 1984a, b, c, 1987; Siebenaller and Somero 1978, 1979, 1982; Somero and Siebenaller 1979; Somero et al. 1983, 1986). In the eastern North Pacific *Sebastolobus alascanus* commonly occurs from 180–440 m and *S. altivelis* from 550–1300 m (Siebenaller 1978). The purpose of our comparison was to characterize a membrane-associated system in a shallow- and a deep-adapted species in order to identify possible loci of adaptation to pressure.

Adenosine regulates numerous physiological processes including platelet aggregation, lipolysis,

coronary vasodilation, and neuronal function in the central nervous system (Burnstock and Brown 1981; Phillis and Wu 1981; Snyder 1985). In the brain the neurophysiologic actions of adenosine are largely inhibitory and appear to involve both presynaptic and postsynaptic sites of action (Fredholm and Hedqvist 1980; Lee et al. 1984). Adenosine influences adenylate cyclase activity via an interaction with at least two distinct membrane-associated receptors. The A_1 adenosine receptor mediates an inhibition of adenylate cyclase, while at A_2 receptors adenosine stimulates adenylate cyclase (Daly et al. 1981). These receptor subtypes have been defined further by their structure-activity profiles of agonists (Daly 1983; Wolff et al. 1981; Stone 1985). At A_1 adenosine receptors the rank order potency of adenosine analogs is N^6-(R-phenylisopropyladenosine (R-PIA) > N-ethylcarboxamidoadenosine (NECA) ≥ 2-chloroadenosine (2ClAdo) > S-PIA. In contrast at A_2 adenosine receptors the potency series for these agonists is NECA > 2ClAdo > R-PIA > S-PIA (Daly 1983; Stone 1985). Adenosine analogs acting on A_1 receptors have been shown to inhibit adenylate cyclase in brain tissue (Cooper et al. 1980), and the adenosine-induced depression of neuronal activity in the rat hippocampus has been shown to involve an interaction with A_1 receptors (Reddington et al. 1982).

The use of radioligands has recently permitted the direct labeling of A_1 adenosine receptors in brain (Bruns et al. 1980), testes (Murphy and Snyder 1981), fat cells (Trost and Schwabe 1981) and heart (Hosey et al. 1984). In these studies tritiated agonist radioligands were demonstrated to label a recognition site with A_1 adenosine receptor selectively. Analogous to numerous other neurotransmitter receptors which are coupled to adenylate cyclase, A_1 adenosine receptors in rat brain can exist in two affinity states for agonists (Lohse et al. 1984). Using the adenosine agonist [^{3}H]cyclohexyladenosine ([^{3}H]CHA) as a radioligand, A_1 adenosine receptors have been shown to have a broad phylogenetic distribution in brains of both primitive and advanced vertebrates (Siebenaller and Murray 1986). The presence of substantial amounts of specific [^{3}H]CHA binding in the brain of primitive vertebrate species such as the hagfish suggested a functional role for adenosine throughout the course of vertebrate evolution.

In the present study we have compared the binding properties of A_1 adenosine receptors in brain membranes of two congeneric marine fishes which experience similar habitat temperatures but differ in their depths of abundance. Agonist interaction with A_1 adenosine receptors has been examined using radioligand binding techniques coupled with computer-assisted data analysis. The rationale for the selection of [^{3}H]CHA as a radioligand probe to characterize the binding properties of A_1 receptors in the two species was based on previous demonstrations of the importance of membrane fluidity in the modulation of agonist interaction between guanine nucleotide binding proteins and the beta-adrenergic receptor (Briggs and Lefkowitz 1980). In contrast, alterations of membrane fluidity had no effect on receptor concentration or the affinity of beta-adrenergic receptors for antagonists such as [^{3}H]dihydroalprenolol (Briggs and Lefkowitz 1980). Thus, the binding of agonists to A_1 adenosine receptors may serve as a model for investigating potential pressure-related adaptations of membrane receptor interaction with guanine nucleotide regulatory proteins and associated effector elements. The results of our studies suggest that agonist interactions with A_1 adenosine receptors in the two species have different affinity characteristics and sensitivities to Mg^{2+} regulation.

Materials and methods

Specimens. Demersal adult *Sebastolobus altivelis* and *S. alascanus* (Scorpaenidae) were taken by otter trawl at their typical depths of abundance on two cruises of the R/V *Wecoma* off the coast of Oregon. Brains were dissected and frozen in liquid nitrogen at sea and transported to the laboratory where they were stored at −80 °C until used.

Chemicals. The radiolabeled [adenine-2,8-^{3}H]CHA (27 Ci/mmol) was purchased from DuPont NEN (Boston, MA). Unlabeled CHA, the R- and S-diastereomers of PIA and NECA were obtained from Research Biochemicals, Inc. (Wayland, MA). Adenosine deaminase (Sigma, Type VI), GTP, N-ethylmaleimide, 2-ClAdo and all other chemicals used were from Sigma Chemical Company (St. Louis, MO).

Preparation of fish brain membranes. Each day's experiment used membranes from both species in parallel. On the day of the experiment, frozen brains were thawed and homogenized using a Tekmar Tissumizer (setting 70 for 20 sec) in 40 volumes of 50 m*M* Tris-HCl buffer (pH 7.6 at 22 °C) containing 1 m*M* EDTA. The resulting homogenates were centrifuged at 48000 *g* for 10 min at 4 °C and the supernatants discarded. The pellets were resuspended in an identical volume of ice-cold 50 m*M* Tris-HCl, 1 m*M* EDTA with a Tekmar Tissumizer as described above. The homogenate was then recentrifuged at 48000 *g* for 10 min and the resultant pellet resuspended in 40 volumes of 50 m*M* Tris-HCl, 1 m*M* EDTA buffer containing 2 IU of adenosine deaminase (Sigma Type VI) per ml. The homogenate was then incubated at 22 °C for 30 min to remove endogenous adenosine (Bruns et al. 1980). Following this incubation, the homogenate was cooled on ice and then recentrifuged as described above. The supernatant was discarded and the final pellet was resuspended in 40 volumes of ice-cold 50 m*M* Tris-HCl buffer with a Tekmar Tissumizer. This suspension was kept on ice until used in the radioligand binding assay.

Binding assay for membrane bound A_1 adenosine receptors. The specific binding of the A_1-selective ligand [^{3}H]CHA to brain membranes was determined using a previously described rapid filtration assay with minor modifications (Bruns et al. 1980; Murray and Cheney 1982). Aliquots (900 µl) of the brain membrane preparations (300–600 µg of protein) were incubated with 50 µl of [^{3}H]CHA and 50 µl of Tris buffer or competing compounds, as indicated in the text, in a total volume of 1 ml. Samples were routinely incubated for 60 min at 22 °C in a water bath as this time was found to be sufficient for the binding reaction to reach equilibrium. The selection of this incubation temperature was dictated by previous demonstrations that agonist binding to A_1 adenosine receptors is entropy-driven and [^{3}H]CHA has a higher affinity at higher temperatures (Murphy and Snyder 1982). The binding reactions were terminated by filtration of the assay tube contents over Whatman GF/B glass fiber filter strips using a Brandel cell harvester (model M-24R; Brandel Instruments, Gaithersburg, MD) under vacuum. Filters were then rinsed with four × 4 -ml washes of ice-cold Tris-HCl buffer to remove unbound radioactivity. Filter disks were placed into counting vials to which was added 8 ml of ACS II (Amersham, Arlington Heights, IL). Filter-bound radioactivity was determined by liquid scintillation spectrometry (Beckman model LS8000) at an efficiency of 53% following overnight extraction at room temperature. The amount of radioligand bound was less than 10% of the total added ligand in all experiments. Specific binding was defined as total binding minus binding occurring in the presence of 30 µ*M* R-PIA, and represented 85 to 90% of the total binding at the K_d values for [^{3}H]CHA in both species.

Analysis of binding data. The estimates for the kinetic parameters for the time course of association and dissociation of [^{3}H]CHA specific binding were obtained using the equations described by Weiland and Molinoff (1981). The association rate constants for [^{3}H]CHA were calculated using linear least-squares regression analyses of pseudo first-order transformations of the binding data as described in the text. The [^{3}H]CHA dissociation rate constants were calculated by linear least-squares regression analysis of the logarithmic tranformation of binding data as described in the text. In saturation experiments [^{3}H]CHA was used in concentrations ranging from 0.08 to 24 n*M*. Analysis of agonist interactions with A_1 adenosine receptors have demonstrated that the A_1 receptor can exist in two affinity states for agonists (Lohse et al. 1984; Ukena et al. 1984). The saturation binding of the adenosine agonist [^{3}H]CHA was therefore analyzed using the iterative public procedure FITFUN on the PROPHET system assuming both a one-site and a two-site model. Individual saturation isotherms were analyzed by nonlinear regression analysis assuming either ligand binding to a single receptor site or binding to two independent species of receptor. These analyses assume that the ligand interactions with one or two species of receptor conform to mass action principles as follows:

One site:

$$B=\frac{[R]\,[L]}{[L]+K_D}$$

Two sites:

$$B=\frac{[R_H]\,[L]}{[L]+K_H}+\frac{[R_L]\,[L]}{[L]+K_L}$$

where B (fmol/mg protein) is the amount of specifically bound [^{3}H]CHA; R, R_H and R_L (fmol/mg protein) are the maximum number of binding sites in either the one- or two-side model; [L] is the concentration of free radioligand; and K_D, K_H and K_L are the respective equilibrium dissociation constants for individual receptor species in the two models. It is assumed in the two-site model that K_H is the equilibrium dissociation constant for the formation of the high-affinity complex and K_L is the equilibrium constant for the formation of the low-affinity complex. Binding to a single site was assumed unless the more complex two-site model better accounted for the experimental data. Data are described as better fit by one model of ligand binding than another when a partial F-test comparing the two models indicated significant improvement in residual sum of squares ($P<0.01$) as described by Hoyer et al. (1984). The F-value for comparing the two models are calculated from an analysis of residuals according to the following equation:

$$F=\frac{(SS_1-SS_2)/(df_1-df_2)}{SS_2/df_2}$$

where SS_1 and SS_2 are residual sums of squares with corresponding degrees of freedom df_1 and df_2 associated with the simpler and more complex model respectively. F-values were calculated using (df_1–df_2) degrees of freedom in the numerator and df_2 degrees of freedom in the denominator (Hoyer et al. 1984). Although a ternary complex model provides a mechanistically more correct quantitative description of agonist-receptor-N protein interactions, the two-independent-site model described above can be used to make tentative conclusions concerning multiple agonist affinity states of a receptor (e.g., Contreras et al. 1986). Parameter estimates for one- and two-site models derived using the Lundon-1 iterative curve-fitting program (Lundeen and Gordon 1985) provided virtually identical results to those obtained using FITFUN on the PROPHET system.

In [^{3}H]CHA competition experiments the IC_{50} values and slope factors were determined using the nonlinear least squares curve-fitting program LIGAND (Munson and Rodbard 1980), and the corresponding dissociation constants (K_i) for inhibitors were calculated using the Cheng and Prusoff equation (1973):

$$K_i=\frac{IC_{50}}{1+([L]/K_d)}$$

where [L] is the total radioligand concentration employed in the competition experiment, K_d is the apparent dissociation constant of [^{3}H]CHA, and the IC_{50} is the concentration of the inhibitor producing 50% inhibition of the specific binding.

Where appropriate, data were compared using a two-tailed Student's *t*-test on paired data.

Protein determination. Membrane protein content was assayed by the method of Lowry et al. (1951) following solubilization of the samples in 0.5 N NaOH. Crystalline bovine serum albumin was used as the standard.

Results

Time course of association and dissociation

[^{3}H]CHA bound specifically and reversibly to *Sebastolobus altivelis* and *S. alascanus* brain membranes. At 22 °C the specific binding of 3.8 n*M* [^{3}H]CHA reached equilibrium within 30 min and remained constant for an additional 90 min (Fig. 1 a and 2 a). Because bound [^{3}H]CHA did not exceed 1.0% of the total free concentration of the radioligand, it can be assumed that the free concen-

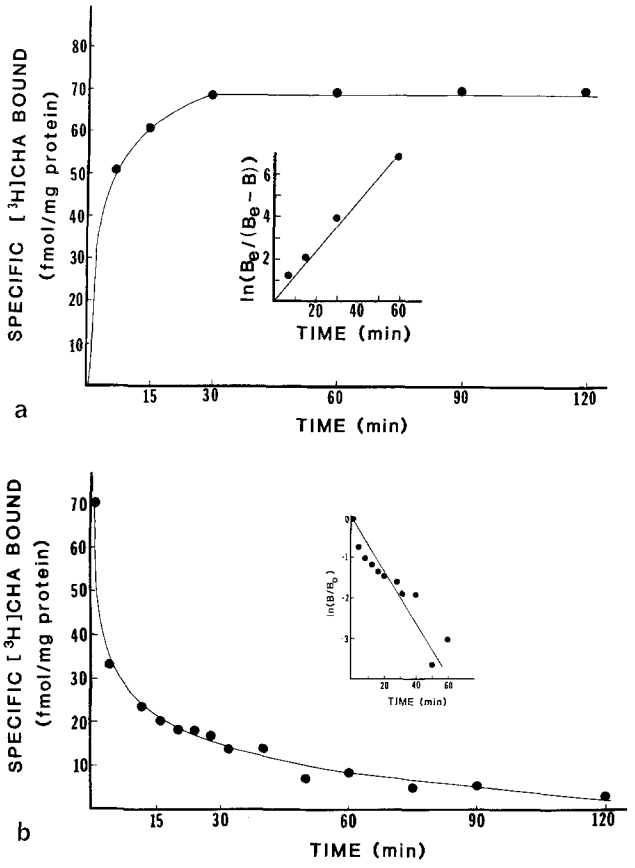

Fig. 1. a Time course of association of specific [^{3}H]CHA binding to *Sebastolobus altivelis* brain membranes. [^{3}H]CHA (3.8 n*M*) was incubated at 22 °C with membranes prepared as described under "Methods". Nonspecific binding was measured in the presence of 30 μ*M* R-PIA and was constant throughout the association reaction. Inset shows the pseudo-first order replot of [^{3}H]CHA association data as explained in text. Data shown are from a single representative experiment. **b** Time course of R-PIA-induced dissociation of specific [^{3}H]CHA binding in *Sebastolobus altivelis* brain membranes. Membranes were first incubated at 22 °C with 3.8 n*M* [^{3}H]CHA for 60 min to allow binding to reach equilibrium. After 60 min 30 μ*M* R-PIA was added in a negligible volume (1% of total incubation volume) to initiate the dissociation reaction. The nonspecific binding, which has been subtracted from each experimental point, was determined throughout the time course in a parallel batch incubation in the presence of 30 μ*M* R-PIA added at the beginning of the incubation. Inset depicts the first-order replot of dissociation data and represents the best least-squares regression line. Data shown are from a single representative experiment

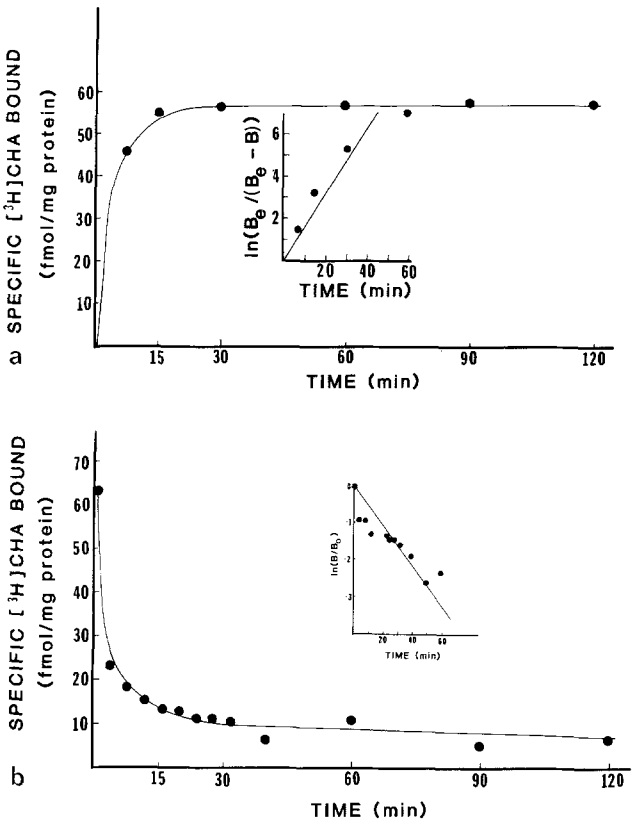

Fig. 2. a Time course of association of specific [^{3}H]CHA binding to *Sebastolobus alascanus* brain membranes. Conditions as in Fig. 1a. Inset shows the pseudo-first order replot of [^{3}H]CHA association data as explained in text. Data shown are from a single representative experiment. **b** Time course of R-PIA-induced dissociation of specific [^{3}H]CHA binding in *Sebastolobus alascanus* brain membranes. Conditions as in Fig. 1b. Inset depicts the first-order replot of dissociation data and represents the best least-squares regression line. Data shown are from a single representative experiment

tration of [^{3}H]CHA remained constant over time. Therefore, the association reaction data can be analyzed as a pseudo-first order reaction as described by the following equation:

$$\ln\frac{[B_{eq}]}{([B_{eq}]-[B])}=[L]k_{+1}+k_{-1}$$

where $[B_{eq}]$ is the amount of [^{3}H]CHA bound at equilibrium, [B] is the amount bound at a given time, t, [L] is the concentration of ligand, k_{+1} is the association rate constant, and k_{-1} is the dissociation rate constant. When $\ln [B_{eq}]/([B_{eq}]-[B])$ is plotted as a function of time (Figs. 1a and 2a insets), the pseudo-first order rate constant, k_{obs}, may be determined from the slope of the line (Weiland and Molinoff 1981; Kitabgi et al. 1977). The mean k_{obs} value for *S. altivelis* was 0.190 ± 0.005 min^{-1} while the corresponding value for *S. alascanus* was 0.228 ± 0.038 min^{-1}. The first order dissociation rate (k_{-1}) was determined directly by allowing [^{3}H]CHA specific binding to reach equilibrium and then monitoring the dissociation of the radioligand after the addition of an excess of cold R-PIA (30 μ*M*). As shown in Figs. 1b and 2b, [^{3}H]CHA binding was readily dissociable in the presence of excess R-PIA. The dissociation data were plotted according to the equation $\ln [B]/[B_o] = k_{-1}t$, in

which $[B_o]$ is the concentration of bound radioligand at time 0 (Figs. 1b and 2b insets). The dissociation rate constant, k_{-1}, determined from the slope of this plot was 0.0339 ± 0.009 min^{-1} for *S. altivelis* and 0.0208 ± 0.005 min^{-1} for *S. alascanus*. The ratio of the rate constants (k_{-1}/k_{+1}) provides an estimate of the equilibrium dissociation constant (K_d) for [^{3}H]CHA binding. Using the data cited above, the kinetically derived K_d values were calculated to be 0.82 n*M* and 0.38 n*M*, respectively for *S. altivelis* and *S. alascanus*. These results suggest that [^{3}H]CHA binds with somewhat greater affinity to the A_1 receptor of *S. alascanus* as compared to that of *S. altivelis*.

Equilibrium saturation analysis

The specific binding of [^{3}H]CHA was found to be saturable in brain membranes from *S. altivelis* and *S. alascanus*. As shown in Fig. 3a and 3b, over the range of 0.08 to approximately 24 n*M* specific binding saturated, while nonspecific binding increased linearly as a function of [^{3}H]CHA concentration. When these data were analyzed using equations based on mass action principles, it was found that the [^{3}H]CHA saturation isotherm in *S. alascanus* membranes was described adequately by an interaction with a single high-affinity state of the receptor in 6 of 6 experiments. Computer-modeling of these data yielded a mean ± SEM K_d value of 1.49 ± 0.18 n*M* with a density of 116 ± 26.3 fmol/mg protein in *S. alascanus* membranes (Table 1). Similarly, in *S. altivelis* the one-site model adequately described the saturation data in 4 of 6 experiments with a mean K_d value of 3.10 ± 0.60 n*M* and a density of 120.4 ± 12.5 fmol/mg protein. This K_d value was significantly ($P < 0.05$) greater than the corresponding one-site K_d value for *S. alascanus*. In contrast to the results with *S. alascanus* membranes, computer-derived parameters for a two-site model could be obtained from [^{3}H]CHA saturation data in *S. altivelis* (Table 1). However, the two-site model did not consistently provide a significant improvement in the fit compared to that obtained from the one-site fit (two-site fits were significantly better in 2 of 6 experiments, $P < 0.001$). The saturation isotherm depicted in Fig. 3a is representative of the experiments in *S. altivelis* brain membranes that were better described by an interaction of [^{3}H]CHA with two-sites rather than one site ($P < 0.001$). These data indicate that [^{3}H]CHA distinguished between two agonist affinity states in this species with a high affinity dissociation constant (K_H) of 0.43 ± 0.09 n*M* and a low affinity dissociation constant (K_L) of 16.3 ± 7.76 n*M*. Although the two-site model did not significantly improve the fit in 4 of 6 experiments in *S. altivelis* membranes, in each case the calculated F-values for the comparison between the one- and two-site models were always greater than the corresponding F-values in the *S. alascanus* isotherms. In addition, in the two experiments in which the data for *S. altivelis* were best fit by a two-site model, the majority of the receptors were in the low-affinity state ($\%R_L = 75$). It is noteworthy that the K_d values for the one-site model in *S. alascanus* and K_H value for the two-site

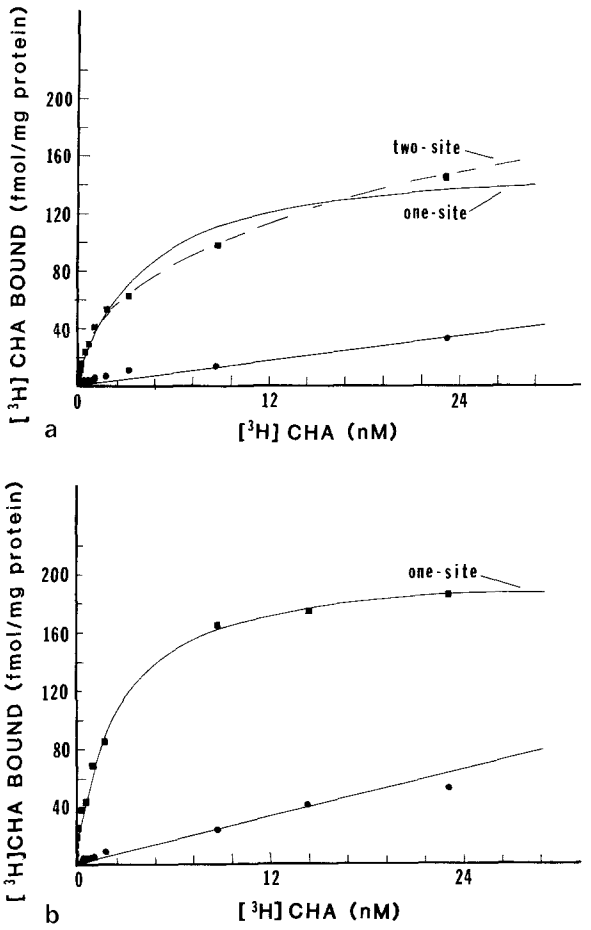

Fig. 3a, b. Equilibrium saturation binding of [^{3}H]CHA to *Sebastolobus altivelis* and *S. alascanus* brain membranes. Membranes from both species were incubated with 11 concentrations of [^{3}H]CHA ranging from 0.08 to 23.3 n*M* in this experiment. The specific binding (square) is defined as the total binding minus nonspecific binding (circle) determined in the presence of 30 μ*M* R-PIA. Values shown are from single experiments which were replicated five times. **a** Saturation isotherms in *S. altivelis* for both the one-site and two-site models generated by computer-assisted analysis as described under 'Methods'. In this experiment the two-site fit described the data significantly better than the one-site fit ($F_{2,8} = 82$, $P < 0.001$). **b** Saturation isotherm in *S. alascanus*. The curve drawn is for a one-site model which adequately described the data in this and five additional experiments

Table 1. Binding parameters derived from computer-assisted analysis of [^{3}H]CHA saturation isotherms in *Sebastolobus altivelis* and *S. alascanus* brain membrane. Membranes were incubated with 8 to 12 concentrations of [^{3}H]CHA ranging from 0.08 to 24 n*M* in six experiments. Values given are the mean ± standard error of the mean for both the 1-site ($n=6$) and 2-site ($n=2$) analyses. K_d is the dissociation constant for the one-site model, while the high-affinity and low-affinity dissociation constants in the two-site model are designated K_H and K_L, respectively. R_T is the maximum number of binding sites for the one-site model, and R_H and R_L are the corresponding densities for the high- and low-affinity states in the two-site model. The percentage of the total receptor population present in the high affinity state in the two-site model is $\%R_H$

Species	1-site		2-site[a]				
	K_d (n*M*)	R_T (fmol/mg protein)	K_H (n*M*)	R_H (fmol/mg protein)	K_L (n*M*)	R_L (fmol/mg protein)	$\%R_H$
S. altivelis	3.10 ± 0.60	120.4 ± 12.5	0.43 ± 0.09	45.3 ± 1.9	16.3 ± 7.76	150.1 ± 52.0	24.8 ± 5.9
S. alascanus	1.49 ± 0.18*	116.0 ± 26.5	—	—	—	—	—

[a] In membranes derived from *S. altivelis* brains, the 2-site model significantly improved the fit compared to the 1-site model in 2 of 6 experiments ($F_{2,8}$ values of 77 and 82, each with $P<0.001$)
* significantly different from the corresponding K_d value of *S. altivelis*, $P<0.05$

model in *S. altivelis* are in reasonable agreement with the kinetically determined K_d values.

Effects of GTP, Mg^{2+} and N-ethylmaleimide on [^{3}H]CHA binding

Guanine nucleotides have been demonstrated to regulate agonist-receptor interactions in a variety of systems which modulate adenylate cyclase activity (Rodbell 1980). Given the ability of A_1 adenosine receptors to mediate an inhibition of adenylate cyclase (Wolff et al. 1981; Cooper et al. 1980), we assessed the influence of GTP on [^{3}H]CHA binding in *S. altivelis* and *S. alascanus* membranes. As shown in Fig. 4, GTP inhibited [^{3}H]CHA binding in a concentration-dependent manner in both species. The maximum degree of inhibition of specific [^{3}H]CHA binding was identical in both species (70–75% inhibition). Similarly, the potencies of GTP were not significantly different, although the IC_{50} value for GTP in *S. alascanus* (8.73 ± 2.46 μ*M*) was somewhat lower than the IC_{50} value in *S. altivelis* (15.5 ± 4.91 μ*M*).

The specific binding of [^{3}H]CHA has been demonstrated to be enhanced in guinea pig and bovine brain membranes by divalent cations such as Mg^{2+} (Goodman et al. 1982). Using a fixed concentration of the radioligand (2.3 n*M*) we examined the sensitivities of specific [^{3}H]CHA binding in *S. altivelis* and *S. alascanus* membranes to varying concentration of $MgCl_2$. The results shown in Fig. 5 indicate that $MgCl_2$ elicited large increases in specific binding of [^{3}H]CHA in both species, with *S. altivelis* displaying a greater sensitivity to this effect. The enhancement of [^{3}H]CHA binding in membranes exposed to 0.1 and 1 m*M* $MgCl_2$ was significantly greater ($P<0.05$) in *S. altivelis* as compared to *S. alascanus* membranes. In *S. altivelis* the 0.1 and 1.0 m*M* concentrations of $MgCl_2$ produced 94.0 ± 5.6% and 131.4 ± 3.2% increases in specific binding, while the corresponding values in *S. alascanus* were 47.8 ± 3.2% and 91.4 ± 6.6%.

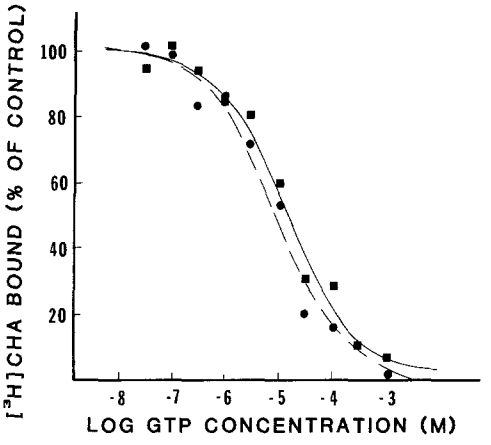

Fig. 4. GTP inhibition of the specific binding of [^{3}H]CHA to *Sebastolobus altivelis* (square) and *S. alascanus* (circle) brain membranes. Membranes were incubated with [^{3}H]CHA (3.1 n*M*) and various concentrations of GTP for 60 min at 22 °C. Data shown are from a representative experiment that was repeated twice. [^{3}H]CHA binding is expressed as a percent of the specific binding observed in the absence of GTP. The mean IC_{50} values for GTP were 15.5 ± 4.91 μ*M* in *S. altivelis* ($n_H=0.72\pm0.17$), and 8.73 ± 2.46 μ*M* in *S. alascanus* ($n_H=0.76\pm0.16$)

The sulfhydryl alkylating reagent N-ethylmaleimide has been reported to reduce [^{3}H]CHA binding to rat brain membranes (Yeung and Green 1983). We therefore determined the effect of the addition of N-ethylmaleimide to the incubation

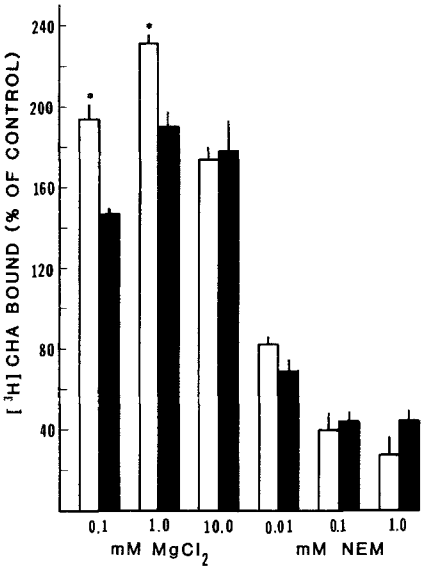

Fig. 5. Effects of $MgCl_2$ and N-ethylmaleimide (NEM) on the specific binding of [^{3}H]CHA to *Sebastolobus altivelis* (open columns) and *S. alascanus* (filled columns) brain membranes. Membranes were incubated with [^{3}H]CHA (3.1 n*M*) and various concentrations of either $MgCl_2$ or NEM for 60 min at pH 7.6, 22 °C. Data shown are means ± SEM of three separate determinations of each. [^{3}H]CHA binding is expressed as a percent of the specific binding observed in the absence of added $MgCl_2$ or NEM. [^{3}H]CHA binding in the presence of 0.1 and 1.0 m*M* $MgCl_2$ was enhanced to a significantly greater extent in *S. altivelis* than in *S. alascanus* ($P<0.05$; paired two-tailed *t*-test)

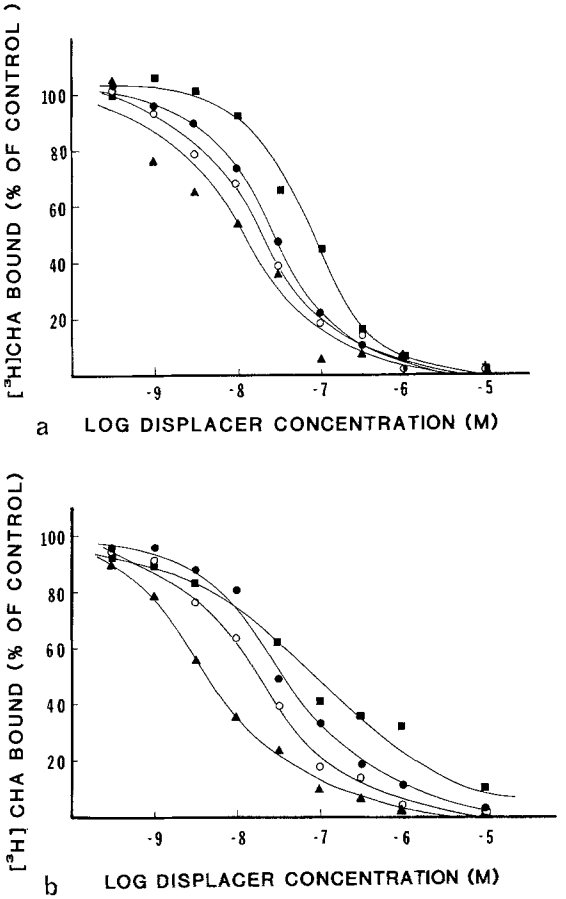

Fig. 6. Inhibition of specific [^{3}H]CHA binding in **a** *Sebastolobus altivelis* and **b** *S. alascanus* brain membranes by adenosine analogs: R-PIA (filled triangles), NECA (open circles), 2ClAdo (filled circles), and S-PIA (filled squares). Nine concentrations of each analog were incubated with membranes and [^{3}H]CHA (3.8 n*M*) for 60 min at 22 °C. Values shown are from a single experiment for each analog which was repeated with results varying less than 15%. The curves drawn are the best fits to the data as determined by nonlinear least-squares repression analysis as described under Methods. The K_i values and Hill slopes for each analog in both species are given in Table 2

medium (pH 7.6) on [^{3}H]CHA binding in *S. altivelis* and *S. alascanus* membranes. The results shown in Fig. 5 indicate that approximately 60–70% of the specific binding was susceptible to inhibition by N-ethylmaleimide in the two species. However, in contrast to the effects of $MgCl_2$, there were no significant differences in the sensitivities of the two species to the N-ethylmaleimide-induced inactivation of [^{3}H]CHA binding sites.

Equilibrium competition profiles of [^{3}H]CHA binding

To determine whether the pharmacological profiles of [^{3}H]CHA binding sites in the two species were similar, we compared the potencies of four selected adenosine analogs as inhibitors of [^{3}H]CHA binding in *S. altivelis* and *S. alascanus* membranes. The competition curves for the adenosine receptor agonists R-PIA, S-PIA, NECA and 2ClAdo are shown in Fig. 6a and 6b. In both species the adenosine analog-inhibition pattern of [^{3}H]CHA binding is consistent with the labeling of A_1 adenosine receptors. The absolute affinities, rank order potencies, and ability to discriminate between the diastereomers of PIA are consistent with the expected characteristics of the A_1 adenosine receptor (Bruns et al. 1980; Daly 1983). Thus, the rank order of agonist affinities in both *S. altivelis* and *S. alascanus* was R-PIA > NECA > 2ClAdo > S-PIA (Table 2). This order of potency is characteristic for A_1 but not A_2 adenosine receptors (Daly 1983). Of particular interest, a comparison of the affinity values (K_i) for the adenosine analogs summarized in Table 2 shows that R-PIA, NECA and S-PIA were significantly ($P<0.05$) more potent as inhibitors of [^{3}H]CHA binding in *S. alascanus* than in *S. altivelis* membranes. The K_i value of R-PIA was 1.37 n*M* in *S. alascanus* which is approximately 3.5 times lower than its K_i value in *S. altivelis*. These findings corroborate the results of the [^{3}H]CHA saturation analysis in which the

Table 2. Relative potencies of adenosine analogs as inhibitors of specific [^{3}H]CHA binding in *Sebastolobus altivelis* and *S. alascanus* brain membranes. The K_i values were calculated using the IC_{50} values derived from logit-log plots of competition experiments using the Cheng-Prusoff equation as described under 'Methods'. Values presented are means ± standard errors of two experiments which differed by less than 15%. Nonspecific binding was determined in the presence of 30 μ*M* R-PIA

Analog	S. altivelis		S. alascanus	
	K_i (n*M*)	Hill slope	K_i (n*M*)	Hill slope
R-PIA	4.85 ± 1.19	0.84 ± 0.21	1.37 ± 0.32*	0.73 ± 0.06
NECA	9.24 ± 0.55	0.80 ± 0.15	6.00 ± 1.02*	0.79 ± 0.11
2ClAdo	11.2 ± 1.32	1.00 ± 0.04	12.6 ± 1.40	0.69 ± 0.11
S-PIA	32.9 ± 4.00	1.05 ± 0.13	21.2 ± 1.25*	0.66 ± 0.18

* indicates K_i values which differ significantly from the corresponding values of *S. altivelis*, $P < 0.05$

[^{3}H]CHA dissociation constant for the one-site model was 2.1 times lower in *S. alascanus* than in *S. altivelis*. Thus, the results of both saturation and competition experiments suggest that selected adenosine analogs have higher affinities for A_1 receptors in *S. alascanus* brain membranes compared to those in *S. altivelis* brain.

The Hill slopes for the least potent analogs, 2-ClAdo and S-PIA were lower in *S. alascanus* than in *S. altivelis* competition curves. Although the explanation for this observation at the molecular level is presently unclear, it is unlikely that this characteristic of 2-ClAdo and S-PIA is related to the formation of the high affinity agonist receptor complex observed in [^{3}H]CHA saturation analysis in *S. alascanus* membranes. The finding that the competition curves for R-PIA and NECA displayed similar Hill slopes in both species suggests that the effects of 2-ClAdo and S-PIA are independent phenomena. Possible mechanisms that could account for the shallow nature of the 2-ClAdo and S-PIA competition curves include interactions with A_2 recognition sites in *S. alascanus* membranes or reduced efficacies of these compounds with respect to the formation of the high affinity agonist-A_1 receptor complex. If either of these two possible interactions between 2ClAdo or S-PIA and adenosine recognition sites in *S. altivelis* were characterized by a lower affinity than that of *S. alascanus* sites, it is possible that such binding would not be detectable with our filtration assay which would result in Hill slopes approaching unity.

Discussion

The effects of hydrostatic pressure on membrane lipid order may have a significant influence on membrane function (e.g., Chong and Cossins 1983), although the magnitude of such effects in marine organisms has not been evaluated. The present study has focused on whether there are differences in the characteristics of adenosine receptor recognition properties which may be indicative of adaptation to hydrostatic pressure. The two scorpaenid congeners which were chosen for this study are closely related phylogenetically (Siebenaller 1978), and thus may not have accumulated many random genetic differences. Because of the similarities in the life histories of these species, their similar habitat temperatures, but different depths of occurrence, the *Sebastolobus* species are a sensitive system with which to detect pressure-related differences (Siebenaller and Somero 1978, 1982; Siebenaller 1978, 1983, 1984a, b, c, 1987).

The results of the present investigation indicate that the A_1 selective radioligand [^{3}H]CHA binds saturably, reversibly and with high affinity to brain membranes prepared from *S. altivelis* and *S. alascanus*. Computer-assisted analysis of [^{3}H]CHA saturation data in the two species revealed that when these data were fit to a one-site model, the mean K_d values differed significantly. These results indicated that the A_1 receptor of *S. alascanus* ($K_d = 1.49 \pm 0.18$ n*M*) possesses a higher affinity for [^{3}H]CHA than does the A_1 receptor of *S. altivelis* ($K_d = 3.10 \pm 0.60$ n*M*). In *S. alascanus* brain membranes the results of computer-modeling demonstrated that this one-site model adequately described saturation data in 6 of 6 experiments. In contrast, in *S. altivelis* membranes parameter estimates for [^{3}H]CHA binding to two sites of receptor could be obtained. Moreover, in 2 of 6 experiments in *S. altivelis* brain membranes the two-site model gave a significantly better fit than the one-site model. The mean dissociation constant values for the high (K_H) and low (K_L) affinity sites for [^{3}H]CHA in *S. altivelis* were 0.43 n*M* and 16.3 n*M*, respectively. These values compare favorably with those obtained for [^{3}H]PIA binding to rat cerebral cortical membranes where the corresponding values for K_H and K_L were reported to be 0.40 n*M* and 13.68 n*M*, respectively (Green and Stiles 1986), as well as the values for [^{3}H]CHA binding to rat cerebral cortical membranes with K_H and K_L values of 0.96 n*M* and 16.8 n*M*, respectively (Szot et al. 1987). Similarly, A_1 adenosine receptors of rat fat cell membranes displayed two binding sites for [^{3}H]PIA with a K_H of 0.3 n*M* and a K_L of 11 n*M* (Ukena et al. 1984). In these previous reports the results were interpreted as representing high- and low-affinity agonist states of the A_1 adenosine receptor. By analogy with other

neurotransmitter receptors which are coupled to adenylate cyclase, adenosine agonists are thought to be capable of inducing a high affinity form of the receptor (Lohse et al. 1984; Ukena et al. 1984; Stiles 1985). These high-affinity states for agonist binding have been demonstrated to represent a ternary complex consisting of the hormone or neurotransmitter, the receptor and a guanine nucleotide binding protein (N protein) (for a review see Limbird 1981). In this ternary complex model guanine nucleotides are thought to bind to the N protein and destabilize the complex yielding a low affinity agonist-receptor complex. Stiles (1985) has reported that the A_1 receptor differs from β and α_2 adrenergic as well as D_2 dopaminergic receptors in that it is tightly coupled to the inhibitory N protein even in the absence of agonists. Given these findings it was proposed that the apparent affinity of agonists for the A_1 receptor will be a function of the extent of A_1 receptor-N protein association (i.e., precoupling) and the tightness of this coupling (Stiles 1985). Thus, it is conceivable that under the conditions of our assay the A_1 receptors of *S. alascanus* brain membranes differ from those of *S. altivelis* in that a higher proportion of the total receptor pool exists associated (precoupled) with the N protein even in the absence of agonist occupation. Alternatively, the agonist-induced interconversion of the A_1 receptors from the low- to the high-affinity state may be enhanced in *S. alascanus* membranes. Either of these possibilities represent potential explanations for our observations of a lower affinity for [^{3}H]CHA in the one-site model in *S. altivelis* and the propensity for the saturation data to fit a two-site model in this species. It is noteworthy that investigation of the specific binding of [^{3}H]diethylphenylxanthine and [^{3}H]quinuclidinyl benzilate, antagonists of adenosine and muscarinic receptors, respectively, revealed no significant differences in receptor density or the affinity of these ligands in the two species (data not shown).

To delineate further the differences in the binding properties of A_1 adenosine receptors in the two species, we have assessed the effects of various agents known to regulate the binding of agonists to A_1 receptors. As expected for a recognition site coupled to an N protein, GTP exerted an inhibition of specific [^{3}H]CHA binding in *S. altivelis* and *S. alascanus* brain membranes. These results are in accordance with previous reports of the effects of guanyl nucleotides on agonist interaction with A_1 adenosine receptors (Murphy and Snyder 1982; Lohse et al. 1984). Although the IC_{50} values for GTP did not differ significantly in the two species, there was a tendency for GTP to be somewhat more potent in *S. alascanus*. This would be consistent with a higher proportion of A_1 receptors existing in a high-affinity state in *S. alascanus* and GTP destabilizing this complex. The inclusion of Mg^{2+} in the binding assay produced a dramatic stimulation of specific [^{3}H]CHA binding with a maximal enhancement occurring at a concentration of 1 m*M* in both species. This effect of Mg^{2+} on [^{3}H]CHA binding has been reported previously in guinea pig and bovine brain membranes (Goodman et al. 1982). The sensitivity of *S. altivelis* to this effect was significantly greater than that of *S. alascanus*. In the beta adrenergic receptor system Mg^{2+} has been proposed to enhance the agonist-induced association of the receptor and N protein by binding to a site on the N protein (Bird and Maguire 1978). It is therefore tempting to speculate that the increased sensitivity of *S. altivelis* to Mg^{2+} is the result of a higher proportion of A_1 receptors existing in the low-affinity agonist state in this species and therefore the greater enhancement in binding is a result of the proportionately larger Mg^{2+}-induced interconversion of the low- to high-affinity state of the receptor. In contrast to the differential sensitivities to the effects of Mg^{2+}, both species displayed equivalent susceptibilities to the N-ethylmaleimide-induced receptor inactivation. These results suggest that the accessibility of the sulfhydryl group(s) to N-ethylmaleimide is similar in the two species. At present we have no information as to whether the sulfhydryl group(s) is on or near the receptor or N protein (for examples of other receptors see Strauss [1984]).

In equilibrium competition experiments adenosine analogs competed for [^{3}H]CHA specific binding with a rank order potency and stereoselectivity expected for the labeling of an A_1 adenosine receptor in both species membranes. The rank order potency in both species was R-PIA > NECA > 2ClAdo > S-PIA, which is characteristic of A_1 but not A_2 receptors (Daly 1983). Consonant with our results from the [^{3}H]CHA saturation experiments, the affinity constants for R-PIA, NECA and S-PIA were 1.5- to 3.5-fold lower ($P<0.05$) in *S. alascanus* as compared to *S. altivelis*. These differences in affinities appeared to be most pronounced for the adenosine agonists possessing the greatest potency and selectivity for A_1 receptors, namely, R-PIA (3.5-fold) and CHA (2.1-fold). Thus, the results of kinetic, equilibrium saturation and equilibrium competition experiments are all consistent with the notion that A_1 adenosine receptors of *S. altivelis* and *S. alascanus* differ with respect to their affinities for selected adenosine agonists. The pos-

sible mechanisms underlying this difference in affinity constants in the two species include differences in the proportions of total receptors existing precoupled to the N protein, and differences in the efficiency of agonist-induced formation of the ternary complex. Further biochemical characterization of A_1 adenosine receptor-N protein interactions in these two congeneric fishes will be required to understand more fully the nature of this difference in affinities. It may tentatively be proposed that membrane receptor coupling and transduction mechanisms have been involved in adaptation to pressure.

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