THE EFFECTS OF HYDROSTATIC PRESSURE ON A₁ ADENOSINE RECEPTOR SIGNAL TRANSDUCTION IN BRAIN MEMBRANES OF TWO CONGENERIC MARINE FISHES

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Summary

To investigate the effects of deep-sea temperatures and hydrostatic pressures on transmembrane signal transduction, the A₁ adenosine receptor/inhibitory G protein/adenyl cyclase complex was studied in brain membranes from two congeneric marine fishes that live at different depths. These scorpaenid species, Sebastolobus alascanus and S. altivelis, have been used as a model system to study adaptations to the deep sea. At 5°C and atmospheric pressure the basal adenylyl cyclase activities of the two species are similar. The inhibition of adenylyl cyclase by the A₁ adenosine receptor-specific agonist, A^cyclopentyladenosine (CPA), was dependent on GTP. The IC₅₀ values for inhibition of adenylyl cyclase by CPA were 2.0±1.14 μmol l⁻¹ and 1.6±1.06 for S. alascanus and S. altivelis, respectively. The A₁ adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine reversed the CPA-induced inhibition of adenylyl cyclase in a concentration-dependent manner. Brain membranes prepared from the Sebastolobus species incubated at 48.1 MPa (0.1 MPa = 1 atmosphere) and 5°C for 2.5 h did not lose basal adenylyl cyclase activity or sensitivity to inhibition by CPA when assayed at atmospheric pressure. In contrast, rat brain membranes lost 59 % of their activity under these conditions. At atmospheric pressure, the Kₘ values of 2-deoxy-ATP were identical for the Sebastolobus species adenylyl cyclases. Increased pressure increased the Kₘ values in both species. However, the Kₘ of 2-deoxy-ATP was less sensitive to pressure for the enzyme from the deeper-living S. altivelis. Basal adenylyl cyclase activity and the inhibitory effect of 100 μmol l⁻¹ CPA were assayed at 0.1, 13.7 and 41.2 MPa. Increased pressure inhibited basal adenylyl

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cylase activity in both species. Basal adenylyl cyclases in brain membranes from the rat and from five additional teleost species were also inhibited by increased pressure. At 13.7 MPa CPA inhibited the adenylyl cyclase from both Sebastolobus species. However, at 41.2 MPa the efficacy of CPA was reversed for S. altivelis, resulting in a stimulation of adenylyl cyclase activity. The phospholipid and fatty acid contents of brain membranes from the two Sebastolobus species did not differ. \[^{32}\text{P} \text{ADP}\] ribosylation by pertussis toxin results in a 10- to 15-fold greater labeling of \(39 \times 10^3\) and \(41 \times 10^3\) M\(_r\) G protein \(\alpha\) subunits in brain membranes of S. altivelis. The G protein complement of these species may play a role in the differential pressure-sensitivity of signal transduction.

**Introduction**

The physical factors of the deep sea have profound effects on the biochemistry and physiology of the organisms that inhabit this environment. This extensive habitat is characterized by low temperatures (1–4 °C) and high hydrostatic pressures (up to about 111 MPa). Pressure increases by 101.3 kPa (=1 atm) for every 10 m depth increase in the water column (Saunders and Fofonoff, 1976). Extremes or variation of both temperature and hydrostatic pressure may potentially disrupt enzymic and metabolic function, and studies have identified evolutionary adaptations in deep-living organisms that permit success in this environment (Siebenaller and Somero, 1989). For example, studies of NAD-dependent dehydrogenases have demonstrated that hydrostatic pressures typical of the bathyal habitat are sufficient to elicit adaptive changes in the cytosolic protein complement of deep-living fishes (Siebenaller, 1978, 1984a; Siebenaller and Somero, 1978, 1979; Somero and Siebenaller, 1979; Hennessey and Siebenaller, 1985, 1987a, b). The evolution of pressure-adapted dehydrogenase homologs has occurred independently in at least four families of deep-sea fishes (Siebenaller and Somero, 1979).

The ordering effects of low temperatures and high hydrostatic pressures on the acyl chains of lipids may cause the most extensive adaptational problems confronting organisms invading the deep sea. For example, the combined effects of the low temperatures and high hydrostatic pressures typical of 3800 m depth, the average depth of the ocean, result in ordering of membrane lipids equivalent to that caused by temperatures of approximately −5 to −7 °C (see, for example, Cossins and Macdonald, 1989). Studies of membranes and membrane-associated enzymes indicate that these components adapt to deep-sea temperatures and hydrostatic pressures. For instance, the relative amounts of total unsaturated fatty acids increase with increasing incubation pressure in cultures of a barophilic bacterium (DeLong and Yayanos, 1985). The composition of membrane lipids differs between deep- and shallow-occurring fishes (Cossins and Macdonald, 1984, 1986; Avrova, 1984). These alterations in lipid composition maintain the ‘fluidity’ of the membrane, preserving an optimal state for membrane function, according to the theory of homeoviscous adaptation (Cossins and Macdonald, 1989). In
many instances, however, these changes do not fully compensate for the change in membrane state due to environmental influences (Cossins and Macdonald, 1989). Membrane-associated systems also display adaptation to the deep-sea environment. For example, the Na⁺/K⁺-ATPase of teleost fish gills (Gibbs and Somero, 1989, 1990) and a sugar transport system in bacteria (DeLong and Yayanos, 1987) are less susceptible to inhibition by high pressure in deeper-occurring species than are homologous systems in shallow-occurring species.

Transmembrane signal transduction may be a critical locus of pressure perturbation in animals invading the deep-water habitat because of the intimate association of components with the cell plasma membrane and the influence of the physical state of the membrane. An example of the general importance of pressure perturbation of membrane function is the high-pressure neurological syndrome, a complex phenomenon with symptoms that include tremors, muscle clonus, loss of motor coordination and convulsions upon exposure to elevated pressures (Halsey, 1982; Brauer, 1984). The high-pressure neurological syndrome has been demonstrated in a variety of chordate taxa, including fishes (Brauer, 1984). Shallow-living fishes are affected by pressures of 2–10.1 MPa, depending on the rate of compression (Brauer, 1984). The syndrome appears to involve multiple effects on excitable membranes (e.g. Grossman and Kendig, 1988; Halsey et al. 1989).

To investigate the effects of hydrostatic pressure on transmembrane signal transduction we have studied the A₁ adenosine receptor/inhibitory guanine nucleotide binding protein (Gᵢ)/adenylyl cyclase complex in brain membranes of two scorpaenid teleost fishes which have served as a model for the study of the effects of hydrostatic pressure (Siebenaller, 1987). These species, Sebastolobus alascanus and S. altivelis, co-occur geographically (Miller and Lea, 1976), are genetically close (Siebenaller, 1978), have similar life histories (Moser, 1974), experience similar temperatures (Hubbs, 1926; Siebenaller, 1984a), but occur at different depths (Hubbs, 1926; Siebenaller and Somero, 1978). Demersal adult S. alascanus are common between 180 and 330 m, and the adults of S. altivelis are found between 550 and 1300 m (Miller and Lea, 1976; Siebenaller and Somero, 1978). These species provide a sensitive system with which to examine fine-scale adaptations to pressure without the potentially confounding effects of other environmental variables or phylogenetic distance (e.g. Siebenaller, 1984a,b, 1987).

The A₁ adenosine receptor is present in the central nervous tissue of vertebrates, but is not detectable in central nervous tissue of the molluscs or arthropods tested (Siebenaller and Murray, 1986). Agonist binding to the A₁ adenosine receptor is adapted during evolution to the cell (body) temperature of the organism (Siebenaller and Murray, 1988) and, at deep-sea temperatures, in a bathyal fish, Antimora rostrata, the receptor is functionally coupled to and modulates adenylyl cyclase (Siebenaller and Murray, 1990). Agonist occupation of the A₁ adenosine receptor inhibits cyclic AMP accumulation in mammalian (Wolff et al. 1981; Londos et al. 1983; Snyder, 1985; Williams, 1987) and teleost (Siebenaller and Murray, 1990) central nervous tissue. The receptor is coupled to
adenylyl cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by an inhibitory guanine nucleotide binding protein (G\textsubscript{i}).

The other major class of adenosine receptor, the A\textsubscript{2} receptor, is coupled to adenylyl cyclase via a stimulatory guanine nucleotide binding protein (G\textsubscript{s}). Agonist occupation of the A\textsubscript{2} receptor increases adenylyl cyclase activity. The A\textsubscript{1} and A\textsubscript{2} adenosine receptors are also distinguished in the rank order potency of adenosine analogs (Daly, 1983\textsuperscript{a,b}; Stone, 1985; Williams, 1987).

We have examined aspects of the coupling of the A\textsubscript{1} adenosine receptor to adenylyl cyclase in brain membranes of the two Sebastolobus species at temperatures and hydrostatic pressures typical of the deep sea. Previously, we have characterized agonist binding to the A\textsubscript{1} adenosine receptors of these species at atmospheric pressure (Murray and Siebenaller, 1987; Siebenaller and Murray, 1986, 1988). Temperature and pressure may affect the function of any of the components of the A\textsubscript{1} adenosine receptor/G\textsubscript{i} protein/adenylyl cyclase complex either directly or through alteration of membrane viscosity. To help distinguish between effects on the membrane lipids and effects on the protein components of this system we have quantified the major classes of phospholipid and fatty acids in brain membranes of these species. To determine whether the patterns observed with the Sebastolobus congeners are characteristic of other species, we have also examined the effects of pressure on the adenylyl cyclase activities of brain membrane preparations from five other teleosts belonging to two predominantly deep-sea families occurring over depths of 400–4500 m and from a mammal, the rat.

**Materials and methods**

**Specimens**

Demersal adult Sebastolobus (Scorpaenidae) were collected by otter trawl off the coast of Oregon on two cruises of the R/V Wecoma. Additional specimens used from these cruises include Antimora microlepis (Moridae, depth range 400–3300 m, Iwamoto, 1975; Small, 1981), Coryphaenoides acrolepis (Macrouridae, 625–2500 m, Iwamoto and Stein, 1974) and C. pectoralis (Macrouridae, 200–2170 m, Iwamoto and Stein, 1974). Additional experiments used brain tissue from the macrourids, Coryphaenoides rupestris (Macrouridae, 550–1960 m, Marshall, 1973; Haedrich and Merrett, 1988) and Chalinura brevibarbis (Macrouridae, 2250–4500 m, Marshall, 1973; Haedrich and Merrett, 1988) taken off the coast of Newfoundland on a cruise of the R/V Gyre. Species were taken at their typical depths of abundance. Brain tissue was dissected, frozen in liquid nitrogen at sea, and transported to the laboratory where tissues were maintained at −80°C until used. The mass of individual Sebastolobus brains was approximately 100 mg. Rat brains were obtained from Pel-Freez (Rogers, Arkansas).

**Preparation of brain membranes**

Sebastolobus brain tissue was disrupted with a Dounce (pestle A) in 100 vols of...
10 mmol\(^{-1}\) Heps, pH 7.6 at 5°C, and centrifuged at 27,000 \(g\) (0–4°C) for 10 min. The pellet was resuspended in buffer, centrifuged at 27,000 \(g\) for 10 min, resuspended in buffer, and brought to 7.5 units of adenosine deaminase per milliliter. One unit of enzyme converts one micromole of adenosine to inosine per minute at 25°C. The homogenate was incubated at 18°C for 30 min, chilled on ice, centrifuged at 27,000 \(g\), and the pellet resuspended in buffer containing 7.5 units of adenosine deaminase per milliliter.

Protein was determined by the method of Lowry et al. (1951) following solubilization of the samples in 0.5 M \(\text{NaOH}\). Bovine serum albumin (Sigma Chemical, St Louis, Missouri) was used as the standard.

For the lipid analyses individual brains were ground into a powder in liquid \(\text{N}_2\). The powder was extracted with 6 \(\text{ml}\) of \(\text{CHCl}_3/\text{CH}_3\text{OH} (1:1, v/v)\), and then reextracted with 3 \(\text{ml}\) of \(\text{CHCl}_3/\text{CH}_3\text{OH} (2:1, v/v)\). The extracts were combined, and phase separation was achieved by adding 3 \(\text{ml}\) of \(\text{CHCl}_3\) and 1.9 \(\text{ml}\) of 0.05% \(\text{CaCl}_2\). The upper phase was discarded and the lower phase was washed once with Folch theoretical upper phase (\(\text{CHCl}_3/\text{CH}_3\text{OH}/0.05\% \text{CaCl}_2, 3:48:47, v/v/v\)). The lipid extracts were stored at \(-20°C\) under \(\text{N}_2\). The brains were processed within 1 month of collection.

**Adenylyl cyclase assays**

The standard adenylyl cyclase assay followed the methods described in Siebenaller and Murray (1990). The assay contained, in a total volume of 150 \(\mu\text{l}\), 10–20 \(\mu\text{g}\) of brain membrane protein, 50 \(\mu\text{mol}\) \(^{-1}\) Heps, pH 7.6 at the assay temperature of 5°C, 50 \(\mu\text{mol}\) \(^{-1}\) 2-deoxy-ATP, approximately \(1\times10^6\) to \(2\times10^6\) cts min\(^{-1}\) [\(\alpha^{32}\text{P}\)]ATP, 10 \(\mu\text{mol}\) \(^{-1}\) GTP, 6.25 \(\mu\text{mol}\) \(^{-1}\) magnesium acetate, 100 \(\mu\text{mol}\) \(^{-1}\) NaCl, 7.5 units of creatine kinase, 5 \(\mu\text{mol}\) \(^{-1}\) phosphocreatine, 1.5 \(\mu\text{g}\) of soybean trypsin inhibitor, 15 \(\mu\text{g}\) of bacitracin and other constituents as indicated below. 2-Deoxy-ATP is employed in assays assessing the effects of adenosine analogs on modulation of adenylyl cyclase because any 2-deoxy-adenosine generated from 2-deoxy-ATP is a weak receptor and P-site agonist, and 2-deoxy-ATP is equivalent to ATP as an adenylyl cyclase substrate (Wolff et al. 1981; Londos et al. 1983). Assays were conducted in a refrigerated water bath for 2 h. The reaction was stopped by adding 250 \(\mu\text{l}\) of 2% sodium dodecyl sulfate, 45 \(\mu\text{mol}\) \(^{-1}\) ATP and 1.3 \(\mu\text{mol}\) \(^{-1}\) cyclic AMP. Each assay condition was replicated in 3–10 tubes. Cyclic \([^{32}\text{P}]\)AMP generated in the assays was determined according to Salomon et al. (1974).

Apparent Michaelis constant (\(K_m\)) values were determined using 6–9 substrate concentrations replicated 6–10 times per experiment. \(K_m\) values were determined using the technique of Johansen and Lumry (1961) with the computer program of Brooks and Suelter (1986). They were determined in at least three independent experiments for each set of conditions.

For assays at elevated hydrostatic pressure, samples were transferred to polyethylene tubing. The tubing was trimmed to exclude air bubbles and sealed using a pipette heat sealer. Cyclic \([^{3}\text{H}]\)AMP (approximately 20,000 cts min\(^{-1}\)) was...
used as an internal standard to monitor the recovery of sample through the sealing, incubation and subsequent column chromatography steps isolating the cyclic $[^{32}\text{P}]$AMP from the $[^{32}\text{P}]$ATP. The $pK_a$ of Hepes, the buffer used in these experiments, is relatively insensitive to pressure (Bernhardt et al. 1988). Samples were incubated in high-pressure vessels maintained at 5°C in a refrigerated circulating water bath. The high-pressure vessels, pump and gauge are described by Hennessey and Siebenaller (1985) and are modeled after those described by Zobell and Oppenheimer (1950). Samples were incubated for 120 min. The time required to seal and pressurize a group of five samples and the time required to remove the samples was less than 6% of the incubation time at elevated pressure. Samples sealed and incubated at atmospheric pressure have adenylyl cyclase activities identical to samples incubated in test tubes.

Concentration–response data for the inhibition of adenylyl cyclase were analyzed by fitting a three-variable logistic equation. The equation used was:

$$Y = \frac{(E - I)}{[1 + (X/IC_{50})]} ,$$

where $Y$ is the adenylyl cyclase activity (pmol min$^{-1}$ mg$^{-1}$ protein) in the presence of a given concentration of adenosine analog ($X$); $E$ is the adenylyl cyclase activity in the absence of adenosine analog; $I$ is the activity in the presence of a maximally inhibiting concentration of adenosine analog, and $IC_{50}$ is the concentration of analog that produces a half-maximal inhibition of adenylyl cyclase activity. The data were analyzed with an unweighted nonlinear regression using FITFUN, a computer modeling program on the PROPHET II computer system.

$[^{32}\text{P}]$ADP ribosylation

Pertussis-toxin-catalyzed $[^{32}\text{P}]$ADP ribosylation of $\alpha$ subunits of the GTP binding proteins $G_\text{i}$ and $G_\text{o}$ followed the procedures described in Siebenaller and Murray (1990). $G_\text{o}$ is an ‘other’ guanine nucleotide binding protein which has been implicated in phosphoinositide turnover. Pertussis toxin was preactivated in 100 mmol$^{-1}$ Tris–HCl, pH 8.0, with 50 mmol$^{-1}$ dithiothreitol for 1 h at room temperature. The incubation mixture contained, in a volume of 100 $\mu$L, 100 mmol$^{-1}$ Tris–HCl, pH 7.5 at the incubation temperature of 5°C, 25 mmol$^{-1}$ dithiothreitol, 2 mmol$^{-1}$ ATP, 100 $\mu$mol$^{-1}$ GTP, 5 $\mu$Ci of $[^{32}\text{P}]$NAD, 1.5 $\mu$g of soybean trypsin inhibitor, 15 $\mu$g of bacitracin, 2 $\mu$g of pertussis toxin and 37–92 $\mu$g of membrane protein. Protein was adjusted so that the concentrations were equal for the two preparations from the $\text{Sebastolobus}$ species. The reaction was stopped by the addition of 50 $\mu$L of stop solution (3% sodium dodecyl sulfate, 42% glycerol, 15% 2-mercaptoethanol, 200 mmol$^{-1}$ Tris–HCl, pH 6.8 at 20°C) followed by boiling for 5 min. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate in 1.5 mm thick 12.5% acrylamide gels followed the procedure of Laemmli (1970). Gels were stained with 0.25% Serva Blue R (Serva Fine Biochemicals, Westbury, New York) in 25% 2-propanol and 10% acetic acid, destained and dried. The dried gels were exposed to Kodak (Rochester, New
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York) X-Omat AR film. The developed autoradiograms were digitized and quantified using an MCID system (Imaging Research, Inc., St Catherine, Ontario, Canada).

Membrane phospholipid and fatty acid composition

For phospholipid analyses, samples equivalent to 25 μg of inorganic phosphate were spotted onto thin-layer chromatography plates (silica gel H +7.5 % magnesium acetate, Analtech, Newark, Delaware). Phospholipids (phosphatidic acid, phosphatidylserine, phosphatidylinositol, sphingomyelin, phosphatidylcholine, phosphatidylethanolamine and cardiolipin) were separated by two-dimensional thin-layer chromatography and quantified by phosphate assay as described by Rouser et al. (1970).

For the fatty acid determinations, samples corresponding to 25 μg of inorganic phosphate were spotted onto silica gel GHL plates (Analtech). The plates were developed in hexane/diethyl ether/glacial acetic acid (65:35:2.3), dried briefly, and visualized under ultraviolet light after spraying with 2',7'-dichlorofluorescein. Fractions corresponding to phospholipids and triglycerides were scraped into glass test tubes, and fatty acid methyl esters prepared by heating the tubes at 100°C for 60 min in the presence of 0.5 ml of benzene and 1.0 ml of 14 % boron trifluoride in methanol. The tubes were then cooled and acidified, and the fatty acid methyl esters were extracted three times with 3 ml of hexane. The esters were stored at −20°C under N₂ until analyzed by gas–liquid chromatography.

Fatty acid methyl esters were analyzed using a Hewlett-Packard model 7942 gas chromatograph equipped with a flame-ionization detector. A capillary column (15 m×0.2 mm) packed with 10 % SP 2330 on 100–200 Chromosorb WAW (Supelco, Bellefonte, Pennsylvania) and helium carrier (1 ml min⁻¹) were used. The oven temperature was programmed from 150 to 210°C at 4° min⁻¹, and the injector and detector temperatures were 220 and 260°C, respectively. The fatty acids were identified by comparing the relative retention times to those of standard mixtures and by semilogarithmic plot and separation analysis (Ackman, 1969).

Reagents

[α⁻³²P]ATP (800 Ci mmol⁻¹), cyclic [³H]AMP (30.5 Ci mol⁻¹) and [adenylate-³²P]-nicotinamide adenine dinucleotide ([³²P]NAD, 31.31 Ci mmol⁻¹) were from DuPont NEN (Wilmington, Delaware). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) and papaverine were obtained from Research Biochemicals, Inc. (Wayland, Massachusetts). Fatty acid methyl ester standards were from Supelco. Pertussis toxin (islet-activating protein) was from List Biological Laboratories, Inc. (Campbell, California). Adenosine deaminase (Sigma, type VI), N⁶-cyclopentyladenosine (CPA), phospholipid standards and all other chemicals used were from Sigma Chemical Co. Water was processed through a four-bowl Milli-Q purification system (Millipore, Bedford, Massachusetts).
Results

Modulation of adenylyl cyclase by an A₁ adenosine receptor agonist

The time course of the basal adenylyl cyclase reaction in brain membranes of each of the *Sebastolobus* species is shown in Fig. 1. At 5°C and atmospheric pressure, the basal adenylyl cyclase activities of the two species are linear for 3 h. The basal adenylyl cyclase activities were similar in both species. Forskolin (3 μmol L⁻¹) increased adenylyl cyclase activity 2.2- to 2.4-fold in each species (data not shown). Adenylyl cyclase assays were routinely conducted for 2 h without forskolin.

The inhibition of basal adenylyl cyclase activity by N⁶-cyclopentyladenosine (CPA) was used as a biochemical index to assess the coupling of the A₁ adenosine receptor to adenylyl cyclase via an inhibitory guanine nucleotide binding protein. CPA is a highly selective A₁ adenosine receptor agonist and was chosen to eliminate possible interactions with the A₂ adenosine receptor (Bruns et al. 1986; Williams et al. 1986) or with the P site on the catalytic subunit of adenylyl cyclase (Londos et al. 1983; Blair et al. 1989; Johnson et al. 1989; Johnson and Shoshani, 1990).

The dependence on GTP concentration of CPA-induced inhibition of basal adenylyl cyclase activity is illustrated in Fig. 2. For both species, at concentrations of added GTP below 0.1 μmol L⁻¹, 100 μmol L⁻¹ CPA had little effect on cyclic AMP accumulation. Maximal inhibition of adenylyl cyclase activity by CPA was observed at GTP concentrations of 1–100 μmol L⁻¹ (Fig. 2). At 5°C, the IC₅₀ values for inhibition of adenylyl cyclase by the A₁-selective agonist CPA were 2.0 ± 1.14 (N=3) and 1.6 ± 1.06 μmol L⁻¹ (N=3) for *S. alascanus* and *S. altivelis*, respectively. Concentration–response curves for representative experiments determining the effects of CPA on the inhibition of basal adenylyl cyclase activity

![Fig. 1](image)

Fig. 1. Time course of the adenylyl cyclase reaction in brain membrane preparations at atmospheric pressure and 5°C. • *Sebastolobus alascanus*; ■ *S. altivelis*. Results from a single representative experiment.
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1.0

Fig. 2. Effect of GTP concentration on the CPA-induced inhibition of brain membrane adenylyl cyclase assayed activity at atmospheric pressure and 5°C. ○ No CPA; ● 100 μmol l⁻¹ CPA. (A) Sebastolobus alascanus; (B) Sebastolobus altivelis. Results from a single representative experiment.

Results from a single representative experiment. are shown in Fig. 3. Inhibition was maximal with CPA concentrations of 10–100 μmol l⁻¹. The maximal inhibition of adenylyl cyclase by CPA was similar in both species and ranged up to 38%. The A₁ adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) (Lohse et al. 1987) reversed the CPA-induced inhibition of adenylyl cyclase in a concentration-dependent manner (Fig. 4). The GTP-dependence of agonist action, the concentration-dependence of the CPA-induced inhibition of cyclic AMP accumulation and the reversal of the CPA-induced inhibition by DPCPX are consistent with A₁ adenosine receptor-mediated inhibition of adenylyl cyclase.

Effects of pressure

The disruption of signal transduction by pressure may result from the loss of
membrane components, which might be ejected from the membrane or denatured as a result of a rigidification of the membrane by a combination of low temperature and high hydrostatic pressure. There are a number of examples of the ejection of membrane proteins upon application of pressure (Muller and Shinitzky, 1981; Deckmann et al. 1985; Lester, 1989). To test whether this might affect adenylyl cyclase activity measured at elevated pressures, brain membrane preparations were incubated in adenylyl cyclase assay mixture, which lacked [α-32P]ATP, at atmospheric or elevated pressure for 2.5 h. After the incubation, the membranes were centrifuged, resuspended in buffer and assayed at 0.1 MPa and 5°C for adenylyl cyclase activity. For both Sebastolobus species, incubation at 48.1 MPa before the assay at atmospheric pressure did not alter the basal adenylyl cyclase activity or inhibition by 100 μmol l⁻¹ CPA (t-test for differences between 0.1 and 48.1 MPa, P>0.05, N=3). In contrast, rat brain membranes lost adenylyl cyclase activity when incubated at 5°C and elevated pressure (Fig. 5). Rat brain membranes lost 28% of their basal adenylyl cyclase activity at 13.7 MPa and 59% of their activity at 48.1 MPa (P<0.05 for each pressure, N=3).

To assess the effects of pressure on substrate binding to adenylyl cyclase, the apparent $K_m$ of 2-deoxy-ATP was determined. 2-Deoxy-ATP is a preferred substrate of adenylyl cyclase (Londos et al. 1983; Johnson and Shoshani, 1990). At atmospheric pressure, the $K_m$ values of 2-deoxy-ATP were identical in the two Sebastolobus species (Fig. 6). The $K_m$ of 2-deoxy-ATP was sensitive to pressure in both species, but the $K_m$ for adenylyl cyclase from S. altivelis was less perturbed by increasing pressure. The $K_m$ of the S. alascanus enzyme was increased fivefold by 48.1 MPa of pressure, the highest pressure tested. In contrast, the $K_m$ for the S. altivelis adenylyl cyclase was doubled by 48.1 MPa.

To determine the effects of pressure on adenylyl cyclase activity and the coupling of the A1 adenosine receptor to adenylyl cyclase in the Sebastolobus species, basal adenylyl cyclase activity and the inhibitory effect of 100 μmol l⁻¹ CPA.
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Fig. 5. Effects of incubation pressure on rat brain membrane basal adenylyl cyclase activity assayed at 5°C and atmospheric pressure. The brain membrane was incubated at the indicated pressure (5°C) for 2.5 h in the adenylyl cyclase assay mixture, except that the mixture lacked [32P]ATP. After the incubation, the membranes were centrifuged, washed, resuspended and assayed for basal adenylyl cyclase activity. The mean and standard errors of three determinations are shown. The data are normalized to the value at 0.101 MPa.

Fig. 6. Effects of hydrostatic pressure on the apparent $K_m$ of 2-deoxy-ATP for the adenylyl cyclase activities of *Sebastolobus alascanus* (●) and *S. altivelis* (■) brain membranes. Assays were conducted at 5°C at the indicated pressures. Values shown are the means of at least three independent determinations. The standard errors of the values are less than 7% of the values.

CPA were examined at atmospheric pressure, 13.7 and 41.2 MPa (Fig. 7). For brain membrane preparations from both species, increased pressure inhibited basal adenylyl cyclase activity. Basal activity in membranes of *S. alascanus* was decreased by 11.4 and 39.8% by 13.7 and 41.2 MPa, respectively. For *S. altivelis* brain membranes, basal adenylyl cyclase activity was inhibited 25 and 35.5% by 13.7 and 41.2 MPa, respectively. Adenylyl cyclase was inhibited by 7 and 25% by
Fig. 7. Effects of hydrostatic pressure on basal adenylyl cyclase activity (open bars: no CPA) and A<sub>1</sub> adenosine receptor-inhibited adenylyl cyclase activity (hatched bars: 100 μmol l<sup>−1</sup> CPA). Assays were conducted at 5°C and the indicated pressures. For each species, data are normalized to the value at 0.1 MPa. (A) Sebastolobus alascanus brain membranes; (B) S. altivelis brain membranes. The values shown are the means and standard deviations of at least five determinations for each species.

100 μmol l<sup>−1</sup> CPA at 13.7 and 41.2 MPa, respectively, in S. alascanus. In S. altivelis brain membranes, CPA inhibited 10% of the basal activity at 13.7 MPa and stimulated adenylyl cyclase activity by 17% at 41.2 MPa. In these experiments, at atmospheric pressure, 100 μmol l<sup>−1</sup> CPA inhibited adenylyl cyclase activity by 15 and 23% in S. alascanus and S. altivelis, respectively. The data shown in Fig. 7 summarize the results of at least five experiments for each species.

The effects of hydrostatic pressure on basal adenylyl cyclase activity were determined in six additional species (Fig. 8). Adenylyl cyclase activity was inhibited most strongly in rat brain membranes. As noted above, rat brain membrane preparations lose adenylyl cyclase during incubation at elevated pressure. 59% of the adenylyl cyclase activity is lost during a 2.5 h incubation at 48.1 MPa (Fig. 5). Rat brain membranes assayed at 27.5 MPa pressure had basal adenylyl cyclase activity only 40% of that at atmospheric pressure. The activity at 41.2 MPa was similar to that measured at 27.5 MPa (Fig. 8). For all the five additional teleost species studied, pressure inhibited basal adenylyl cyclase activity (Fig. 8). Adenylyl cyclase was inhibited to a greater extent by 41.2 MPa than by 27.5 MPa in membrane preparations from Coryphaenoides rupestris (45% inhibition at 41.2 MPa, 21% at 27.5 MPa) and C. acrolepis (53% inhibition at 41.2 MPa, 36% at 27.5 MPa). Adenylyl cyclase was inhibited to a similar extent by 27.5 and 41.2 MPa in preparations from C. pectoralis (25% inhibition at 27.5 MPa, 26% at 41.2 MPa), Chalinura brevibarbis (41% at 27.5 MPa, 44% at 41.2 MPa) and Antimora microlepis (40% at 27.5 MPa, 49% at 41.2 MPa).

[^32P]ADP ribosylation

To determine whether the α subunits of G<sub>i</sub> and G<sub>o</sub> in S. alascanus and S. altivelis have a differential susceptibility to pertussis-toxin-catalyzed ADP ribosylation, we
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Fig. 8. Basal adenylyl cyclase activity in brain membrane preparations assayed at 5°C at 0.1 MPa (open bar), 27.5 MPa (filled bar) and 41.2 MPa (hatched bar) for five teleost fishes and the rat. For each species, data are normalized to the value at 0.1 MPa. The species are identified by number: (1) the rat, *Rattus rattus*, (2) *Coryphaenoides rupestris*, (3) *C. acrolepis*, (4) *C. pectoralis*, (5) *Antimora microlepis* and (6) *Chahnura brevibarbis*. The means and standard errors of three determinations for each species are shown.

assessed the level of $[^{32}\text{P}]$ADP ribosylation produced by a fixed concentration of pertussis toxin in both species. Fig. 9 shows a representative autoradiogram of a 12.5% acrylamide sodium dodecyl sulfate gel of membrane preparations incubated with $[^{32}\text{P}]$NAD with 2 μg of pertussis toxin and without pertussis toxin. Proteins of approximately $39 \times 10^3$ to $41 \times 10^3 \text{Mr}$ are specifically labeled in the presence of pertussis toxin. The incorporation of label in the membranes from the two species was compared by densitometry. The brain membrane preparations from *S. altivelis* show 10- to 15-fold greater incorporation of $[^{32}\text{P}]$ADP $\mu\text{g}^{-1}$ protein than do the preparations from *S. alascanus*.

Membrane lipid composition

To determine whether the differences in the effects of pressure on $K_m$ of 2-deoxy-ATP values between the species and whether the patterns of pressure inhibition of adenylyl cyclase and coupling to the $\alpha_1$ adenosine receptor might reflect differences in the lipid composition of the brain membranes of the *Sebastolobus* species, a comparison was made of the major phospholipid groups and fatty acids of the brain membranes. The phospholipid compositions of brain membranes of the two species are given in Table 1. The values shown are the means of determinations on five brains from each species. There are no statistically significant differences between the species ($P>0.05$, $t$-test). Half of the brain membrane phospholipid in each species was phosphatidylcholine. Phosphatidylethanolamine constituted 32% of the total in each species. The fatty acid composition of the *Sebastolobus* brain membranes are given in Table 2. The
Fig. 9. A representative autoradiogram of *Sebastolobus* brain membrane preparations \[^{32}\text{P}]\text{ADP-ribosylated by pertussis toxin at } 5^\circ\text{C and atmospheric pressure. Preparations were incubated in the presence of }^{[32\text{P}]\text{NAD for 2 h without (A) or with (B) pertussis toxin. The preparations were denatured and subjected to electrophoresis in 12.5\% acrylamide gels in the presence of sodium dodecyl sulfate following Laemmli (1970). The gels were dried and exposed to X-ray film. Lane 1, *Sebastolobus alascanus* membranes without pertussis toxin; lane 2, *S. altivelis* membranes without pertussis toxin; lane 3, *S. alascanus* membrane with pertussis toxin; lane 4, *S. altivelis* membrane with pertussis toxin.}

Table 1. *Phospholipid content of Sebastolobus brain membranes*

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th><em>S. alascanus</em> ((\mu g\text{ P}_i\text{ mg}^{-1}\text{ protein}))</th>
<th><em>S. altivelis</em> ((\mu g\text{ P}_i\text{ mg}^{-1}\text{ protein}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidic acid</td>
<td>0.71±0.15</td>
<td>0.77±0.11</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>1.68±0.06</td>
<td>1.62±0.15</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>0.50±0.12</td>
<td>0.54±0.06</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>0.19±0.07</td>
<td>0.25±0.09</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>10.36±1.63</td>
<td>10.85±1.90</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>6.65±1.41</td>
<td>6.89±0.13</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>0.56±0.06</td>
<td>0.49±0.13</td>
</tr>
<tr>
<td>Total</td>
<td>20.46±3.66</td>
<td>21.35±3.12</td>
</tr>
</tbody>
</table>

Data are means±s.d. of determinations on five individuals.
None of the phospholipid classes differs between the membranes of the two species \((P>0.05, \text{Student's } t\text{-test})\).
Table 2. Fatty acid composition of Sebastolobus brain membranes

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>S. alascanus (% mass)</th>
<th>S. altivelis (% mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>17.0±0.2</td>
<td>18.4±0.7</td>
</tr>
<tr>
<td>16:1n7</td>
<td>4.8±0.4</td>
<td>5.2±0.5</td>
</tr>
<tr>
<td>18:0</td>
<td>9.5±0.5</td>
<td>8.4±0.5</td>
</tr>
<tr>
<td>18:1n9</td>
<td>19.4±0.8</td>
<td>19.8±1.0</td>
</tr>
<tr>
<td>18:1n7</td>
<td>5.6±1.2</td>
<td>6.1±1.0</td>
</tr>
<tr>
<td>18:2n6</td>
<td>0.5±0.0</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>20:1</td>
<td>1.0±0.2</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>20:4n6</td>
<td>3.5±0.2</td>
<td>4.0±0.7</td>
</tr>
<tr>
<td>22:1</td>
<td>0.9±0.2</td>
<td>0.9±0.2</td>
</tr>
<tr>
<td>20:5n3</td>
<td>5.4±0.4</td>
<td>5.2±0.4</td>
</tr>
<tr>
<td>22:4n6</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>24:1</td>
<td>6.6±1.0</td>
<td>5.8±0.7</td>
</tr>
<tr>
<td>22:5n3</td>
<td>0.9±0.2</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>22:6n3</td>
<td>24.6±2.1</td>
<td>22.8±2.9</td>
</tr>
</tbody>
</table>

The values are the means±s.d. of determinations on five individuals.

Only the data for the 20:1 fatty acids differ between the membranes of the two species ($P<0.05$, Student's $t$-test on $\sin^{-1}\sqrt{(p/100)}$ transformed data). None of the other fatty acid classes differ ($P>0.05$).

predominant fatty acids in both species were 18:1n9 and 22:6n3. Only one fatty acid class differed statistically between the two species, 20:1 ($P<0.05$). For the other classes, the $\sin^{-1} \sqrt{(p/100)}$ transformed data do not differ by $t$-test ($P>0.05$). It is not unexpected to find one significant difference by chance given the number of $t$-tests performed, and no importance is attached to the single statistically significant difference.

Discussion

As exemplified by the high-pressure neurological syndrome, the function of neural membranes may be highly susceptible to disruption by hydrostatic pressure, and, hence, pressure effects on membranes and membrane-associated processes may be an important selective force in evolutionary adaptation to the deep sea. The experiments reported here provide insight into the effects of environmental factors on one facet of membrane function, transmembrane signal transduction. Both deep-sea temperatures and pressures may affect transmembrane signaling. Based on the observation that, in warm-bodied homeotherms, agonist binding is entropy-driven (Bruns et al. 1980; Trost and Schwabe, 1981; Murphy and Snyder, 1982; Lohse et al. 1984), the low temperatures characteristic of the deep sea could potentially disrupt agonist binding to the A<sub>1</sub> receptor in ectotherms. However, agonist recognition and binding are maintained in temperature adaptation
This conservation of ligand binding characteristics at a species' body temperature is analogous to the evolutionary conservation of apparent $K_m$ values in adaptation to temperature and pressure (Siebenaller and Somero, 1989).

At 5°C, the $A_1$ adenosine receptors in brain membrane preparations from the Sebastolobus species are coupled to adenylyl cyclase. CPA, an $A_1$ receptor agonist, inhibits cyclic AMP accumulation in these preparations. The IC$_{50}$ values for inhibition of adenylyl cyclase by CPA are similar in the two species, 2.0 and 1.6 $\mu$mol$^{-1}$ for $S. alascanus$ and $S. altivelis$, respectively (Fig. 3). Maximal inhibition of adenylyl cyclase by 100 $\mu$mol$^{-1}$ CPA at atmospheric pressure does not differ between the species and is reversed by the $A_1$ adenosine receptor antagonist DPCPX (Fig. 4). The inhibitory action of CPA is dependent on GTP concentration (Fig. 2). These characteristics are consistent with $A_1$ adenosine receptor negative modulation of adenylyl cyclase. At atmospheric pressure, these characteristics are similar in the two Sebastolobus species.

The Sebastolobus species differ in the pressure sensitivity of $K_m$ of 2-deoxy-ATP values (Fig. 6). At 5°C and atmospheric pressure, the $K_m$ values are similar. The $K_m$ values for both species are increased by increasing hydrostatic pressure; however, the adenylyl cyclase from the shallower-living $S. alascanus$ is more susceptible to pressure perturbation (Fig. 6). Compared to the atmospheric pressure value, 48.1 MPa causes a fivefold increase in the $K_m$ of 2-deoxy-ATP for the $S. alascanus$ adenylyl cyclase homolog. For the $S. altivelis$ homolog, 48.1 MPa doubles the $K_m$ value. The apparent volume changes associated with the perturbation of the $K_m$ values in the two species are 72.3±7.1 and 42.4±6.7 ml mol$^{-1}$ for $S. alascanus$ and $S. altivelis$, respectively. This difference in pressure sensitivity between the adenylyl cyclase homologs of the two species is similar to the difference in pressure-sensitivity of their NAD-dependent dehydrogenase homologs (Siebenaller and Somero, 1978; Siebenaller, 1984a). For the four dehydrogenase homologs studied, the $K_m$ of coenzyme values are much more pressure-sensitive for the enzymes from $S. alascanus$ than for the homologs from $S. altivelis$ (Siebenaller, 1987). This pattern is also observed in comparisons of NAD-dependent enzyme homologs from other shallow- and deep-living fishes (Siebenaller and Somero, 1979; Siebenaller, 1984a, 1987).

Incubation of the brain membrane preparations from the two Sebastolobus species at 48.1 MPa and 5°C does not result in the loss of basal adenylyl cyclase activity or sensitivity to modulation by CPA upon subsequent assay at atmospheric pressure. Although there is no loss of membrane components affecting the $A_1$ adenosine receptor/$G_i$ protein/adenylyl cyclase complex in Sebastolobus brain membranes under these conditions, incubation of rat brain membranes at elevated pressures results in a marked loss of adenylyl cyclase activity (Fig. 5). Several studies report that pressure induces shedding of membrane-associated proteins from mammalian preparations (Muller and Shinitzky, 1981; Deckmann et al. 1985; Lester, 1989). The loss of adenylyl cyclase activity in rat brain membranes may result either from pressure-induced ejection of components or from pressure-
induced denaturation. The pressures employed in these experiments are, however, much less than those typically required to induce protein denaturation (Jaenicke, 1981; Hennessey and Siebenaller, 1985, 1987a, b; King and Weber, 1986). The sensitivity of the rat brain preparations may result from an incubation temperature 32°C below the body temperature of the species, which will affect the structure of the membrane lipids. In such an organized lipid milieu, the protein components may be readily ejected by pressure increases. Basal adenylyl cyclase activity, assayed at elevated pressure and 5°C, is also markedly inhibited in rat brain (Fig. 8). Of the species surveyed, rat brain adenylyl cyclase is most sensitive to increased pressure (Fig. 8). The marked inhibition undoubtedly results from loss of adenylyl cyclase activity during the incubation as well as from pressure inhibition of the enzymic activity.

Basal adenylyl cyclase activity of both Sebastolobus species is inhibited when assayed at elevated pressure in an assay cocktail chosen to obtain an optimal velocity at atmospheric pressure (Fig. 7). The decrease in adenylyl cyclase activity induced by increased pressure is similar in the two species. At atmospheric pressure and 13.7 MPa, the coupling of the A1 adenosine receptor to adenylyl cyclase, as measured by the inhibition caused by 100 μmol 1⁻¹ CPA, is similar in the Sebastolobus species. The efficacy of CPA is reduced in both species by 13.7 MPa pressure. At 41.2 MPa, CPA inhibits cyclic AMP accumulation in S. alascanus. In contrast, the efficacy of CPA is reversed in the S. altivelis membranes at this pressure, resulting in stimulation of adenylyl cyclase in five of six experiments (Fig. 7). This suggests that there may be a pressure-induced change in the coupling of G proteins to adenylyl cyclase, which effects an unmasking of CPA activation of the A2 adenosine receptor/Gs pathway at this pressure. It should be noted that 41.2 MPa is beyond the pressure range experienced by either of the Sebastolobus species. As discussed below, the extent of ADP ribosylation of G proteins by pertussis toxin differs between the two species (Fig. 9).

Pressure also inhibits adenylyl cyclase activity in brain membranes prepared from five teleost species from two typically deep-dwelling families (Fig. 8). Among these fishes there are two patterns of inhibition of adenylyl cyclase. Increasing pressure decreases enzyme activity in Coryphaenoides rupestris and C. acrolepis. In the species that live at rather deeper levels, C. pectoralis, Chalinura brevibarbis and Antimora microlepis, adenylyl cyclase was inhibited to the same extent by 27.5 and 41.2 MPa. In contrast, our previous study of the effects of hydrostatic pressure on the adenylyl cyclase of the deep-living fish Antimora rostrata, which commonly occurs at depths of 2500 m, found that the basal adenylyl cyclase activity of this species was unaffected by 27.5 MPa of hydrostatic pressure (Siebenaller and Murray, 1990). This pressure is similar to the pressures experienced by this species at the deeper end of its depth distribution. In addition, the efficacy of the agonists CPA and N-ethylcarboxamidoadenosine in inhibiting adenylyl cyclase in A. rostrata brain membranes was unimpaired by 27.5 MPa.

The phospholipid and fatty acid contents of brain membranes do not differ in the two Sebastolobus species (Tables 1 and 2). The phospholipid and fatty acid
composition of the brain membranes in the two species is similar to that reported for membranes from other cold-adapted species (Patton, 1975; Avrova, 1984; Cossins and Macdonald, 1986). As expected, the saturated fat content is reduced and the monoenoic and polyunsaturated contents increased relative to those in warm-adapted species (compare with Patton, 1975; Avrova, 1984; Cossins and Macdonald, 1986). Increased pressure increases membrane order by an amount equivalent to a temperature decrease of $1.5-2.5^\circ\text{C} \cdot \text{MPa}^{-1}$, based on the Clayperon relationship (Cossins and Macdonald, 1989). The additional apparent temperature difference experienced by the membranes of the two species due to the different pressure regimes is less than approximately $3^\circ\text{C}$. This apparent temperature difference between the habitats of *S. alascanus* and *S. altivelis* is not sufficient to require or elicit compositional changes to alter membrane viscosity.

Our results implicate two loci at which pressure may perturb transmembrane signaling. The activity of the effector component may be altered, owing to an effect of pressure on substrate binding (Fig. 6) or to an effect on $V_{\text{max}}$. These are also the predominant effects of pressure on cytosolic enzymes (Siebenaller and Somero, 1989). The second locus of pressure perturbation is the coupling of the effector to modulatory receptors via $G$ proteins. Pressure may alter the pool of $G$ proteins that interact with the effector enzyme. This possibility is supported by the decreased efficacy of CPA at increasing pressures in the *Sebastolobus* brain membranes and the reversal of the efficacy of CPA in *S. altivelis* membranes at 41.2 MPa. We have found striking differences in the susceptibility of $G$ protein $\alpha$ subunits to pertussis-toxin-catalyzed ADP ribosylation in brain membranes of the *Sebastolobus* species. There is a 10- to 15-fold difference in the extent of $[^{32}\text{P}]\text{ADP}$ ribosylation of the $\alpha$ subunits of $G_1/G_0$ in the two species (Fig. 9). *S. altivelis* brain membranes, therefore, either have an increased amount of $G$ proteins or an increased susceptibility to pertussis-toxin-catalyzed ADP ribosylation (Fig. 9). Pressure alteration of $G$ protein coupling of receptors to effectors could pose problems for neuromodulation. However, as exemplified by at least one deep-living species, *Antimora rostrata*, pressure resistance may evolve (Siebenaller and Murray, 1990).

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References


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