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To assess the effects of hydrostatic pressure on transmembrane signal transduction, we have examined the A_1 adenosine receptor - G protein - adenylyl cyclase complex in brain tissue of marine teleost fishes which occur at different depths. We have used two congeneric species, Sebastolobus alascanus and S. altivelis and the deeper-living Antimora rostrata. The function of adenylyl cyclase and modulation of basal adenylyl cyclase activity by A_1 adenosine receptor agonists is unaffected by pressures of 272 atm in brain membranes from A. rostrata. The K_m of ATP for adenylyl cyclase is pressure sensitive in the Sebastolobus species. However, the adenylyl cyclase of the deeper-living S. altivelis is less affected by pressure increases. Pertussis toxin-catalyzed [^{32P}]ADP-ribosylation of G proteins differs between the Sebastolobus species. These differences are related to occupancy of the muscarinic cholinergic and adenosine receptors. The phospholipid and fatty acid compositions of the Sebastolobus species are similar.

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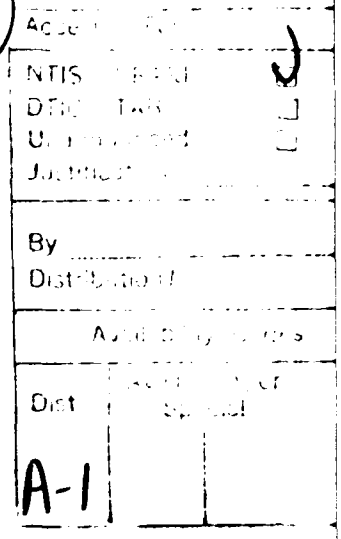

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ANNUAL REPORT ON GRANT N00014-89-J-1869

PRINCIPAL INVESTIGATOR: Thomas F. Murray
(In collaboration with Dr. Joseph F. Siebenaller, Louisiana State University)

GRANTEE: Oregon State University

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INTRODUCTION

To understand the effects of hydrostatic pressure on transmembrane signal transduction, we have been examining the A_1 adenosine receptor - inhibitory guanine nucleotide binding protein (G_i protein) - adenylyl cyclase complex in brain tissue of marine teleost fishes which occur at different depths. Agonist occupation of the A_1 adenosine receptor inhibits cAMP accumulation in mammalian central nervous tissue preparations (e.g., Wolff et al., 1981; Londos et al., 1983; Snyder, 1985; Williams, 1987). We have used two congeneric scorpaenid species, Sebastolobus alascanus and S. altivelis which have been used as a model system for the study of pressure adaptations (Siebenaller and Somero, 1989; Siebenaller, 1990), and a deeper-living morid species, Antimora rostrata. This past year we have continued our efforts defining the effects of hydrostatic pressure on the A_1 adenosine receptor-mediated modulation of adenylyl cylase activity, we have continued characterization of the inhibitory guanine regulatory proteins in brain tissue using pertussis toxin-catalyzed ADP-ribosylation and GTPase assays, and determined the phospholipid and fatty acid composition of the two Sebastolobus species brain membranes.

MATERIALS AND METHODS

Specimens. Demersal adult Sebastolobus alascanus and S. altivelis (Scorpaenidae) were collected by otter trawl at their depths of typical abundance off the coast of Oregon on cruises of the R/V Wecoma. Tissues were dissected and frozen in liquid nitrogen at sea and transported to the laboratory where the tissues were maintained at -80°C until used. S. alascanus is common between 180 and 330 m; S. altivelis is typically found between 550 and 1300 m. Antimora rostrata (Moridae) was taken by otter trawl between 850 and 2500 m, off the coast of Newfoundland, Canada. Rat and chicken brains were obtained from Pel Freez (Rogers, Ark.).

Preparation of brain membranes. For adenylyl cyclase and GTPase assays, brain tissue was disrupted with a Dounce (pestle A) in 100 volumes of 10 mM HEPES, pH 7.6 at 5°C, centrifuged at 27,000 x g (0-4°C) for 10 min. The pellet was resuspended in buffer, centrifuged at 27,000 x g for 10 min, resuspended in buffer, and 7.5 units/ml of adenosine deaminase were added. The homogenate was incubated at 18°C for 30 min, chilled on ice, centrifuged at 27,000 x g, and the pellet resuspended in buffer and 7.5 units/ml adenosine deaminase. Fifty µl of this homogenate was used in the adenylyl cyclase assays. Twenty µl were used in the GTPase assays.

For ADP-ribosylation experiments, membranes were homogenized with a Dounce (pestle A) in 40 volumes of 50 mM Tris-HCl, pH 7.6 at 5°C. The homogenate was centrifuged at 27,000 x g for 10 min. The pellet was resuspended in 40 volumes of Tris-HCl buffer. Fifty µl of this were used for the ribosylation experiments.

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

[^{32}P]ADP-ribosylation. Pertussis toxin - catalyzed [^{32}P]ADP-ribosylation of GTP binding proteins followed the procedures described in Ribeiro-Neto et al. (1985) and Greenberg et al. (1987). The 100-µl incubation mixture contained 100 mM Tris-HCl, pH 7.5 at the incubation temperature of 5°C, 25 mM dithiothreitol, 2 mM ATP, 0.1 mM GTP, 5 µCi [^{32}P]-NAD, 1.5 µg soybean trypsin inhibitor, 15 µg bacitracin, 2 µg pertussis toxin and 37 to 92 µg membrane protein. After 3 h, the reaction was stopped by addition of 50 µl of stop solution (3% sodium dodecyl sulfate, 42% glycerol, 15% 2-mercaptoethanol, 200 mM Tris-HCl, pH 6.8 at 20°C) and boiled for 5 min. The denatured samples were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis in a 1.5 mm thick 12.5% acrylamide gel following Laemmli (1970). The gel was stained with 0.25% Serva Blue R (Serva Fine Biochemicals, Westbury, New York) in 25% 2-propanol, 10% acetic acid, destained and dried. The dried gels were exposed to Kodak (Rochester, New York) X-Omat AR film. DuPont Cronex Lightning Plus intensifying screens were used.

Adenylyl cyclase assays. The standard adenylyl cyclase assay contained in a total volume of 150 µl, 10 to 20 µg of A. rostrata brain membrane protein, 50 mM HEPES, pH 7.6 at the assay temperature of 5°C, 50 µM 2-deoxy-ATP, approximately 1 to 1.5 x 10^6 cpm [α-^{32}P]ATP, 10 µM GTP, 6.25 mM Mg acetate, 100 mM NaCl, 7.5 units creatine kinase, 5 mM phosphocreatine, 1.5 µg soybean trypsin inhibitor, 15 µg bacitracin and other constituents as indicated below. Assays were conducted in a refrigerated water bath for 2 h. The reaction was stopped by adding 250 µl of 2% sodium dodecyl sulfate, 45 mM ATP and 1.3 mM cAMP. Assays were in triplicate. [^{32}P]cAMP generated in the assays was determined according to Salomon et al. (1974).

Pressure apparatus. For assays of the effects of hydrostatic pressure on

adenylyl cyclase activity and inhibition, samples were transferred to polyethylene tubing. The tubing was trimmed to exclude air bubbles and sealed using a pipet heat sealer. [^{3}H]cAMP (approximately 20,000 cpm) was used as an internal standard to monitor the recovery of sample through the sealing and incubation, and through the subsequent column chromatography steps isolating the [^{32}P]cAMP from the [^{32}P]ATP following Salomon et al. (1974). 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 apparatus is described in Hennessey and Siebenaller (1985). Samples were incubated for 120 min. The time required to seal and pressurize a group of four 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 which are incubated in test tubes.

GTPase assays. Assays of the GTPase activity associated with the α subunit of G_i followed the methods described by Hausleithner et al. (1985). Assays were conducted at 5°C in a volume of 50 µl. The standard assay mixture contained 0.1 mM ATP;, 5 mM creatine phosphate, 1.2 mg/ml creatine phosphokinase, 2 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM EGTA, 100 mM NaCl, 1 mM dithiothreitol, 1.25 mM AppNHp, 50 mM Tris-HCl, pH 7.5 at 5°C and varying concentrations of GTP. Assays were started by the addition of membrane preparation (2 to 9 µg protein per tube). The assay was stopped by the addition 1 ml of 20 mM phosphoric acid with 5% activated charcoal. The suspension was centrifuged for 10 min at 5000 x g and 0.5 ml of the supernatant liquid taken for scintillation counting.

Reagents. [adenylate-^{32}P]-nicotinamide adenine dinucleotide ([^{32}P]NAD, 31.31 Ci/mmol), [^{3}H]DPCPX (34.4 Ci/mmol), [α-^{32}P]ATP (800 Ci/mmol), [γ-^{32}P]GTP (30 Ci/mmol) and [^{3}H]cAMP (30.5 Ci/mol) were from DuPont NEN (Wilmington, Delaware). The R-diastereomer of N^6-phenylisopropyladenosine (PIA), 5'-N-ethylcarboxamidoadenosine (NECA) and papaverine were obtained from Research Biochemicals, Inc. (Wayland, Massachusetts). Pertussis toxin was from List Biological Laboratories (Campbell, California). Electrophoresis reagents and molecular weight standards were from Bio-Rad (Richmond, California). Adenosine deaminase (Sigma, Type VI), N^6-cyclopentyladenosine (CPA), 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

We completed our characterization of the modulation of adenylyl cyclase by the A_1 adenosine receptor in brain membranes of the deep-sea fish Antimora rostrata which is common between 850 and 2500 m (Siebenaller and Murray, 1990). Adenylyl cyclase of A. rostrata functions, and is modulated by the A_1 adenosine receptor, at the in situ temperatures and hydrostatic pressure which this species experiences. Basal adenylyl cyclase in this species is unaffected by 272 atm pressure, a pressure close to that experienced by this species in situ. Modulation of the adenylyl cyclase by adenosine analogs is unimpaired in this species by increased pressure (Fig. 1). This is in contrast to our findings for shallower occurring species. Deep-living species do display adaptation at the level of membrane function to the increased hydrostatic pressures of their habitat.

To assess the effects of hydrostatic pressure on basal adenylyl cyclase activity, the effects of pressure on the K_m of 2-deoxy-ATP were determined using brain membrains from the Sebastolobus congeners. K_m values were determined using six to nine ATP concentrations. Six replicates of each ATP concentration were used. The K_m values were determined using the technique of Johansen and Lumry (1961) with the computer program of Brooks and Suelter (1986). K_m values at each pressure were determined in at least three independent experiments for each species. For the two species adenylyl cyclase activities, increasing measurement pressure increases the K_m values. However, the adenylyl cyclase of the deeper living Sebastolobus altivelis is less perturbed by pressure increases than is the enzyme from S. alasacanus (Fig. 2). The apparent volume changes associated with the perturbation of K_m are 42.4 ± 6.7 and 72.3 ± 7.1 ml mol^{-1} for S. altivelis and S. alascanus, respectively.

A possible mechanism of pressure disruption of signal transduction is the loss of membrane components, which might be ejected or denature due to a rigidification of the membrane by a combination of low temperature and high hydrostatic pressure. There are a number of examples of the loss membrane proteins due to membrane rigidification, e.g., Muller and Shinitzky (1981); Deckmann et al. (1985) and Lester (1989).

The disruption of signal transduction by pressure ejection of membrane components or irreversible pressure denaturation was tested for Sebastolobus altivelis and S. alscanus brain membranes and brain membranes from rat and chicken. Brain membranes were incubated at atmospheric pressure and elevated pressure in the adenylyl cyclase reaction mixture minus [^{32}P]ATP. After the incubation, the homogenate was centrifuged and the membranes assayed at 5°C and atmospheric pressure. For the two Sebastolobus species, incubation at pressure before the assay did not alter the basal adenylyl cylcase activity. In contrast, the chicken and rat brain membrane preparations incubated at 5°C and atmospheric pressure had higher activities than the preparations princubated at 476 atm and 5°C for 2.5 h in two out of three experiments. In the third experiment, there was no significant difference between the treatments.

In collaboration with Dr. Arthur Hagar, School of Medicine, Louisiana State University, New Orleans, we have determined the fatty acid composition (Table 1) and phospholipid composition (Table 2) of brain membranes from the Sebastolobus species. There were no statistically significant differences in relative compositions of either the fatty acids or phospholipids between the two species. The differences in the depth distributions of these species are apparently not large enough to elicit adaptive adjustment of the membrane composition. The possibility exists that there may be differences in some other component which was not measured, such as cholesterol content. The differences in the pressure effects on brain membrane associated components in these two species may most likely stem from differences in the primary sequence of the protein components, or in their posttranslational modifications.

Table 1. Fatty acid composition of Sebastolobus brain membranes. Data are presented as area percent. The values are the means ± S.D. of determinations on 5 individuals.

Fatty Acid	S. alascanus	S. altivelis
16.0	14.72 ± 0.50	15.87 ± 0.71
16:1n7	4.15 ± 0.41	4.52 ± 0.44
18:0	8.25 ± 0.62	7.24 ± 0.47
18:1n9	16.89 ± 0.86	17.10 ± 0.68
18:1n7	5.36 ± 0.25	5.20 ± 0.87
18:2n6	0.38 ± 0.03	0.47 ± 0.06
20:1	0.90 ± 0.13	1.60 ± 0.32
20:4n6	3.09 ± 0.23	3.48 ± 0.61
22:1	0.73 ± 0.15	0.84 ± 0.20
20:5n3	4.68 ± 0.30	4.53 ± 0.36
22:4n6	0.28 ± 0.04	0.28 ± 0.06
24:1	5.69 ± 0.75	5.00 ± 0.58
22:5n3	0.84 ± 0.07	0.77 ± 0.10
22:6n3	21.43 ± 1.63	19.75 ±2.62
Total	86.88 ± 2.00	86.65 ± 0.77

Table 2. Phospholipid content of Sebastolobus brain membranes. Data are means ± S.D. of determinations on five individuals (μg P_i/mg protein).

Phospholipid	S. alascanus	S. altivelis
Phosphatidic Acid	0.71 ± 0.15	0.77 ± 0.11
Phosphatidylserine	1.68 ± 0.06	1.62 ± 0.15
Phosphatidylinositol	0.50 ± 0.12	0.54 ± 0.06
Sphingomyelin	0.19 ± 0.07	0.25 ± 0.09
Phosphatidylcholine	10.36 ± 1.63	10.85 ± 1.90
Phosphatidylethanolamine	6.65 ± 1.41	6.89 ± 0.13
Cardiolipin	0.56 ± 0.06	0.49 ± 0.13
Total	20.46 ± 3.66	21.35 ± 3.12

The differences in the extent of pertussis toxin-catalyzed ADP-ribosylation of G proteins in the two Sebastolobus species (Siebenaller and Murray, 1990) have been confirmed and extended by delineating the pertussis toxin concentration-response relationships in brain membranes of S. alascanus and S. altivelis. Furthermore, we have demonstrated that occupancy of either muscarinic cholinergic or adenosine receptors amplifies this difference in the susceptibility to pertussis toxin catalyzed [^{32}P]ADP-ribosylation of the α subunits of G_i/G_o. The oxotremorine- and N-ethylcarboxamidoadenosine-induced increases in the extent of [^{32}P]ADP-ribosyltion are antagonized by atropine and 8-para-sulfophenyltheophylline, respectively. This suggests that the enhancement of the pertussis toxin-catalyzed response is a receptor-mediated event.

As a further probe of G_i protein function we have begun characterization of the low-K_m GTPase activity in the two Sebastolobus species. The reaction is linear for at least two hours at 5°C under the conditions we have employed (Fig. 3). Thus, this assays is suitable for studies at pressure using the techniques which we have developed for adenylyl cyclase. The A_1 adenosine receptor agonist R-PIA stimulates the low K_m GTPase activity, but not the non-specific (high K_m) GTPase activity of fish brain membranes (Fig. 4). The ATPase inhibitor AppNHP used in these assays may potentially compete with A_1 adenosine receptor analogs. To assess this possibility we examined the competition of AppNHP and the A_1 adenonsine receptor antagonist ^{3}H-DPCPX. AppNHP is a poor displacer of ^{3}H-DPCPX binding, and will not interefere with our studies employing adenosine agonists. K_i values measured at 5°C using standard binding techniques (Siebenaller and Murray 1988) are 4.1 mM and 2.9 mM for S. altivelis and S. alascanus, respectively.

We have identified components of the A_1 adenosine receptor - G_i protein - adenylyl cyclase system in fish brain membranes which differ among species adapted to different depth regimes, and at least some components of these systems appear to differ in their responses to hydrostatic pressure. Further studies are planned to clarify the pressure adaptations of this signal transduction system.

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FIGURE LEGENDS

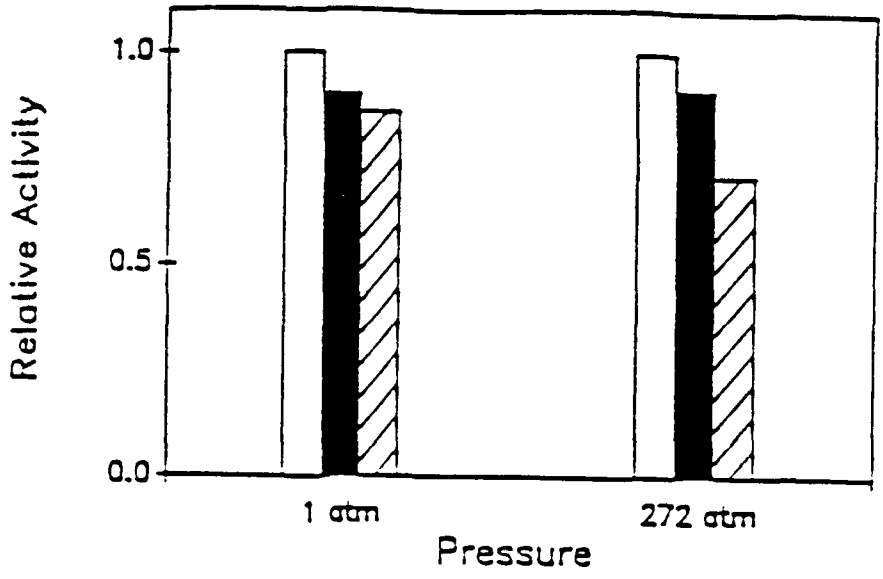
Fig. 1. The effects of hydrostatic pressure on Antimora rostrata basal adenylyl cyclase activity (open bar) and inhibition of basal adenylyl cyclase activity by the adenosine analogs cyclopentyl adenosine (100 µM; filled bar) and N-ethylcarboxamidoadenosine (100 µM; hatched bar). Membranes were incubated at atmospheric pressure or 272 atm pressure for 2 h at 5°C. All values are standardized to the 1 atm basal adenylyl cyclase activity. The 1 atm and 272 atm basal activities were 3.3 pmol min^{-1} mg $protein^{-1}$. The 1 atm data are the mean of three replicates; the 272 atm values are the mean of six replicates. The average standard errors are 11.7% of the values of the mean. From Siebenaller and Murray (1990).

Fig. 2. The effects of hydrostatic pressure on the K_m of 2-deoxy-ATP for the adenylyl cyclase activities of Sebastolobus altivelis (filled circles) and S. alascanus (open circles) brain membranes. Assays were conducted at 5°C and the indicated pressures following the techniques described in Materials and Methods. Values shown are the means of at least three independent determinations. The apparent volume changes associated with the pressure-perturbation of the values are 42.4 ± 6.7 and 72.3 ± 7.1 ml mol^{-1} for S. altivelis and S. alascanus, respectively.

Fig. 3. Time course of the GTPase reaction at 5°C in brain membranes from Sebastolobus alascanus. Open circles: low-K_m GTPase activity in the absence of added agonist. Filled circles: low-K_m GTPase activity stimulated by the addition of 100 µM R-phenylisopropyladenosine. The low-K_m GTPase activity was determined by subtracting the [^{32}P]GTP hydrolyzed at a concentration of 30 µM GTP from the [^{32}P]GTP hydrolyzed at 0.3 µM GTP following Hausleithner et al. (1985). Assays were performed in triplicate.

Fig. 4. [^{32}P]GTP dilution curve determined at 5°C in brain membranes from S. alascanus. Incubations were conducted for 1 h with the addition of 30,000 cpm of [^{32}P]GTP per tube. Assays were performed in triplicate. Open circles: GTPase activity in the absence of added agonist. Filled circles: GTPase activity the in the presence of 100 µM R-phenylisopropyladenosine.

Fig. 1


Relative Activity
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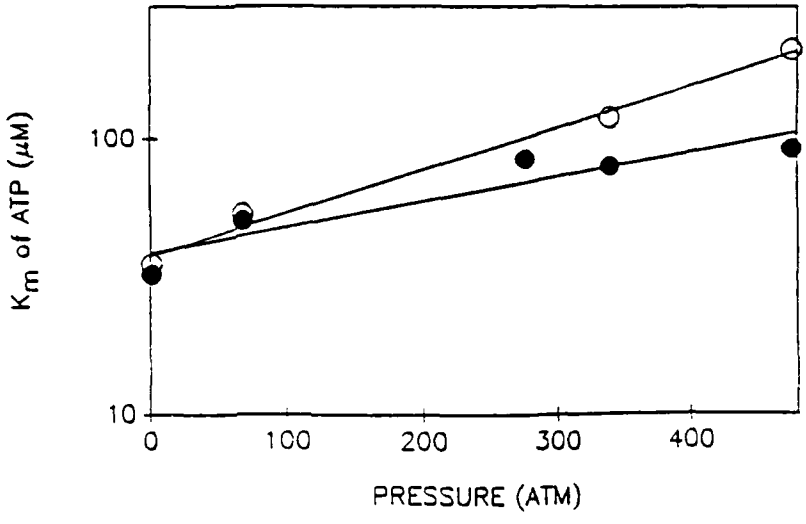
0.0
1 atm
272 atm
Pressure

Fig. 2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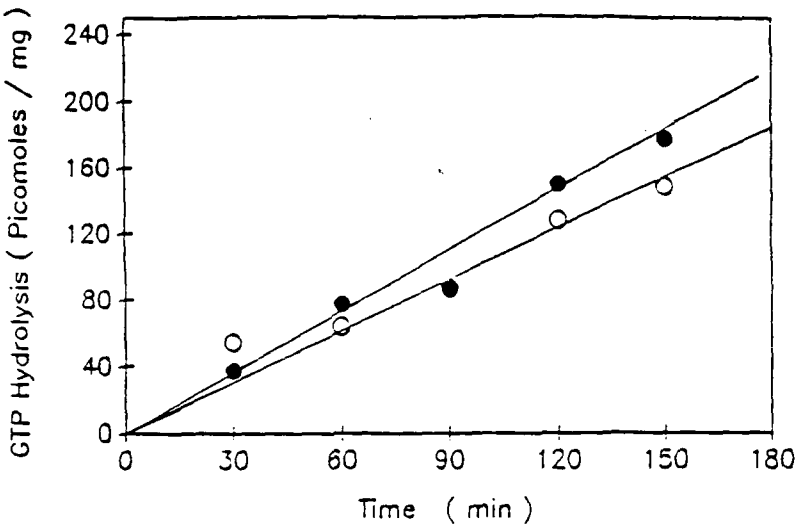
100
10
0
100
200
300
400
PRESSURE (ATM)

Fig. 3


GTP Hydrolysis (Picomoles / mg)
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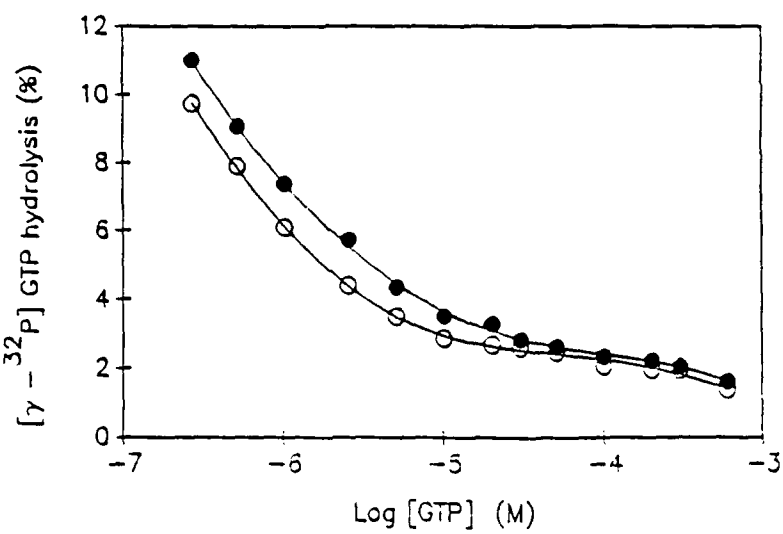
200
160
120
80
40
0
0
30
60
90
120
150
180
Time (min)

Fig. 4


[γ - 32P] GTP hydrolysis (%)
12
10
8
6
4
2
0
-7
-6
-5
-4
-3
Log [GTP] (M)