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CHARACTERIZATION OF ADENYLYL CYCLASE IN GOLDFISH BRAIN

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Abstract—The rate of production of cAMP by the adenylyl cyclase enzyme from goldfish brain was linear with time and with protein concentration. In agreement with mammalian adenylyl cyclase systems the enzyme is divalent cation dependent, being activated in the presence of either Mg^{2+} or Mn^{2+}. Forskolin also stimulated the rate of reaction in a dose-dependent manner with a half-maximal effect of 1 μM. The activated enzyme was inhibited by high concentrations of Ca^{2+} but was independent of Na^+ concentration. The presence of guanine nucleotide binding proteins (G-proteins) was demonstrated by the fact that both NaF and guanosine 5'-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) stimulated the basal rate. In addition, the p[NH]ppG dose–response curve of the forskolin-stimulated enzyme was biphasic, similar to that observed for other systems. At low concentrations of p[NH]ppG a small inhibition was observed while higher concentrations produced a stimulation. These data suggest that the goldfish brain adenylyl cyclase enzyme complex includes both stimulatory and inhibitory G-proteins in addition to the catalytic unit. A series of known and putative goldfish neurotransmitter substances failed to either stimulate or inhibit the adenylyl cyclase activity. The endogenous neurotransmitters which interact with this second messenger system remain to be determined.

Since the discovery of cAMP as a second messenger, its synthesis by adenylyl cyclase enzymes has been described in a wide phylogenetic distribution of organisms ranging from bacteria to higher vertebrates [1]. Modulation of the activity of adenylyl cyclase is used by a variety of hormones and neurotransmitters to elicit their intracellular responses. By increasing or decreasing the level of cAMP, the activity of protein kinase A is altered which in turn regulates the activity of protein substrates including receptors and ion channels through phosphorylation [2]. This cascade of events has been found to underpin a number of physiological events both in neuronal and non-neuronal tissue. In the central nervous system cAMP is known to be involved in regulating ion channel activity [3], neuronal survival [4] and in the underlying mechanisms of learning [5].

Goldfish, and in particular their retinotectal pathway, are a widely used model system for the study of synaptic plasticity and neuroregeneration [6]. Kainate binding sites, which are expressed in high abundance in goldfish brain [7, 8], have been strongly implicated in some of these processes [9]. Recently, a pharmacologically distinct kainate binding site in goldfish brain has been proposed as a novel form of metabotropic glutamate receptor (mGluR) based on its interaction with guanine nucleotides [10, 11]. By analogy to the metabotropic receptors in rat [12], it may play a significant part in synaptic plasticity of the goldfish retinotectal system.

The aim of this study was to characterize the goldfish brain adenylyl cyclase system in order to investigate whether the putative goldfish metabotropic receptor is coupled either positively or negatively to that enzyme. To our knowledge, there have been no previous reports of this enzyme in goldfish.

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MATERIALS AND METHODS

Membrane preparation. Membranes were prepared essentially as described previously [8]. In preliminary experiments, a Tris–citrate buffer (50 mM Tris, pH 7.4 with citric acid at 4°) was used; however, citrate was found to interfere with the adenylyl cyclase assay, probably by chelating Mg^{2+}, so 50 mM Tris–HCl (pH 7.4 at 4°) was used in all subsequent experiments.

Frozen whole goldfish brains were thawed in ice-cold Tris–HCl buffer containing 320 mM sucrose (~1 mL/brain) and homogenized in a glass–Teflon homogenizer. The homogenate was centrifuged at 1500 *g* for 10 min at 4° and the pellet discarded. The supernatant was centrifuged for 30 min at 48,000 *g* 4°. The resultant pellet was resuspended in 5 mL ice-cold 50 mM Tris–HCl (minus sucrose) and rapidly frozen. The frozen suspension was thawed, diluted to 35 mL and re-centrifuged for 20 min at 48,000 *g*. This freeze–thaw cycle was repeated a further two times to ensure complete elimination of endogenous glutamate. The final pellet was resuspended to approximately 0.5 mg/mL protein and used either immediately or stored frozen at −70°. These membranes gave binding characteristics in agreement with previously published data [8, 13].

In some experiments, to determine the effects of the standard freeze–thaw protocol described above, rat liver and goldfish brain membranes were prepared by a less rigorous alternative procedure [14]. Very

briefly, a 1 mM $NaHCO_3$ buffer was used throughout and the homogenized tissue was centrifuged at 1500 *g* for 15 min at 4°, resuspended and centrifuged again without any freeze–thaw steps. The final membrane suspension was used immediately in the adenylyl cyclase assay.

Adenylyl cyclase assays. Adenylyl cyclase was assayed essentially by the method of Salomon *et al.* [15] with minor modifications. The reaction mixture contained 0.1 mM ATP, 0.1 mM GTP, 1 mM cAMP, 0.25 mM Ro 20-1724, 20 mM creatine phosphate, 200 U/mL creatine kinase, 5 mM $MgCl_2$, 50 mM Tris–HCl pH 7.4 unless stated in figure legends. Thirty microlitres of membranes were added to start the reaction. The production of cAMP was measured over 15 min at 37°. The reaction was terminated with the addition of 100 μL of 2% w/v sodium dodecyl sulphate, 40 mM ATP, 1.4 mM cAMP pH 7.4 (including approx. 20,000 cpm [^{3}H]cAMP).

Protein assays. Protein concentrations were determined using the BioRad assay kit using bovine serum albumin as standard.

Materials. All adenylyl cyclase components were from Boehringer except Ro 20-1724 which was from TBI; bovine calmodulin and forskolin from Sigma. All ligands were from Tocris Neuramin or Sigma. [2,3^{3}H]cAMP was from Amersham and [α-^{32}P]ATP was from ICN Flow. Ecoscint A was from National Diagnostics. General reagents were from Fisons.

Data analysis. Data shown in the figures are given as the means ± SD of triplicates from typical experiments. Where error bars are not drawn they fall within the symbols used in the figure.

RESULTS

The rate of cAMP production for both basal and maximal forskolin (100 μM)-stimulated cyclase is linear with time (Fig. 1a) and with protein concentration over the range used (Fig. 1b). From these data an assay time of 15 min and a protein concentration of 10–15 μg protein/assay were selected for the subsequent experiments.

The divalent cation dependence of cAMP production is shown in Fig. 2. As observed for mammalian adenylyl cyclase, there is an absolute dependence on the presence of Mg^{2+} or Mn^{2+} [16]. A concentration of 100 μM Mg^{2+} or 10 μM Mn^{2+} is required to see significant activity while 5 mM Mg^{2+} or 1 mM Mn^{2+} is maximal. As the final assay concentration 5 mM Mg^{2+} was chosen and corresponds to basal activity in Fig. 1. Mg^{2+} is a cofactor for the enzyme and is required in excess of the ATP concentration [16]. Consistent with other systems Mg^{2+} promotes far higher activity than Mg^{2+} producing greater than twice the rate of maximal stimulation.

The diterpene forskolin is a potent stimulant of adenylyl cyclase from a range of species and tissues [17]. As shown in Fig. 1 forskolin stimulates the goldfish enzyme approximately 3-fold above basal activity. The half-maximal stimulation by forskolin is 1 μM (Fig. 3a) and is maximal at 10 and 100 μM. The forskolin (100 μM)-stimulated rate is susceptible to significant Ca^{2+} inhibition at concentrations equal to that of ATP (i.e. 100 μM) but lower than the

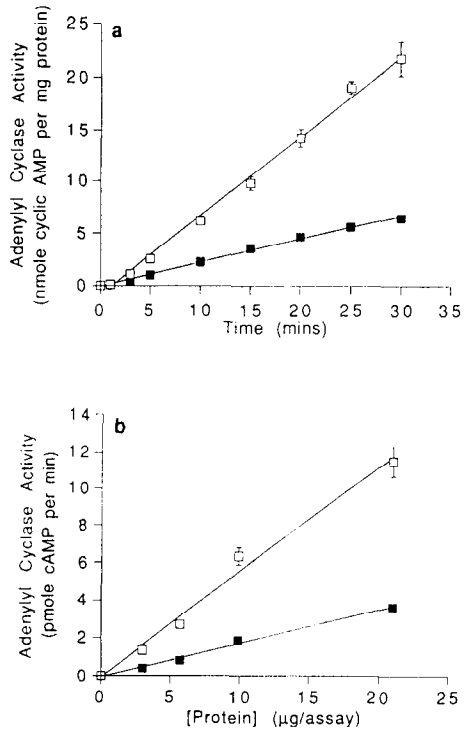

Fig. 1. (a) Linearity of assay conditions with time. The reaction was initiated by the addition of membranes (11.2 μg protein/assay) and cAMP production was measured over the times shown in the absence (■ = n) or presence (□ = o) of 100 μM forskolin. (b) Linearity of the assay with protein concentration. The reactions were started by addition of membranes and cAMP was measured for 15 min in the absence (■ = n) or presence (□ = o) of 100 μM forskolin.

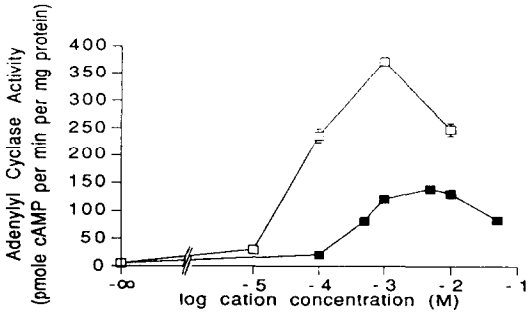

Fig. 2. Effects of divalent cations on goldfish brain adenylyl cyclase activity. cAMP production was measured under standard conditions with increasing concentrations of Mg^{2+} (■ = n) or Mn^{2+} (□ = o).

Mg^{2+} concentration (Fig. 3b). This inhibition is similar to that seen for adenyl cyclase from other species [18]. NaCl which does not inhibit mammalian adenylyl cyclase had no effect up to 100 mM (data not shown).

It has been reported that NaF can stimulate both

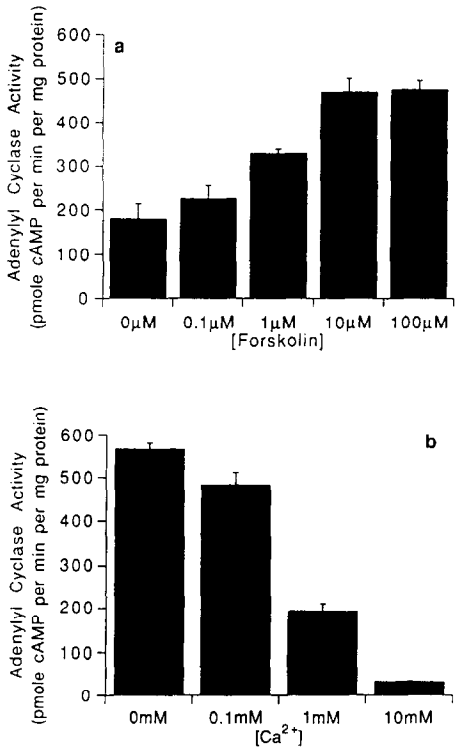

Fig. 3. (a) Dose–response of goldfish brain adenylyl cyclase activity to forskolin. Membranes were incubated with increasing concentrations of forskolin. (b) Effects of Ca^{2+} on forskolin (100 μM)-stimulated adenylyl cyclase activity.

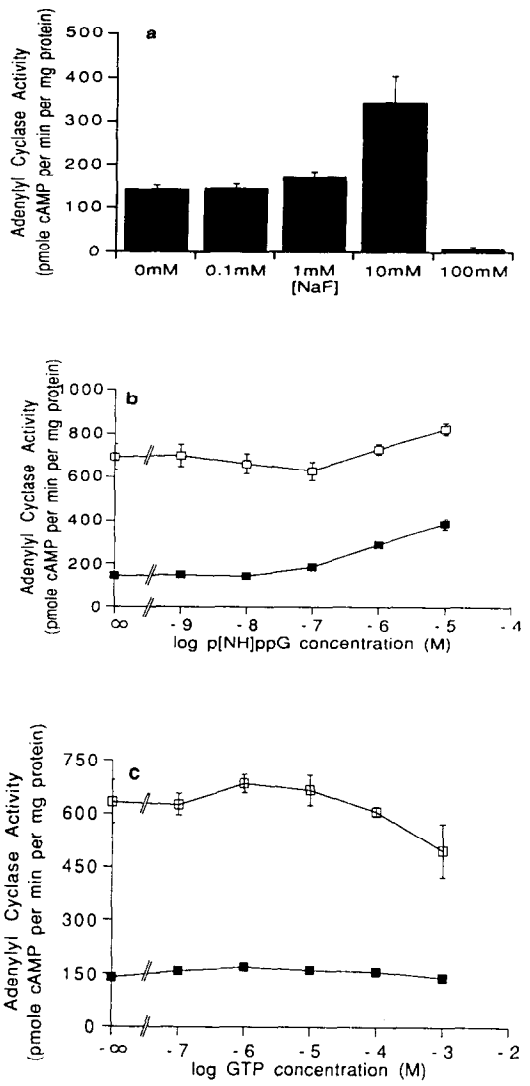

Fig. 4. (a) Effects of NaF on goldfish brain adenylyl cyclase activity. (b) Effects of the nucleotide analogue p[NH]ppG on goldfish brain adenylyl cyclase activity in the absence (■ = n) or presence (□ = o) of 100 μM forskolin. (c) Effects of GTP on goldfish brain adenylyl cyclase activity in the absence (■ = n) or presence (□ = o) of 100 μM forskolin.

inhibitory and stimulatory G-proteins (G_i and G_s*) by mimicking the terminal phosphate of GTP and thereby maintaining a stimulated conformation of the protein [19]. As shown in Fig. 4a 1 and 10 mM NaF produce a stimulation of the basal rate of cAMP production suggesting the involvement of G_s. NaF at 100 mM reduced activity to levels well below basal rates possibly by competitive inhibition by F^- at the ATP binding site (data not shown).

Guanine nucleotides were also used to investigate the presence or absence of G-proteins in this enzyme preparation. The effect of guanosine 5'-[$\beta\gamma$-imido]-triphosphate (p[NH]ppG) and GTP on both basal and forskolin-stimulated rates of cAMP production were assessed. p[NH]ppG produced a dose-dependent stimulation of the basal rate (Fig. 4b) at concentrations above 0.1 μM. At 0.1 μM p[NH]ppG a small inhibition of the forskolin-stimulated enzyme was observed followed by stimulation at higher concentrations. In contrast, GTP had little effect on the basal rate, giving rise only to a small stimulation of basal activity at a concentration of 1 μM which was seen on the forskolin-stimulated rate also. GTP inhibited the forskolin-stimulated rate at 1 mM but not at lower concentrations (Fig. 4c). Thus 100 μM GTP was used routinely in our assays, a concentration which should maximize agonist-stimulated effects but which had no effect on basal or forskolin-stimulated cyclase. Control experiments using glucagon to stimulate rat liver adenylyl cyclase elicited a 4-fold increase in cAMP production thereby confirming that our assay system was competent to show receptor-mediated changes in enzyme activity.

A wide range of agonists which are known to either stimulate or inhibit cAMP production in a variety of other species were tested for their effect on the goldfish brain adenylyl cyclase. Isoprenaline (β-adrenoceptors), dopamine (D1 and D2 receptors), 5-hydroxytryptamine (5-$HT_{1a,1d}$ and 5-HT_4 receptors), carbachol (M2 and M4 receptors), morphine

* Abbreviations: p[NH]ppG, guanosine 5'-[$\beta\gamma$-imido]-triphosphate; G_i, inhibitory guanine nucleotide binding protein; G_s, stimulatory guanine nucleotide binding protein.

(μ and δ opioid receptors) and glutamate, (1*S*, 3*R*)1-aminocyclopentane-1,3-dicarboxylic acid and kainate (putative matabotropic receptor) gave no more than 10% stimulation of basal or inhibition of forskolin-stimulated cAMP formation at 100 μM (data not shown).

Since Ca^{2+}/calmodulin has been shown to activate adenylyl cyclases especially from brain and in some cases has been cited as essential for agonist-dependent effects [18] we investigated whether 1 μM bovine calmodulin and 10 μM Ca^{2+} would support hormone effects on the goldfish brain adenylyl cyclase. The goldfish enzyme was not stimulated by Ca^{2+}/calmodulin and, furthermore, we observed no effects of either 100 μM carbachol, morphine, glutamate, kainate or (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid on either basal or forskolin-stimulated rates in the presence of Ca^{2+}/calmodulin (data not shown).

DISCUSSION

Previous studies have shown that there is a very high level of kainate binding sites in goldfish brain [8]. More recently, it has been reported that these binding sites are coupled to a pertussis toxin-sensitive G-protein, suggesting that they may represent a unique type of metabotropic glutamate receptor [10]. As many of the rat metabotropic receptors are known to be coupled to adenylyl cyclase [20, 21], we hypothesized that there might be a link between the kainate binding protein and this enzyme in goldfish. However, to our knowledge there have been no previous reports of adenylyl cyclase from goldfish brain. We have, therefore, characterized this enzyme from the membrane preparation commonly used for binding assays and have examined the effects of kainate and other glutamatergic ligands.

Using the conditions established in this study, the enzyme assay yielded a linear time course for at least 30 min. Similarly, assays were linear for the protein concentrations in the range 0–20 μg protein/assay. In agreement with adenylyl cyclase from other tissues the enzyme required divalent cations for activity and its activity is further enhanced when Mg^{2+} is present in excess of ATP [16]. Mn^{2+} can replace Mg^{2+} and will stimulate the enzyme to a greater extent but not as strongly as forskolin (Figs 2 and 3a).

Forskolin stimulated the rate of cAMP production by a mechanism that mainly involves the catalytic unit but which also has a G_s component and can be modulated by the presence of guanine nucleotides [17]. Consistent with other systems the goldfish brain membranes showed a dose-dependent cyclase response to forskolin (Fig. 3a). NaF also stimulated the basal rate of cAMP production. NaF is commonly used to stimulate G-proteins and binds to the G-proteins such that it remains permanently activated. The raised basal rate shown in Fig. 4a therefore represents G_s activity. The rate of cAMP production stimulated by forskolin was increased further by the addition of high concentrations of p[NH]ppG, and a low concentration of GTP. Conversely enzyme activity was decreased slightly by low concentrations of p[NH]ppG or high concentrations of GTP (Fig. 4b and c). This pattern of activity, particularly the biphasic nature of the forskolin-stimulated p[NH]-ppG dose–response curve, strongly suggests that both G_s and inhibitory guanine nucleotide binding protein (G_i) are present in goldfish brain membranes [17].

It is possible that freeze–thaw cycles in the membrane preparation may reduce the size of G_i inhibition of adenylyl cyclase. We therefore used a membrane preparation without freeze–thaw cycles to test the effects of this treatment. No greater reduction in forskolin-stimulated cAMP production by p[NH]ppG was observed over that displayed by the standard preparation (data not shown). Thus, only a small G_i activation can be demonstrated in goldfish brain membranes. Furthermore, lower temperatures have been reported to promote G_i effects [19]; however, in our system lowering the assay temperature to 25° did not result in the facilitation of G_i-coupled agonist effects (data not shown).

Calcium and calmodulin have a more important role in CNS adenylyl cyclase enzymes than in the periphery [22, 23]. Both calmodulin-dependent and -independent enzymes are present in the CNS [24]. High concentrations of Ca^{2+} did inhibit the production of cAMP in the goldfish CNS, as in mammalian CNS membrane preparations [18]. Nevertheless, it is possible that this is due to direct inhibition of the enzyme rather than a calmodulin-related event [Fig. 2b]. Unlike the effects observed in mammalian adenylyl cyclase systems, low concentrations of Ca^{2+} did not stimulate the enzyme even in the presence of 1 μM calmodulin. It is important to stress, however, that bovine calmodulin was used and the adenylyl cyclase in goldfish may require goldfish calmodulin.

The reasons for the lack of agonist-induced changes in goldfish adenylyl cyclase activity are unclear. The freeze–thaw washes that were used in the membrane preparation effectively removed all endogenous glutamate because these membranes bind kainate with characteristics similar to those reported previously [8] (data not shown). Thus, it is unlikely that "tone" due to endogenous glutamate could be masking the effects of applied agonists. Furthermore, control experiments were performed under identical assay conditions using the well-characterized adenylyl cyclase system in rat liver membranes. In those experiments glucagon raised the basal rate of cAMP production 4-fold with a half-maximal stimulation at 10 nM. The liver membranes were prepared by a protocol that avoids freeze–thaw washes which might affect receptor–G-protein coupling. However, goldfish brain membranes prepared in this way did not support agonist stimulation of the basal rate nor inhibition of forskolin-stimulated adenylyl cyclase (data not shown).

In conclusion, we have characterized a novel adenylyl cyclase in goldfish brain which is composed of both catalytic and modulatory components. Although goldfish G_s and G_i are functional we have not, as yet, been able to identify any neurotransmitter-mediated responses. These results suggest that some co-factor essential for the proper agonist-evoked

function of the adenylyl cyclase system in goldfish brain may have been removed or inactivated. One intriguing possibility is that this co-factor could be a goldfish calmodulin.

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