Alpha₁-adrenoceptor-mediated inhibition of cellular cAMP accumulation in neonatal rat ventricular myocytes*

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Summary. We studied adrenergic regulation of cellular cAMP in neonatal rat ventricular myocytes. Since cAMP content depends on synthesis, breakdown and egress, the contribution of each of these mechanisms was assessed. In the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, cAMP accumulation stimulated by the β-adrenoceptor agonist (-)-isoprenaline was diminished when the mixed α + β adrenoceptor agonist (-)-noradrenaline was coincubated with (-)-isoprenaline. Moreover, adenylyl cyclase activation stimulated by (-)-isoprenaline was decreased by (-)-noradrenaline and by the selective α₁-adrenoceptor agonists (-)-phenylephrine and methoxamine, suggesting that α-adrenoceptor agonism regulates cAMP metabolism through its effect on the synthetic pathway. Evidence for α₁-adrenoceptor mediation of this response was enhancement of (-)-noradrenaline-induced cAMP generation by the selective α₁-adrenoceptor antagonist terazosin (10 nmol/l). The selective α₂-adrenoceptor antagonist yohimbine (10 nmol/l) had no effect. The α₁-adrenoceptor mediated depression of (-)-isoprenaline-stimulated cAMP generation and adenylyl cyclase activation was prevented by terazosin and in separate experiments markedly enhanced by pertussis toxin pretreatment, suggesting involvement of a guanine-nucleotide regulatory protein in this process.

We conclude that in rat neonatal cardiac myocytes agonist occupation of the α₁-adrenoceptor inhibits β-adrenoceptor stimulated cAMP accumulation most likely by coupling to a guanine nucleotide inhibitory protein.

Key words: α₁-adrenoceptors – cAMP – Adenylyl cyclase – Rat myocytes

Introduction

Cyclic AMP is an important second messenger in numerous physiologic systems and is under careful moment-to-moment regulation. Agonists which stimulate an increase in cellular cAMP occupy β-adrenergic, prostaglandin E1, vasoactive intestinal peptide, adenosine A2 or glucagon receptors (Levitzki 1987). Conversely, occupation of α₂-adrenergic, muscarinic cholinergic, adenosine A1 or opiate receptors causes a decrease in cAMP accumulation in many cell systems (Levitzki 1987; Limbird 1988).

Adenylyl cyclase, the enzyme responsible for cAMP synthesis, is linked to its activating and inhibitory receptors through a family of guanine-nucleotide regulatory proteins, commonly termed Gs and Gi (Neer and Clapham 1988; Freissmuth et al. 1989; Simon et al. 1991). The involvement of these G proteins in the regulation of cAMP can be demonstrated experimentally by treatment of cells or membranes with either cholera toxin or pertussis toxin, both of which irreversibly ADP-ribosylate the α subunits of these regulatory proteins (Moss 1987). Pertussis toxin treatment prevents the inhibition of cAMP generation by α₂-adrenoceptor (Weiss et al. 1987) or muscarinic cholinergic receptors (Levitzki 1987) or adenosine A1 or opiate receptors causes a decrease in cAMP accumulation in many cell systems (Levitzki 1987; Limbird 1988).

It is generally accepted that α₂-adrenoceptors are important in the regulation of cAMP whereas α₁-adrenoceptors are thought to play no role in this process in most tissues (Limbird 1988). Cardiac tissue, particularly rat myocardium, is richly endowed with α₁-adrenoceptors (Karliner et al. 1979, 1982, 1985; Han et al. 1989; Endoh

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et al. 1991) which may be important in the regulation of myocardial growth and inotropy (Scholz et al. 1988; Endo et al. 1991; Simpson et al. 1991) and in the pathogenesis of ventricular arrhythmias (Culling et al. 1987). The role of the α₁-adrenoceptor in the regulation of cAMP in cardiac tissue is controversial. Watanabe and co-workers (1977) reported that α₁-adrenoceptor stimulation lowers the amount of cAMP generated through β-adrenoceptor agonist stimulation. They also observed that (−)-noradrenaline, a mixed α + β adrenoceptor agonist, generates less cAMP than the potent β-adrenoceptor agonist (−)-isoprenaline, suggesting a role for the α₁-adrenoceptor in the inhibition of cAMP accumulation.

Decreased quantities of cellular cAMP could be due either to diminished synthesis or increased breakdown. The inability of some investigators to demonstrate an effect of either methoxamine or pertussis toxin on cAMP accumulation in adult rat heart ventricular myocytes has led to the conclusion that the α₁-adrenoceptor is not linked directly to adenyl cyclase activity (Buxton and Brunton 1985). Furthermore it has been suggested that the effect of α₁-adrenoceptor agonism is via activation of a cyclic nucleotide phosphodiesterase resulting in enhanced breakdown of cAMP (Buxton and Brunton 1985). These conflicting observations have left the following questions unanswered: 1) is the α₁-adrenoceptor directly linked to cAMP regulation? 2) what is the mechanism for decreased cAMP accumulation when the α₁-adrenoceptor is occupied? and 3) is the guanine-nucleotide inhibitory protein (Gi) involved in the regulation of the cardiac myocyte cAMP generating system? In this report we demonstrate that the α₁-adrenoceptor is linked via a pertussis toxin inhibitable substrate to the regulation of cAMP. Furthermore, we show that accelerated breakdown of cAMP is not promoted by occupation of the α₁-adrenoceptor, nor does α₁-adrenoceptor agonism affect cyclic nucleotide phosphodiesterase activity in neonatal rat ventricular myocytes.

Materials and methods

Materials. Bromodeoxyuridine, acetic anhydride, dithiothreitol, 5'-guanylylimidodiphosphate, phosphocreatine, creatine phosphokinase, guanosine triphosphate, K₂PO₄, NAD, EDTA, sodium acetate, triethylamine, vitamin B₁₂, penicillin G, 3-isobutyl-l-methylxanthine, (-)-isoprenaline, (-)-noradrenaline, (-)-phenylephrine, methoxamine, gamma globulin, succinyl-cAMP, ATP, cAMP, ascorbic acid, snake venom (Ophiophagus hannah), ammonium acetate, glucagon, and sucrose were purchased from Sigma, St. Louis, Mo., USA. Neutral alumina (AG7, 100–200 mesh) was from BioRad. Trypsin was from Difco. Minimal essential medium (MEM) Eagle was from Gibco. Pertussis toxin was from List Biologicals. [¹²⁵I]-sodium iodide, [¹²³I]-iodocyanopindolol (ICYP), [⁴⁻¹⁵]₂⁻β-(4-hydroxyphenyl) ethylaminomethyl) tetralone (BE2254), [⁴⁻¹⁵]²⁻H-cAMP, [¹²⁵I]-adenosine, and [¹²³I]-NAD were from New England Nuclear. Adenosine 5'-[³²P] triphosphate, tetratriethylammonium salt was from Amersham. Calf serum was from Difco. Unlabeled BE2254, (–)-propranolol and terazosin were gifts from New England Nuclear, Ayerst and Abbott Laboratories, respectively. Antibodies to cAMP were a generous gift from Dr. Hunter Heath.

Cell culture. Cultures were composed of single, isolated cells prepared from hearts of day-old rats as described previously (Simpson 1983; Karliner et al. 1985). In summary, cells were obtained by brief, alternating cycles of room-temperature trypsinization and mechanical disaggregation. Cells were combined, washed, and preplated in the presence of 5% calf serum to reduce the number of contaminating nonmyocardial mesenchymal cells. After 30 min the still suspended muscle cells were removed from the attached nonmuscle cells, counted and diluted to ~86,000 viable cells per ml in culture medium with 5% calf serum. One ml of this cell suspension was distributed per well of 24-well culture tray (#3047, surface area 200 mm²/well). Additionally, 3 x 10⁶ cells were plated in 100 mm plastic culture dishes. Bromodeoxyuridine (BrDU) 100 µmol/l was present for the first 3 days of culture to prevent the proliferation of nonmyocardial cells. Greater than 90% of the culture consisted of cardiac myocytes with 85% viability as assessed by trypan blue dye exclusion. All cultures were maintained at 37 °C in humidified air containing 1% CO₂. The standard culture medium was minimal essential medium (MEM) Eagle with Hanks’ salt solution supplemented with 1.5 µmol/l vitamin B₁₂, 50 units/ml penicillin G, and 5% calf serum.

Addition of adrenergic agents. Cells (5–7 days in culture) were washed and incubated in 1 ml MEM without serum or bromodeoxyuridine. In experiments in which cAMP was measured, cells incubated in medium containing 1 mmol/l 3-isobutyl-l-methylxanthine (IBMX). Ten µl aliquots of each adrenergic agent, diluted to the appropriate concentration in 0.01 mol/l ascorbic acid (final concentration 100 µmol/l), were added to each well. At the end of 5 min, a time at which preliminary experiments demonstrated that stimulation had reached a plateau, medium was removed and cells processed for cAMP content.

Determination of cAMP. A modification of the radioimmunoassay method of Steiner et al. (1972) was used to detect cAMP in the range of 0.01 – 100 picomoles. Briefly, cellular cAMP was extracted with 3 ml 95% ethanol which was then evaporated in air. The residue was stored at −20 °C until assayed at which time it was dissolved in 300 µl 0.05 mol/l sodium acetate buffer, pH 6.2. One hundred µl samples in duplicate were assayed from each well and each test condition was represented by 3 wells; data are expressed as pmol/well. To detect cAMP in the range of 0.01 – 10 picomoles, the more sensitive acetylated assay was used. Prior to the addition of tracer and antibody, each sample was treated with 5 µl of acetylation mixture (200 µl triethylamine and 100 µl acetic anhydride). The acetylated assay was used for all experiments in which no phosphodiesterase inhibitor (IBMX) was present.

cAMP breakdown experiments. Washed cells were incubated in serum-free medium containing 100 µmol/l (−)-isoprenaline for 2 or 3 min. Further β-adrenoceptor stimulation was prevented by the addition of (−)-propranolol, final concentration 10 µmol/l. β₁-adrenoceptor agonists or antagonists were added and samples taken at various time intervals.

Phosphodiesterase assay. A modification of the method of Filburn and Karn (1973) was used to detect phosphodiesterase (PDE) activity. Cultured cells from three 100 mm plastic plates were washed and then rapidly frozen at −70 °C. After 5 min, the plates were thawed at room temperature; 1 ml of SET buffer (0.25 mol/l sucrose, 1 mmol/l EDTA, 5 mmol/l Tris, pH 7.4) was added, the cells were removed by scraping and suspended in a final volume of 1.5 ml SET and stored at −70 °C until assayed. Prior to assay, they underwent two additional freeze-thaw cycles to release any bound PDE. One-hundred µl of each cell preparation in triplicate was then used for the PDE assay. After preincubation at 30 °C for 5 min in PDE buffer (0.1 mol/l Tris-HCl, pH 7.7; 20 mmol/l D-glucose, 0.137 mol/l NaCl, 0.5 mmol/l MgCl₂), the substrate H⁻cAMP (−16,000 dpm) with 1 µmol/l cold cAMP (final concentration) in the buffer was added. The mixture was incubated for 15 min at 30 °C and the reaction stopped by the addition of 10 µl of 10 mmol/l Tris-EDTA, pH 7.4 and boiling for 90 s. The H⁻adenosine formed was then hydrolyzed by incubation with 100 µl of snake venom (Ophiophagus hannah, 1 mg/ml) at 30 °C for 30 min. The reaction was stopped by boiling for 60 s. Samples were cooled and 14C-adenosine (3–6,000 dpm) was added to monitor recovery. The reaction mixture (250 µl) was then placed on a neutral alumina column (1.5 – 1.7 g) which