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ANGIOTENSIN II RECEPTORS NEGATIVELY COUPLED TO ADENYLATE CYCLASE IN RAT MYOCARDIAL SARCOLEMMA

INVOLVEMENT OF INHIBITORY GUANINE NUCLEOTIDE REGULATORY PROTEIN*

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Abstract—The effect of angiotensin II (AII) on adenylate cyclase was studied in the rat and rabbit heart sarcolemma. AII inhibited adenylate cyclase activity in the rat and rabbit sarcolemma in a concentration-dependent manner. Maximal inhibition of about 35–40% was observed in the rat, with an apparent K_i of about 3 nM; about 30% inhibition, with an apparent K_i of about 6 nM, was noted in rabbit sarcolemma. The inhibitory effect of AII was dependent on the presence of guanine nucleotides and was blocked by saralasin. In addition, AII also inhibited the stimulatory effects of isoproterenol and glucagon on adenylate cyclase. Ninhibin, a sperm factor which has been shown to modify the characteristics of inhibitory guanine nucleotide regulatory protein (G_i), attenuated the inhibitory effects of AII on basal and hormone-sensitive adenylate cyclase. Furthermore, pertussis toxin (PT) treatment of the sarcolemma in the presence of [^{32}P]NAD resulted in ADP-ribosylation of a single 41-kD protein. PT also attenuated the AII-mediated inhibition of basal and hormone-sensitive adenylate cyclase and enhanced the magnitude of the stimulatory effects of isoproterenol and glucagon on adenylate cyclase activity. These data suggest that the rat myocardial sarcolemma contains AII receptors that are negatively coupled to adenylate cyclase through G_i protein.

The adenylate cyclase system is composed of three components: receptor, catalytic subunit, and stimulatory (G_s‡) and inhibitory (G_i) guanine nucleotide regulatory proteins. These binding proteins act as transducers and, in the presence of guanine nucleotides, transmit the signal from the hormone-occupied receptor to the catalytic subunit. The stimulation and inhibition of adenylate cyclase by hormones are mediated through the G_s and G_i proteins of adenylate cyclase respectively [1]. The guanine nucleotide regulatory proteins are also targets of bacterial toxins that are useful probes for defining the interaction of the regulatory proteins with other components of the adenylate cyclase system. Bacterial toxins, such as cholera toxin and pertussis toxin (PT), have been shown to ADP-ribosylate in the presence of [α-^{32}P]NAD, the α-subunits of guanine nucleotide regulatory proteins [G_s (45,000), G_i (40,000–41,000) and G_o (39,000)] and, thereby, modify the characteristics of these proteins [2–10]. Cholera toxin irreversibly activates G_s protein, mediating the stimulation of adenylate cyclase, whereas PT in addition to G_o, acts on G_i protein, which regulates inhibition, and attenuates the GTP-dependent and receptor-mediated inhibition of adenylate cyclase [9]. The functions of G_o protein are not yet known, but it may interact with enzymes associated with Ca^{2+} mobilization and not with adenylate cyclase [11].

Angiotensin II (AII), a vasoactive peptide, has been shown to produce vasoconstriction of vascular and non-vascular smooth muscle and exerts positive inotropic and chronotropic effects in mammalian heart muscle [12, 13]. Various studies have demonstrated that the effect of AII on target tissues is mediated through its interaction with receptor sites on the membranes [14]. Angiotensin receptors have been found in several tissues [15–19]. The inhibition of adenylate cyclase by AII has been demonstrated in the kidney [20], adrenal cortex [19], liver [18, 21] and vascular smooth muscle [22]. By using [^{125}I]AII, two classes of AII-binding sites have been reported in the rabbit ventricular myocardium [23]. Although the affinities of both these binding sites for AII were shown to be decreased by guanine nucleotides [23], suggesting the association of the receptor with guanine nucleotide regulatory proteins, no effect of AII on adenylate cyclase could be demonstrated. We undertook the present studies to investigate whether angiotensin receptors in the heart are also coupled to the adenylate cyclase system and whether guanine nucleotide regulatory proteins are involved in the

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‡ Abbreviations: AII, angiotensin II; PT, pertussis toxin; G_s, stimulatory guanine nucleotide regulatory protein; G_i, inhibitory guanine nucleotide regulatory protein; SDS, sodium dodecyl sulfate; GTPγS, guanosine 5′-O-(thiotriphosphate); NAD, nicotinamide adenine dinucleotide; and EGTA, ethyleneglycolbis(amino-ethylether)tetraacetate.

coupling of AII receptors to adenylate cyclase. We report that: (1) AII inhibited adenylate cyclase in the rat myocardial sarcolemma; and (2) by using ninhibin [24] and PT, we demonstrated that AII receptors are coupled to adenylate cyclase through G_i protein.

MATERIALS AND METHODS

Isolation of rat and rabbit heart sarcolemma

The heart sarcolemma was isolated essentially according to the method described earlier [25]. Male Sprague–Dawley rats (250–300 g) or rabbits were decapitated, and their hearts were quickly removed and placed in ice-cold 10 mM Tris–HCl buffer, pH 7.4. The ventricles were washed thoroughly, cut into small pieces, and homogenized in a Virtis blender for 30 sec in 10 vol. of 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA. The homogenate was filtered through cheesecloth and centrifuged at 1000 *g* for 10 min. The sediment was suspended in 20–25 vol. of 10 mM Tris–HCl buffer, pH 7.4, stirred in a cold room for 30 min, and centrifuged at 1000 *g* for 10 min. This process was repeated two more times, first by suspending the sediment in 10 mM Tris–HCl buffer, pH 8.0, and then in the same buffer but at pH 7.4. The sediment was then suspended in 20–25 vol. of 10 mM Tris–HCl, pH 7.4, extracted with 0.4 M LiBr for 45 min, and centrifuged at 1000 *g* for 10 min. The sediment was again suspended in 10 mM Tris–HCl, pH 7.4, stirred for 15–20 min, and centrifuged at 1000 *g* for 10 min. The sarcolemmal fraction thus obtained was suspended in a buffer containing 10 mM Tris–HCl, 1 mM dithiothreitol (DTT) and 1 mM EDTA, pH 7.4, and used for adenylate cyclase determination.

As reported earlier [26], the sarcolemmal fraction prepared by the hypotonic shock–LiBr treatment contained negligible amounts (2–4%) of contamination by other subcellular organelles, such as mitochondria, sarcoplasmic reticulum, and myofibrils. In addition, this preparation was also devoid of endothelial cells, since angiotensin-converting enzyme activity (a marker for endothelial cells) was absent in this sarcolemmal fraction [25].

ADP-ribosylation of heart sarcolemma

ADP-ribosylation of heart sarcolemma was performed, as described previously [27], by the method of Burns *et al.* [28]. The heart sarcolemma (20–30 μg protein) was incubated in 25 mM glycylglycine buffer, pH 7.5, containing 15 μM [α^{32}P]NAD (~20 μCi/ml), 0.4 mM ATP, 0.4 mM GTP, 15 mM thymidine, 10 mM DTT and ovalbumin (0.1 mg/ml) with and without PT (5 μg/ml) for 30 min at 37° in a total volume of 100 μl. The reaction was terminated by the addition of 20 μl of a stop mix containing 5% SDS and 50% β-mercaptoethanol. The contents were heated for 10 min in a boiling water bath. The labeled proteins were analyzed by subjecting the aliquots to SDS-polyacrylamide gel electrophoresis by the method of Laemmli [29] with 12% SDS-polyacrylamide gels. After electrophoresis, the gels were fixed, stained, destained, dried and autoradiographed by exposure to Kodak XAR-5 film, as described previously [30]. They were calibrated by using molecular weight standards (Pharmacia): phosphorylase *b* (M_r = 94,000), albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), trypsin inhibitor (M_r = 20,000), and α-lactalbumin (M_r = 14,400).

To study the effects of PT on adenylate cyclase activity, PT treatment was performed in two different ways, as described previously [27]:

In vivo *studies.* Male Sprague–Dawley rats (100 g, 40 days old) were injected intraperitoneally with PT (1.5 μg) in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.05 M NaCl or vehicle, and killed 48 hr later. The hearts were removed, and the sarcolemma was prepared as described above.

In vitro *studies.* The heart sarcolemma was pretreated with or without (control) PT for 30 min at 30° in the same reaction mixture as described above for ADP-ribosylation except that 1 mM NAD was used instead of [α^{32}P]NAD. It was washed two to three times with 10 mM Tris, 1 mM EDTA buffer, pH 7.5, finally suspended in the same buffer, and used for adenylate cyclase activity determination. Preincubation of heart sarcolemma at 30° for 30 min in the absence or presence of PT resulted in a significant loss of enzyme activity (~60%) which was independent of the presence of PT in the incubation medium (adenylate cyclase activity before and after preincubation was 220 ± 15 and 76 ± 4 pmol cAMP (mg protein · 10 min)$^{-1}$). However, the percent inhibition of adenylate cyclase by AII remained unchanged (data not shown).

Adenylate cyclase activity determination

Adenylate cyclase activity was determined by measuring [^{32}P]cAMP formation from [α^{32}P]ATP, as described previously [27, 31]. The assay medium contained 50 mM glycylglycine, pH 7.5, 0.5 mM MgATP, [α^{32}P]ATP (1–1.5 × 10^6 cpm), 5 mM $MgCl_2$ (in excess of the ATP concentration), 100 mM NaCl, 0.5 mM cAMP, 1 mM 3-isobutyl-1-methylxanthine, 0.1 mM EGTA, 10 μM GTPγS, and an ATP-regenerating system consisting of 2 mM creatine phosphate, 0.1 mg creatine kinase/ml, and 0.1 mg myokinase/ml in a final volume of 200 μl. Incubations were initiated by the addition of the heart sarcolemma (30–70 μg) to the reaction mixture which had been thermally equilibrated for 2 min at 37°. The reactions, conducted in triplicate for 10 min at 37°, were terminated by the addition of 0.6 ml of 120 mM zinc acetate. cAMP was purified by coprecipitation of other nucleotides with $ZnCO_3$, by the addition of 0.5 ml of 144 mM Na_2CO_3 and subsequent chromatography by the double column system, as described by Salomon *et al.* [32]. Under the assay conditions used, adenylate cyclase activity was linear with respect to protein concentration and time of incubation.

Protein was determined essentially as described by Lowry *et al.* [33], with crystalline bovine serum albumin as standard.

Materials

ATP, cAMP and isoproterenol were purchased from Sigma (St. Louis, MO, U.S.A.); creatine kinase (EC 2.7.3.2), myokinase (EC 2.7.4.3) and GTPγS were from Boehringer Mannheim, Montreal,

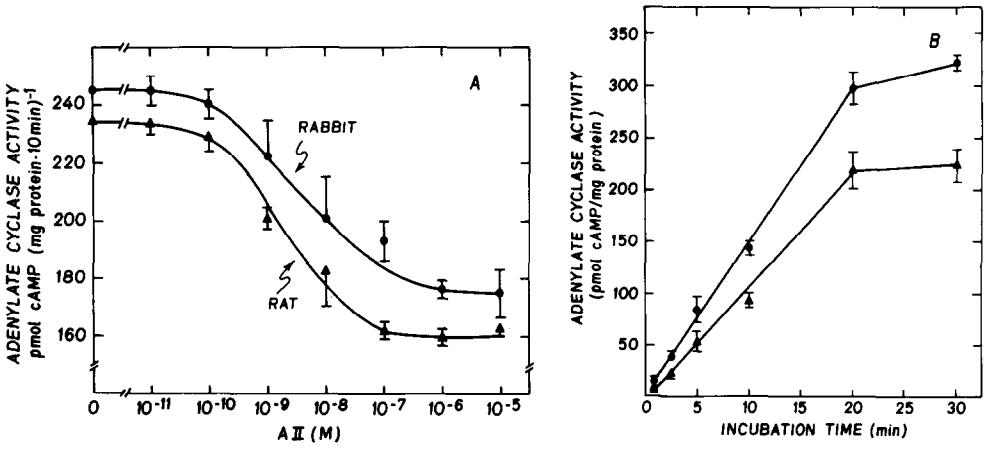

Fig. 1. (A) Effect of various concentrations of angiotensin II on adenylate cyclase activity in heart sarcolemma from rat (▲—▲) and rabbit (●—●). Adenylate cyclase activity was measured as described in Materials and Methods. Values are the means ± SEM of three separate experiments. (B) Effect of time of incubation on adenylate cyclase activity in the absence (●—●) and presence of 10 μM AII (▲—▲). Adenylate cyclase activity was measured as described in Materials and Methods. Values are the means ± SEM of three separate experiments.

Quebec, Canada. [α^{32}P]ATP was from Amersham, Oakville, Ontario, Canada, and [α-^{32}P]NAD from DuPont Canada, Mississauga, Ontario, Canada. The electrophoresis chemicals were obtained from Bio-Rad Laboratories, Mississauga, Ontario, Canada; AII and saralasin were from Peninsula Laboratories Inc., Belmont, CA, U.S.A., and PT was from List Biochemicals, Campbell, CA, U.S.A. Ninhibin was donated by Dr. Roger A. Johnson of the State University of New York, Stony Brook, NY, U.S.A.

RESULTS

Effect of AII on adenylate cyclase

As shown in Fig. 1A, AII inhibited adenylate cyclase activity in the rat and rabbit sarcolemma in a concentration-dependent manner. Maximal inhibition of about 35–40% was observed in the rat with an apparent K_i of about 3 nM, whereas about 30% inhibition with an apparent K_i of about 6 nM was noted in the rabbit sarcolemma. The inhibition of adenylate cyclase by AII was reversed completely by 10 μM saralasin, an AII antagonist (Table 1). These data suggest that AII receptors are present in the rat and rabbit myocardium and are negatively coupled to adenylate cyclase.

To investigate if the AII-induced inhibition of adenylate cyclase was dependent on the time of incubation, enzyme activity in the absence and presence of AII (10^{-5} M) was determined at various time intervals. The results in Fig. 1B indicate that enzyme activity was linear up to 20 min, but the percent inhibition (~30–35%) by AII remained unaltered for up to 30 min.

Requirement of guanine nucleotides in mediating the inhibitory effect of AII on adenylate cyclase

Figure 2 demonstrates the effect of AII on adenylate cyclase in the absence and presence of various concentrations of GTPγS. Basal adenylat cyclase activity was increased with up to 10 μM GTPγS (~4-fold stimulation), and then declined. In the absence of GTPγS, AII was unable to elicit any inhibitory effect on adenylate cyclase, whereas in the presence of GTPγS it suppressed enzyme activity. Maximal inhibition (~35%) was observed at 10 μM GTPγS (inset) decreasing at higher concentrations of GTPγS. Only ~10% inhibition was observed at 50 μM, with complete abolition occurring at 100 μM. The suppressive effect of higher concentration of GTPγS on AII-mediated inhibition may have been due to the presence of NaCl in the assay mixture, as

Table 1. Effect of saralasin [Sar^1 Ala^8]-angiotensin II on angiotensin-induced inhibition of adenylate cyclase in rat myocardial sarcolemma

Addition	Adenylate cyclase activity [pmol cAMP (mg protein · 10 min)$^{-1}$]
None	210 ± 5
Angiotensin II (AII) (10 μM)	145 ± 10
AII + saralasin (10 μM)	212 ± 2

Adenylate cyclase activity was determined in the presence of 100 mM NaCl and 10 μM GTPγS as described in Materials and Methods. Values are the means ± SEM of three experiments each done in triplicate.

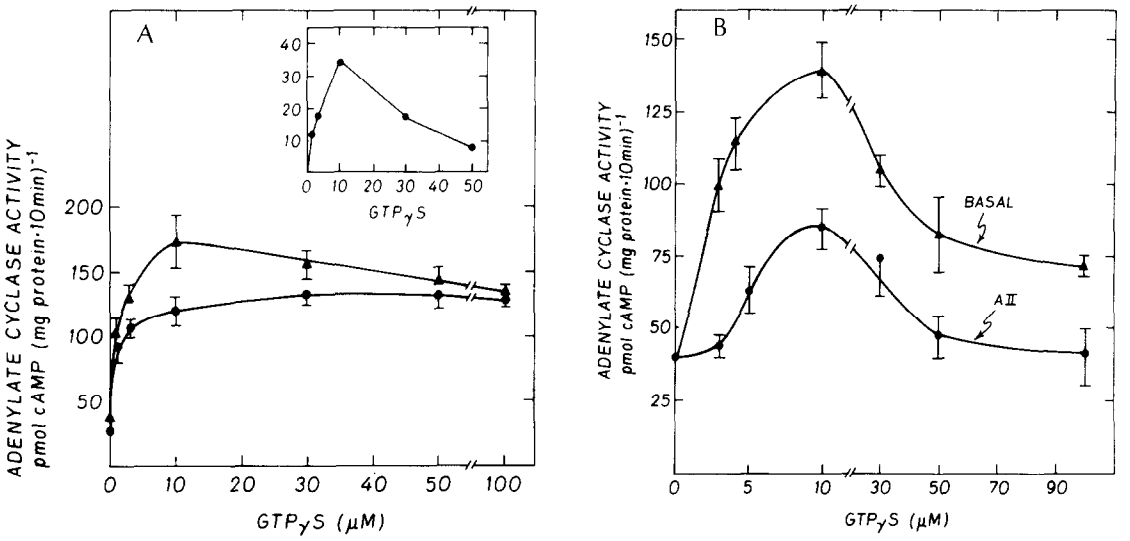

Fig. 2. Dependence on guanine nucleotide of adenylate cyclase inhibition by angiotensin II in rat heart sarcolemma. Adenylate cyclase activity was determined as described in Materials and Methods in the presence of increasing concentrations of GTPγS alone (▲—▲) or in combination with 10 μM angiotensin II (●—●) in the presence (A) or absence (B) of 100 mM NaCl. Inset shows the percent inhibition of adenylate cyclase by angiotensin II in the presence of various concentrations of GTPγS. Values are the means ± SEM of three separate experiments.

such inhibition was not evident in the absence of NaCl, as shown in Fig. 2B. These data suggest that guanine nucleotide regulatory protein may be required for the coupling of AII receptors to adenylate cyclase in the heart sarcolemma.

Effect of monovalent cations on AII-mediated inhibition of adenylate cyclase

The requirement of monovalent cations has been demonstrated for the expression of the inhibitory actions of hormones on adenylate cyclase [18–20, 22]. In the heart sarcolemma, AII was able to depress adenylate cyclase activity in the absence of monovalent cations, but in the presence of NaCl, the degree of inhibition was increased to a small extent (by about 5–10%, data not shown).

Effect of AII on hormone-stimulated adenylate cyclase activity

Isoproterenol and glucagon stimulated adenylate cyclase activity to various extents. However, AII markedly reduced isoproterenol- and glucagon-sensitive adenylate cyclase activity as shown in Table 2. The ability of AII to suppress hormone-sensitive adenylate cyclase activities has also been noted in other tissues [20–22].

Effect of ninhibin on myocardial adenylate cyclase activity

Ninhibin, a factor isolated from bovine sperm [24], has been shown recently to activate adenylate cyclase activity in platelets, brain membranes [34, 35] and aortic vascular smooth muscle [31]. In addition, it has also been found to attenuate the α-adrenergic inhibition of platelet adenylate cyclase [35] and atrial natriuretic factor-mediated inhibition of aortic adenylate cyclase [31] by blocking the G_i protein of adenylate cyclase.

Since guanine nucleotides are required to elicit the inhibitory effect of AII on adenylate cyclase in the heart, it is likely that G_i protein is involved in the coupling of AII receptors to adenylate cyclase. To

Table 2. Interaction of angiotensin II with some agonists

Additions	Adenylate cyclase activity [pmol cAMP (mg protein · 10 min)$^{-1}$]	Percent inhibition by AII
None	148 ± 6	
AII (10 μM)	86 ± 4	42
Isoproterenol (50 μM)	313 ± 6	
Isoproterenol + AII	232 ± 31	26
Glucagon (1 μM)	259 ± 11	
Glucagon + AII	216 ± 9	17

Adenylate cyclase activity was determined in the presence of 100 mM NaCl and 10 μM GTPγS as described in Materials and Methods. Values are the means ± SEM of three separate experiments each done in triplicate.

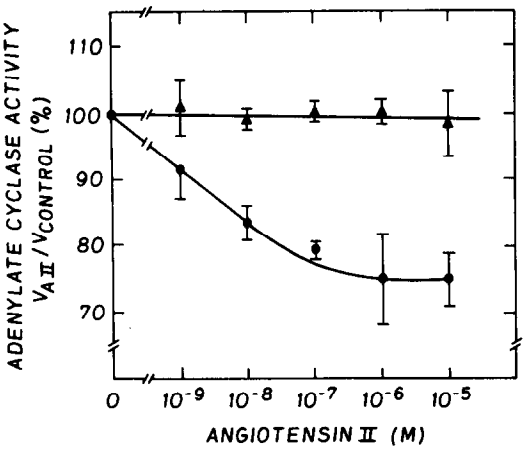

Fig. 3. Effect of ninhibin on angiotensin II-mediated inhibition of adenylate cyclase in rat heart sarcolemma. Adenylate cyclase activity was determined in the presence of various concentrations of angiotensin II alone (●—●) or in combination with 2 μg ninhibin (▲—▲). The heart sarcolemma was preincubated with and without ninhibin for 5 min at 37°. The determination of enzyme activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min as described under Materials and Methods. Indicated on the ordinate is the adenylate cyclase activity as % of control activity measured in the absence of angiotensin II. In the absence of angiotensin II, adenylate cyclase activity was 163 ± 5 and 295 ± 7 pmol cAMP formed per mg protein per 10 min for control and ninhibin-treated membranes respectively. The values are the means ± SEM of three separate experiments.

determine the above possibility, we studied the action of ninhibin on adenylate cyclase activity inhibited by AII. Figure 3 shows that AII suppressed adenylate cyclase in a concentration-dependent manner in control heart sarcolemma, with maximal inhibition (~25%) being observed at 1 μM. However, in membranes treated with ninhibin, the inhibitory effect of AII was abolished completely. These data suggest that AII receptors may be coupled to adenylate cyclase through G_i protein.

Table 3 illustrates the effects of ninhibin treatment on the AII-mediated inhibition of hormone-stimulated adenylate cyclase activity. Ninhibin, as reported previously [31, 34, 35], stimulated basal enzyme activity by about 2-fold, and also potentiated the stimulatory effects of isoproterenol and glucagon on adenylate cyclase activity in the rat heart sarcolemma. However, the AII-mediated inhibition of basal, isoproterenol- and glucagon-stimulated enzyme activity in control membranes was blocked almost completely by ninhibin treatment. These data are in agreement with our previous findings in the rat aorta [31] and indicate that AII receptors in the rat myocardial sarcolemma are coupled to adenylate cyclase through G_i protein. Since the mechanism by which ninhibin inactivates G_i protein is not yet clear, we used PT, which has been shown to ADP-ribosylate G_i protein and thereby inactivates it.

Effect of PT on adenylate cyclase

Figure 4 depicts the effect of PT on AII-mediated inhibition of adenylate cyclase. AII, as shown before (Fig. 1), inhibited enzyme activity in a concentration-dependent manner in control (untreated) membranes, but this action was blocked completely in PT-treated membranes. These data strongly suggest that G_i protein mediates the ability of AII to inhibit adenylate cyclase. Similar results were also observed when PT was administered under *in vivo* conditions (data not shown).

Table 4 demonstrates the effect of PT on isoproterenol- and glucagon-stimulated adenylate cyclase activity in the absence and presence of AII. Isoproterenol and glucagon elevated adenylate cyclase activity in control heart sarcolemma by about 100 and 60%, respectively, whereas, in PT-treated membranes, basal enzyme activity was increased markedly, and the stimulatory effects of isoproterenol and glucagon were enhanced by about 75 and 225% respectively. A similar enhancement of hormone-stimulated adenylate cyclase activity by PT

Table 3. Effect of ninhibin on angiotensin II-mediated inhibition of basal, isoproterenol- and glucagon-stimulated adenylate cyclase activity in rat heart sarcolemma

Additions	Adenylate cyclase activity [pmol cAMP (mg protein · 10 min)$^{-1}$]	
	Control	Ninhibin
None	161 ± 11	349 ± 4
Isoproterenol (50 μM)	268 ± 16	557 ± 26
Glucagon (1 μM)	264 ± 27	481 ± 32
AII (10 μM)	113 ± 2	335 ± 6
Isoproterenol + AII	220 ± 2	540 ± 31
Glucagon + AII	185 ± 2	539 ± 23

The heart sarcolemma was preincubated with and without ninhibin (2 μg) for 5 min at 37°. The determination of enzyme activity was initiated by the addition of cyclase reaction mixture. The reaction was terminated after 10 min as described in Materials and Methods. Values are the means ± SEM of three experiments each done in triplicate.

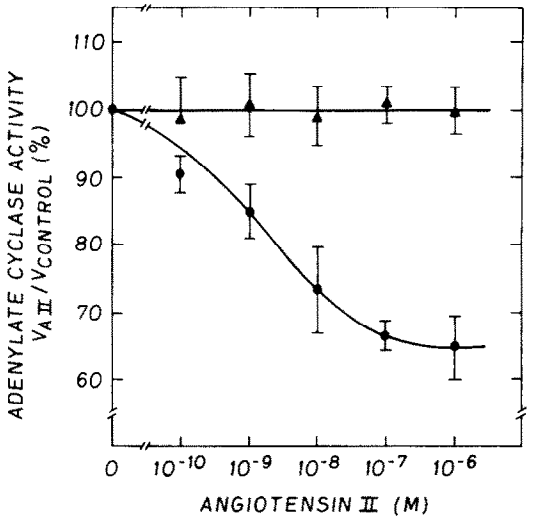

Fig. 4. Effect of PT on angiotensin II-mediated inhibition of adenylate cyclase in rat heart sarcolemma treated without (control) or with PT, as described in Materials and Methods. Adenylate cyclase activity was determined in the absence and presence of various concentrations of angiotensin II in untreated control (●—●) and PT-treated (▲—▲) heart sarcolemma in the presence of 10 μM GTPγS. The values are the means ± SEM of three separate experiments. Basal enzyme activity in control and PT-treated heart sarcolemma was 101 ± 9 and 107 ± 6 pmol of cAMP (mg protein · 10 min)$^{-1}$ respectively.

treatment has been reported previously [27–36]. In addition, AII inhibited basal, isoproterenol- and glucagon-stimulated enzyme activity in control membranes, and this inhibitory action was abolished in PT-treated membranes. Similar results were obtained under *in vitro* conditions (data not shown). These results also suggest the involvement of G_i protein in the coupling of AII receptors to adenylate cyclase.

Table 4. Effect of pertussis toxin on the stimulatory and inhibitory response of hormones in rat myocardial sarcolemma

Additions	Adenylate cyclase activity (% activity)	
	Control	PT-treated
None	100	100
Isoproterenol (50 μM)	211	295
Glucagon (1 μM)	161	298
Angiotensin II (10 μM)	58	119
Isoproterenol + AII	156	285
Glucagon + AII	145	296

Adenylate cyclase activity was determined in the presence of GTPγS as described in Materials and Methods. Rats were injected intraperitoneally with pertussis toxin (1.5 μg) (PT-treated) in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.05 M NaCl or vehicle (control), and killed 48 hr later. The hearts were removed, and heart sarcolemma was isolated as outlined in Materials and Methods. Values are the means of three separate experiments. Basal enzyme activity was taken as 100%. The basal enzyme activity in control and PT-treated (injected) rats was 87 ± 7 and 148 ± 6 pmol cAMP (mg protein · 10 min)$^{-1}$ respectively.

It should be noted that the effect of PT treatment on basal adenylate cyclase activity *in vivo* (Table 4) was different from that observed under *in vitro* conditions (Fig. 4). PT stimulated basal adenylate cyclase activity by about 75% *in vivo*, whereas it did not exert any significant action *in vitro*. This difference may have been due to the fact that, under *in vitro* conditions, the membranes were treated with PT for a short period of time (30 min as compared to 48 hr), which may not have been sufficient to alter the functions of G_i protein to the same extent as may have occurred *in vivo*.

ADP-ribosylation of guanine nucleotide regulatory protein by PT

To establish the presence of PT substrate in heart membranes, ADP-ribosylation of heart sarcolemma was studied, and the results are shown in Fig. 5. Incubation of heart sarcolemma with [α-^{32}P]NAD and PT resulted in the predominant labeling of a protein band with an apparent molecular weight of 41,000 (Fig. 5) which was essentially the only toxin substrate detected under our experimental conditions. On the other hand, ADP-ribosylation of this protein was not observed when heart sarcolemma from PT-injected rats was treated with PT and [α-^{32}P]NAD (data not shown), indicating that it had been already ADP-ribosylated.

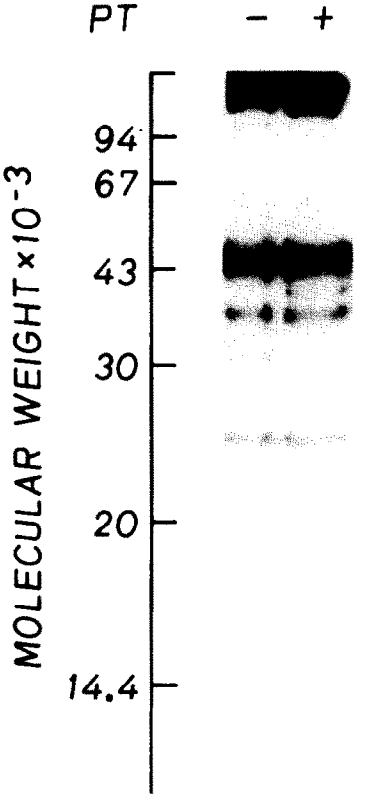

Fig. 5. Autoradiograph showing pertussis toxin-catalyzed ADP-ribosylation of rat heart sarcolemma incubated with [α-^{32}P]NAD in the absence [lane 1, (−)] or presence of 5 μg/ml PT [lane 2, (+)]. The ^{32}P-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography as described in Materials and Methods.

DISCUSSION

The presence of angiotensin receptors has been demonstrated in several tissues [15–19]. In the liver, anterior pituitary and adrenal cortex, angiotensin can stimulate phosphatidylinositol breakdown [37–40] and inhibit adenylate cyclase. These data indicate that angiotensin receptors in membranes are coupled to at least two transducing systems, adenylate cyclase complex and phosphatidylinositol 4,5-bisphosphate phosphodiesterase (phosphalipase C) which splits phosphatidylinositol 4,5-bisphosphate into inositol triphosphate and diacylglycerol. The presence of two populations of angiotensin receptors (high and low affinity) has also been noted in the rabbit ventricular myocardium by radioligand-binding techniques [23], which suggests that angiotensin receptors in the heart may also be coupled to two distinct transducing systems. In the current studies, we demonstrated that one of these two systems to which angiotensin receptors are coupled is the adenylate cyclase system. Its inhibition by AII in heart sarcolemma suggests that angiotensin receptors are negatively coupled to adenylate cyclase. The high-affinity angiotensin receptors with a K_d of 2.4×10^{-9} M, determined by radioligand-binding studies [23], may be coupled to the adenylate cyclase system since the apparent K_i for adenylate cyclase inhibition was also found to be in the same range (1–5 nM) in the present studies. The inability of other investigators [23] to demonstrate the coupling of angiotensin receptors to adenylate cyclase could be due to differences in membrane isolation techniques and assay conditions. However, our results are in agreement with reports on other tissues [18–22] in which AII also inhibited adenylate cyclase activity. Furthermore, the inhibition by AII was specific, since it was antagonized by saralasin, which is an antagonist of angiotensin action. The inhibition of hormone-stimulated adenylate cyclase and cAMP by AII has been reported by several investigators [20–22]. We demonstrated in our studies that AII also inhibited isoproterenol- and glucagon-sensitive adenylate cyclase activity in myocardial sarcolemma. The inhibition of myocardial adenylate cyclase was also dependent on the presence of guanine nucleotides, suggesting that G_i protein may be involved in the coupling of angiotensin receptors to adenylate cyclase.

Ninhibin treatment, which has been shown to inactivate or inhibit G_i protein, resulted in the enhancement of basal and hormone-stimulated enzyme activity. These data are consistent with earlier reports on platelet and aorta membranes [31, 34] in which ninhibin treatment augmented basal, GTP- and agonist-stimulated enzyme activity. These results indicate that ninhibin treatment in heart sarcolemma also inactivates or modifies G_i protein function. This notion is further substantiated by the fact that ninhibin was also able to attenuate the AII-mediated inhibition of basal as well as isoproterenol- and glucagon-stimulated enzyme activity. The mechanism by which ninhibin inactivates G_i protein is not yet clear, but it has been shown that ninhibin treatment clips off a small fragment ($M_r \sim 2000$) from $G_i\alpha$* which may be an important factor for the coupling of inhibitory hormone receptors to adenylate cyclase. However, the $\beta\gamma$ complex of G_i protein is not affected by such treatment.* From these data, it can be concluded that G_i protein is involved in the coupling of AII receptors to adenylate cyclase in heart sarcolemma.

Furthermore, when G_i protein was inactivated by PT treatment, AII was no longer able to inhibit adenylate cyclase activity stimulated by isoproterenol or glucagon. In addition, the ability of AII to interact with its receptor and inhibit adenylate cyclase was abolished completely. These results are consistent with those obtained in other systems [40, 41] and indicate that AII inhibition of adenylate cyclase in rat heart sarcolemma is also mediated via G_i protein.

PT-catalyzed ADP-ribosylation of heart sarcolemma resulted in the labeling of a single protein band with an apparent molecular weight of 41,000, indicating that the heart ventricular sarcolemma has only one toxin substrate. Our data are not in agreement with the previous results of Malbon *et al.* [42] and Pobiner *et al.* [41], showing the presence of two toxin substrates of 40,000 and 41,000 molecular weight in the rabbit heart and 41,000 and 39,000 in the chick heart respectively. These discrepancies may be due to differences in species or in their preparations: the whole heart was used, whereas in our studies only the ventricular sarcolemma was employed.

In conclusion, the present studies demonstrate that angiotensin receptors in heart sarcolemma were negatively coupled to adenylate cyclase through an inhibitory guanine nucleotide regulatory protein (G_i). It is suggested that the positive inotropic effect of AII on the heart may not be mediated through an increase in cAMP levels and may involve the PI turnover system. Alternatively, the decrease in cAMP by AII may be associated with the regulation of Ca^{2+} movements which exert positive inotropic effects on the heart.

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