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BETA$_1$-ADRENERGIC SELECTIVITY OF THE NEW CARDIOTONIC AGENT DENOPAMINE IN ITS STIMULATING EFFECTS ON ADENYLATE CYCLASE

MASANORI INAMASU, TETSUYA TOTSUKA, TOMIHIRO IKEO, TAKU NAGAO and SHIGEYUKI TAKEYAMA

Biological Research Laboratory, Tanabe Seiyaku Co., Ltd., Toda, Saitama 335, Japan

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Abstract—Effects of the new selectively β_1-adrenergic cardiotonic drug denopamine (TA-064), (−)-(*R*)-1-(*p*-hydroxyphenyl)-2-[(3,4-dimethoxyphenethyl)amino]ethanol, on the adenylate cyclase-adenosine-3′,5′-monophosphate (c-AMP) system of various tissues and cells in rats and guinea pigs were investigated in comparison with those of isoproterenol.

Denopamine at concentrations above 10^{-6} M stimulated lipolysis *in vitro*, and, above 10^{-5} M, elevated the c-AMP level in isolated rat fat cells. The c-AMP level of guinea-pig heart ventricular muscle was also elevated when the heart was perfused with 3×10^{-6} M denopamine or when slices of ventricular muscle were incubated with 10^{-6} M denopamine. These changes were abolished in the presence of β-adrenergic antagonists. Incubation with denopamine did not cause substantial elevation of c-AMP levels in rat reticulocytes and diaphragm.

Denopamine activated adenylate cyclase of the rat fat cell membranes in a concentration-dependent manner. Although dose dependence was less apparent, denopamine also activated adenylate cyclase of the membrane fraction from guinea pig cardiac muscle, but it hardly activated the same enzyme from rat reticulocytes. Isoproterenol, on the other hand, showed marked concentration-dependent activation of adenylate cyclase in all these preparations. Denopamine did not inhibit c-AMP phosphodiesterase of both particulate and supernatant fractions of guinea-pig cardiac muscle.

The stimulation of lipolysis by denopamine was observed even when elevation of the c-AMP level was not detected, while the stimulation of lipolysis by isoproterenol was always accompanied with an elevation of c-AMP. When guinea-pig hearts were perfused with 3×10^{-6} M denopamine or 10^{-7} M isoproterenol, their cardiotonic effects were of the same magnitude whereas the degree of c-AMP elevation in the ventricular tissue by denopamine was significantly less than that by isoproterenol.

It was concluded that stimulation of the adenylate cyclase-c-AMP system by denopamine was restricted to the tissues whose receptors were predominantly of the β_1-type, and that the elevation of c-AMP levels in these tissues by denopamine was less marked than by isoproterenol, suggesting that the stimulation of lipolysis and heart by denopamine may be mediated by a special pool of c-AMP or some other unknown factor(s).

Plasma membrane adenylate cyclase is activated by stimulation of β-adrenergic receptors, resulting in an elevation of intracellular adenosine-3′,5′-monophosphate (cyclic AMP, c-AMP) which mediates many hormone-induced responses [1]. Beta-receptors have been divided into two subtypes, β_1 and β_2, as proposed by Lands *et al.* [2, 3], and distribution of these subtypes among various tissues and cells has been determined by several investigators. Thus, the subtype of the β-receptors of cardiac muscle [2–6] and fat cells [2, 3, 7–9] is predominantly β_1, while that of skeletal muscle [10–12] and reticulocytes [5, 13, 14] is exclusively β_2.

Effects of non-selective β- and selective β_2-agonists on tissue adenylate cyclase activity and c-AMP levels have been extensively investigated. Non-selective β-agonists such as isoproterenol induce activation of adenylate cyclase and elevate c-AMP levels in tissues with β-receptors of either subtype at similar agonist concentrations [15, 16], and selective β_2-agonists such as salbutamol [17] and procaterol [17, 18] do so in purely or predominantly β_2-receptor-carrying tissues at lower drug concentrations than in tissues of the β_1-subtype. On the other hand, reports on similar studies with selective β_1-agonists are very few, because highly selective β_1-agonists have not been available.

The cardiotonic drugs prenalterol [19] and dobutamine [20, 21] have been reported to elicit cardiotonicity through stimulation of β_1-receptors. However, it has been reported that prenalterol was a partial agonist from pharmacological studies [22, 23] and exerted a β_2-antagonistic action [24], and that dobutamine elicited α- and β_2-adrenergic actions [21]. It has also been reported that prenalterol was bound equally well to both subtypes of β-receptors, but it did not activate adenylate cyclase [25]. It has also been shown that dobutamine binds to both subtypes of β-receptors [26] and activates adenylate cyclase of either subtype [15], and it also binds to α-receptors with a higher affinity than to β-receptors [26].

The new cardiotonic drug denopamine (TA-064), (−)-(*R*)-1-(*p*-hydroxyphenyl)-2-[(3,4-dimethoxy-

phenethyl)amino]ethanol, has been reported to show selectively β_1-adrenergic properties in its pharmacological actions [27]. This drug, like other known β-agonists, elevated circulating levels of free fatty acids (FFA), glycerol, insulin and c-AMP, but it was unique among the β-agonists in that it decreased blood glucose levels and did not elevate blood lactate levels [28]. The changes in circulating glycerol, insulin and glucose levels after administration of denopamine were abolished by pretreatment with the selective β_1-antagonist practolol [28].

In the present study, we investigated *in vitro* effects of denopamine in comparison with isoproterenol on the adenylate cyclase-c-AMP system of rat fat cells and guinea-pig cardiac muscle as β_1-type-predominant tissues and rat reticulocytes and diaphragm as β_2-type tissues. We confirmed β_1-selectivity of denopamine by observing the activation of adenylate cyclase only in the β_1-predominant tissues. The degrees of elevation of c-AMP levels by denopamine, however, were smaller than those by isoproterenol under the conditions in which these two drugs exerted similar magnitudes of physiological, i.e. cardiotonic and lipolytic, actions.

MATERIALS AND METHODS

Reagents

Denopamine hydrochloride was synthesized at the Organic Chemistry Research Laboratory, Tanabe Seiyaku Co., Ltd. (Toda, Saitama, Japan). Isoproterenol hydrochloride was purchased from Nakarai Chemicals Ltd. (Kyoto, Japan), and propranolol hydrochloride and practolol hydrochloride from I.C.I. Pharmaceuticals (Wilmslow, Cheshire, U.K.). The β-adrenergic drugs except denopamine were in racemic form. Other reagents and their sources were: lactate dehydrogenase, glycerokinase, creatine kinase, creatine phosphate disodium salt, β-NAD, β-NADH disodium salt, phosphoenolpyruvate sodium salt and pyruvate kinase from Boehringer Mannheim GmbH. (Mannheim, F.R.G.); ATP disodium salt, GTP trisodium salt, adenosine-3',5'-monophosphate monosodium salt (c-AMP), and "YAMASA Cyclic AMP Assay Kit®" from Yamasa Shoyu Co., Ltd. (Choshi, Chiba, Japan); forskolin from Calbiochem-Behring Corp. (La Jolla, CA) bovine serum albumin (fraction V) and collagenase (Type II) from Sigma Chemical Co. (St. Louis, MO); phenol reagent from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan); 8 - ^{3}H - adenosine - 3',5' - monophosphate ammonium salt from New England Nuclear (Boston, MA); Bio Rad AG1-X2 (−400 mesh) from Bio Rad Laboratory (Richmond, CA); theophylline from Nakarai Chemicals, Ltd.

Animals

Male Sprague–Dawley rats (6–10 weeks old, 180–270 g in body wt) were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan). Male Hartley guinea pigs (230–360 g in body wt) were purchased from Shizuoka Laboratory Animal Center or from Japan Laboratory Animal Co. (Tokyo, Japan).

Anemic rats were obtained by intraperitoneal injection, once a day for 3 days, of 50 mg/kg/day of phenylhydrazine hydrochloride, dissolved in saline and neutralized with sodium bicarbonate to pH 7.0, and were sacrificed 5 days after the last injection for preparation of reticulocytes.

Animals except the anemic rats and guinea pigs for perfusion experiments were fasted overnight (about 20 hr) before use.

Composition of the basal medium for incubation or perfusion

Krebs Ringer bicarbonate (KRB) buffer, consisting of 117 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.26 mM $CaCl_2$ and 5.5 mM glucose, was saturated with a mixed gas (95% O_2–5% CO_2) and adjusted to pH 7.4 before use. The glucose concentration of the buffer for incubation of rat diaphragm was 11.1 mM. Bovine serum albumin (4% in a final concentration) was added to the buffer for preparation and incubation of rat fat cells.

Krebs Henseleit solution, consisting of 118 mM NaCl, 4.7 mM KCl, 2.55 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 24.9 mM $NaHCO_3$ and 11.1 mM glucose, was used as the basal medium for perfusion of isolated guinea-pig hearts.

Preparation of fat cells and their membranes

Fat cells were isolated from the rat epididymal adipose tissue by the method of Rodbell [29]. Thirty milligrams of collagenase in 10 ml of KRB buffer containing 4% bovine serum albumin was used for digestion of 5 g of the tissue.

The membrane fraction of fat cells was prepared by the procedure of Rodbell [30] and used for the assay of adenylate cyclase activity. The yield of the membranes was 3.6 mg as protein from 10 g adipose tissue.

Preparation of reticulocytes and their membranes

Anemic rats were anesthetized and blood was collected from the abdominal aorta. The blood was mixed with a one-ninth volume of 10 mg/ml of EDTA disodium salt and centrifuged at 300 *g* for 5 min. The plasma and buffy coat were removed, and the cells were suspended in 1 vol. of ice-cold 150 mM NaCl–5 mM Tris–HCl buffer (pH 8.0) and centrifuged. This process was repeated three times. Over 90% of the erythrocytes from the anemic rats were reticulocytes on microscopic observation after staining with new methylene blue.

Membranes of the reticulocytes were prepared by the method of Charness *et al.* [31], and used for the assay of adenylate cyclase activity. The yield of the membranes was 2.4 mg as protein from 1 ml blood.

Preparation of supernatant and particulate fractions of guinea-pig heart ventricle

Apical portions of the guinea-pig ventricle (about 1.5 g from two hearts) were homogenized in 20 vol. of ice-cold 0.25 M sucrose containing 5 mM Tris at pH 7.4 with a Polytron® (Kinematika GmbH., Kriens, Luzern, Switzerland). The homogenate was filtered through gauze and centrifuged at 12,000 *g* for 30 min at 4°. The pellet (particulate fraction)

was used for the assay of adenylate cyclase and phosphodiesterase. Phosphodiesterase activity was also measured with the supernatant fraction.

Lipolysis

Isolated rat fat cells (1.4×10^5 cells/vessel) were incubated in 1 ml KRB containing 4% bovine serum albumin in the presence or absence of test drug at 37° with shaking under the gas phase of 95% O_2–5% CO_2. Beta-antagonist was added 10 min before the start of incubation. After 60 min, incubation was stopped by addition of perchloric acid (5% in a final concentration). Then, the mixture was centrifuged at 1000 *g* for 15 min. The supernatant was neutralized with 5 M potassium carbonate and centrifuged again to remove the potassium perchlorate prior to determination of glycerol. All the procedures after addition of perchloric acid were carried out at 4°.

In vitro *effects on tissue c-AMP levels*

Fat cells. Aliquots of one and the same batch of isolated fat cells were incubated for 3 min by the same procedure as above except that the scale was half. The supernatant after neutralization with potassium carbonate was used for determination of c-AMP by radioimmunoassay.

Heart slices. The ventricle of guinea-pig or rat heart was sliced with a YH-Slicer (Hotta Rika Co. Ltd., Tokyo, Japan). The slices, 0.5 mm in thickness and 0.6–2.4 mg as protein per slice, were preincubated at 37° for 20 min in 30 ml KRB aerated with 95% O_2–5% CO_2, and four or five slices were transferred to 5 ml fresh medium containing test drug. After incubation for 1 (for rat) or 5 (for guinea pig) min, reaction was stopped by transferring each slice quickly to 1 ml ice-cold 5% perchloric acid. The slices were immediately homogenized and the homogenates were centrifuged. Each supernatant was neutralized with potassium carbonate and centrifuged again. The final supernatants and the initial pellets were used for determination of c-AMP and protein, respectively.

Reticulocytes. About 10^9 reticulocytes were incubated in 1 ml KRB containing test drug for 3 min at 37°. Incubation was stopped by addition of 1 ml ice-cold 10% perchloric acid. The mixture was centrifuged and the supernatant was further processed for c-AMP assay.

Diaphragm. Three rats were decapitated to remove blood and the diaphragms were excised. Each tissue was dissected to 8 pieces (2.5–6.0 mg as protein/piece) after 45 min-preincubation in 10 ml KRB at 37°. Three pieces of the tissue were transferred to 5 ml fresh medium containing test drug. After 1 min-incubation, each piece was homogenized in 2 ml ice-cold 5% perchloric acid and centrifuged. The supernatant and pellet were further processed for determination of c-AMP and protein, respectively.

Perfusion of guinea-pig heart

The heart was isolated from a guinea pig (236–322 g in body wt) anesthesized with sodium pentobarbital (70 mg/kg i.p.) and perfused by the Langendorff technique [32] at a flow rate of 12 ml/min at 37°. The medium was gassed with 95% O_2–5% CO_2. The heart was preperfused with the basal medium for 30 min to stabilize the basal tension before perfusion with a β-agonist. Addition of the β-antagonist propranolol was initiated 5 min before the start of agonist perfusion and continued throughout the agonist perfusion period. Contractile force was measured isometrically with a strain gauge transducer (U-gauge, Shinkoh, Tokyo, Japan) and a carrier amplifier (RP-5, Nihon Kohden, Tokyo, Japan). The initial tension was 2 g. The first differentiation of contractile force, dF/dt, was measured by an analogue differentiator (S-5151, Nihon Kohden) and recorded on a polygraph (RM-45, Nihon Kohden). The heart was removed from the strain gauge 55 sec after the initiation of agonist-perfusion, and then an apical portion (about 500 mg in wet wt) of the heart was cut off, frozen in liquid nitrogen, and stored at −70° until c-AMP determination. About 8 sec were required for this process. The stored tissue was homogenized in 5 vol. of ice-cold 5% perchloric acid with a Polytron®, and centrifuged at 1500 *g* for 30 min. The supernatant and pellet were further processed for determination of c-AMP and protein, respectively.

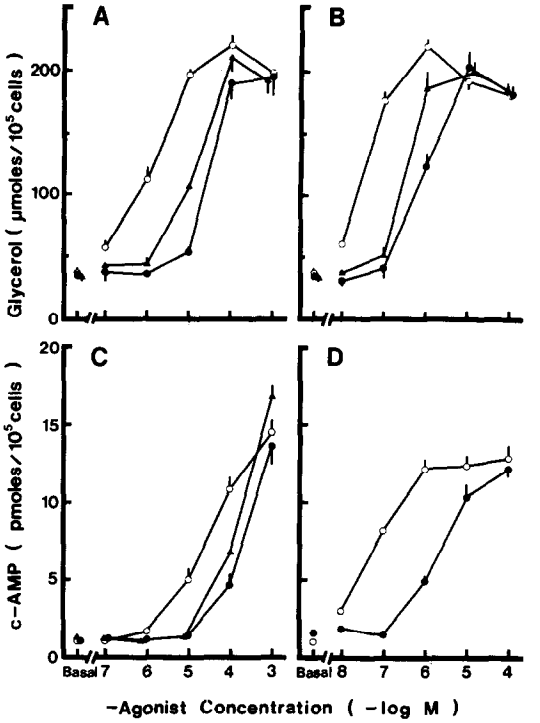

Fig. 1. Effects of denopamine and isoproterenol on lipolysis and c-AMP levels in isolated rat fat cells and their antagonism by propranolol and practolol. Rat epididymal fat cells were incubated with denopamine (A and C) and isoproterenol (B and D) for 60 (lipolysis, A and B) or 3 (c-AMP, C and D) min after 10 min-preincubation without antagonist (○) or with propranolol (3×10^{-6} M, ●) or practolol (5×10^{-4} M, ▲). See the text for other conditions. Each point and bar represent the mean and SE of three observations.

Assays for adenylate cyclase and phosphodiesterase activities

The reaction mixture for the assay of adenylate cyclase was composed of (in final concentrations)

Table 1. Effects of denopamine and isoproterenol on c-AMP levels of cardiac muscle slices from guinea pigs and rats

Drug	Concentration (M)	c-AMP (pmoles/mg protein) —	c-AMP (pmoles/mg protein) with forskolin
(A) Guinea pig (N = 5)			
—		1.71 ± 0.15	
Denopamine	10^{-6}	2.70 ± 0.18*	
	10^{-5}	2.73 ± 0.70	
	10^{-4}	2.25 ± 0.39	
Isoproterenol	10^{-7}	2.45 ± 0.40	
	10^{-6}	3.01 ± 0.20†	
	10^{-5}	5.49 ± 0.50†	
(B) Rat (N = 4)			
—		1.44 ± 0.13	2.99 ± 0.09
Denopamine	10^{-5}	1.65 ± 0.15	6.77 ± 0.87*
Isoproterenol	10^{-6}	3.15 ± 0.22†	12.85 ± 1.03†

Slices of the guinea-pig (A) or rat (B) ventricle were incubated with agonist for 5 (A) or 1 (B) min at 37°. Forskolin (10^{-5} M) was added at the start of incubation. Each value represents the mean ± SE. Significant differences from the control (without agonist): * $P < 0.01$, † $P < 0.001$.

Table 2. Effects of denopamine and isoproterenol on dF/dt and c-AMP levels of perfused guinea-pig hearts

Agonist	Conc. (M)	Propranolol 10^{-6} M	dF/dt (g/sec)	c-AMP (pmoles/mg protein)
—		—	111.2 ± 17.7	3.80 ± 0.29
Denopamine	3×10^{-6}	—	227.5 ± 22.5*	4.56 ± 0.16‡§
Isoproterenol	10^{-7}	—	204.8 ± 28.4‡	5.70 ± 0.19†
—	—	+	110.2 ± 16.7	3.34 ± 0.35
Denopamine	3×10^{-6}	+	111.8 ± 17.4	3.38 ± 0.19
Isoproterenol	10^{-7}	+	106.3 ± 17.4	3.32 ± 0.25

See the text for detailed procedures. Values represent means and SE of six hearts. Significant differences from control (without agonist): ‡ $P < 0.05$, * $P < 0.01$, † $P < 0.001$. Significant difference from isoproterenol: § $P < 0.05$.

40 mM Tris–HCl buffer (pH 7.4), 5 mM $MgCl_2$, 5 mM theophylline, 0.01 or 0.25 mM GTP trisodium salt, 1 mM ATP disodium salt, 20 mM creatine phosphate, 5 U/ml creatine kinase, test drug and enzyme in a volume of 0.25 ml. After the mixture excluding ATP was preincubated for 3 min at 30°, the assay was started by addition of ATP. Incubation was continued for 5 min at 30°, and was stopped by addition of perchloric acid (5% in a final concentration). The mixture was centrifuged and the supernatant was neutralized to determine c-AMP.

Phosphodiesterase activity was determined by the procedure of Thompson and Appleman [33] in the presence of 2×10^{-7} M c-AMP as substrate. The reaction was run for 3 min in a volume of 0.2 ml.

Chemical and statistical analyses

Cyclic AMP was determined by radioimmunoassay using "YAMASA Cyclic AMP Assay Kit®". Glycerol was enzymatically determined [34]. Protein was determined by the method of Lowry *et al.* [35] using bovine serum albumin as standard. Data are shown as the mean and SEM. Statistical significance of differences was determined by Student's *t*-test. In some experiments, incubation was performed in duplicate or quadriplicate and observed values were presented as such. Effects of drug on adenylate cyclase activity of the particulate fraction of guinea-pig heart were analyzed by the orthogonal comparison [36].

RESULTS

Effects on lipolysis and c-AMP levels in isolated rat fat cells

Denopamine and isoproterenol stimulated lipolysis and elevated c-AMP levels in isolated fat cells concentration-dependently, and these effects were competitively antagonized by propranolol and practolol (Fig. 1). The potencies of stimulation of lipolysis and elevation of c-AMP levels by denopamine were about 30- and 300-fold less, respectively, than those

by isoproterenol (Fig. 1). The degrees of maximal stimulation of lipolysis and elevation of c-AMP levels by denopamine were similar to those by isoproterenol (Fig. 1).

There was a parallel relationship between the stimulation of lipolysis and the elevation of c-AMP levels by isoproterenol, but the elevation of c-AMP levels by denopamine required higher concentrations of the agonist than the increase in lipolysis did: i.e. there was substantial stimulation of lipolysis in the absence of detectable elevations of c-AMP (Fig. 1).

Effects on c-AMP levels of guinea-pig and rat heart slices

Denopamine and isoproterenol both elevated c-AMP levels of guinea-pig heart slices *in vitro*, the increase by isoproterenol being moe marked and concentration-dependent than that by denopamine (Table 1A). With rat heart slices, isoproterenol was again much more active than denopamine, whose c-AMP-elevating activity becomes appreciable only in the presence of forskolin (Table 1B).

Effects on cardiotonicity and c-AMP levels of perfused guinea-pig heart

When a guinea-pig heart was perfused by the Langendorff technique, both denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M) in the perfusion medium increased cardiotonicity immediately after the start of perfusion, and gave a peak tension at 15–30 sec after the start, which lasted until the end of observation. The increases in the first differentiation of cardiac force, dF/dt, at 55 sec after the start of perfusion with drug, i.e. immediately before the heart was removed from the strain gauge, were of almost the same degree for denopamine and isoproterenol (Table 2). On the other hand, the degree of c-AMP elevation against the control by denopamine was 40% of that by isoproterenol (Table 2). The increases in dF/dt and ventricular c-AMP levels by the two agonists were both suppressed by pretreatment with propranolol (Table 2).

Effects on c-AMP levels in rat reticulocytes and diaphragm

Isoproterenol at concentrations above 10^{-8} M elevated reticulocyte c-AMP levels, while denopamine showed only a slight increase at 10^{-4} M (Fig. 2).

The "β_1"-cardiotonic drug prenalterol has been reported to exert a β_2-antagonistic action [24]. Therefore, effect of denopamine on isoproterenol-induced elevation of c-AMP levels in reticulocytes was tested. Denopamine suppressed the isoproterenol-induced elevation of reticulocyte c-AMP levels only at 10^{-3} M (Fig. 3). The potency of the β_2-antagonism by denopamine was about four orders less than that by propranolol (Fig. 3).

Isoproterenol at 10^{-6} M markedly elevated c-AMP levels of rat diaphragm, but denopamine at 10^{-4} M showed no such effect (Table 3).

Effects on adenylate cyclase and c-AMP phosphodiesterase

Effects of denopamine and isoproterenol on adenylate cyclase activities of the membranes of rat fat cells and reticulocytes and the particulate fraction of guinea-pig ventricular muscle were investigated.

Isoproterenol markedly and concentration-dependently activated the enzyme of all preparations investigated (Fig. 4). Denopamine also concentration-dependently activated adenylate cyclase of fat cell membranes, although its potency was two orders lower than that of isoproterenol (Fig. 4A). The degree of maximal activation by denopamine was

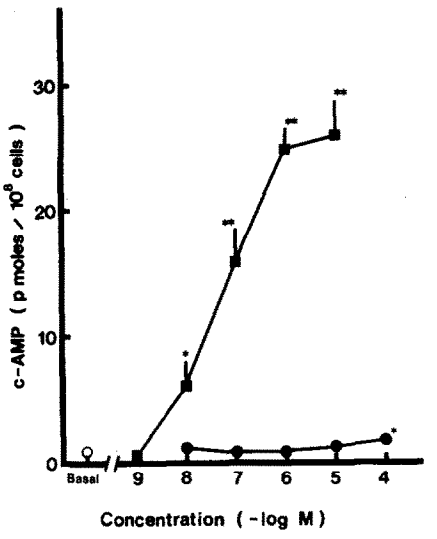

Fig. 2. Effects of denopamine and isoproterenol on rat reticulocyte c-AMP levels. Rat reticulocytes (8.6×10^8 cells/ml) were incubated with denopamine (●), isoproterenol (■) or without agonist (○) for 3 min at 37°. Each point and bar represent the mean and SE of four observations. Significant differences from the control: * $P < 0.05$, ** $P < 0.01$.

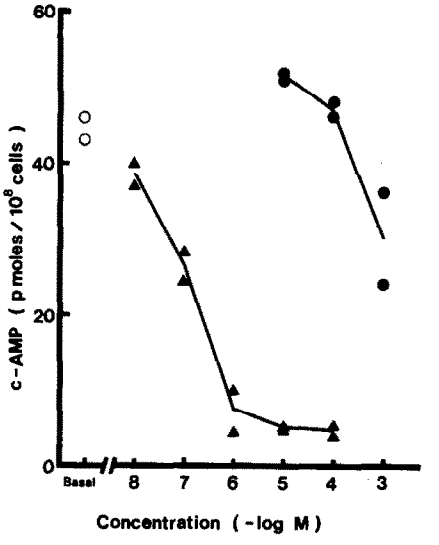

Fig. 3. Effects of denopamine and propranolol on isoproterenol-induced elevation of c-AMP of rat reticulocytes. Rat reticulocytes (1.12×10^9 cells/ml) were incubated with isoproterenol (10^{-6} M) for 3 min at 37° after 10 min preincubation without antagonist (○) or with denopamine (●) or propranolol (▲) at concentrations indicated on the graph.

Table 3. Effects of denopamine and isoproterenol on c-AMP levels of rat diaphragm

Drug	Conc. (M)	c-AMP (pmoles/mg protein)
—		2.40 ± 0.32
Denopamine	10^{-6}	2.05 ± 0.65
	10^{-4}	2.36 ± 0.63
Isoproterenol	10^{-6}	11.20 ± 2.50*

Pieces (2.5–6.0 mg protein/piece) of rat diaphragm were incubated with denopamine or isoproterenol for 1 min at 37°. Values represent means and SE of 3 experiments. Significant effect of agonist: * $P < 0.01$.

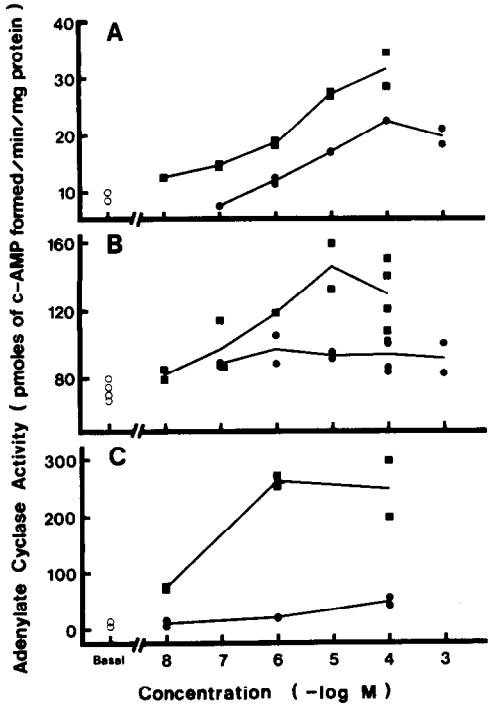

Fig. 4. Effects of denopamine and isoproterenol on adenylate cyclase activity of the membrane fractions from rat fat cells, guinea-pig ventricular muscle and rat reticulocytes. Rat fat cell membranes (A), the particulate fraction of guinea pig ventricle (B), and rat reticulocyte membranes (C) were incubated with isoproterenol (■) or denopamine (●) at concentrations indicated on the graph, or without β-agonist (○) for 5 min at 30°. See the text for detailed procedures of enzyme preparation and adenylate cyclase assay. Each point represents individual observations of duplicate or quadruplicate vessels. The method of orthogonal comparison was applied to analyze the result of Experiment B.

about 60% of that by isoproterenol (Fig. 4A). With the particulate fraction of guinea-pig ventricular muscle, although dose dependency was less apparent, denopamine showed marginal but statistically significant activation (Fig. 4B). With the reticulocyte membranes, denopamine showed only a slight activation at 10^{-4} M (Fig. 4C).

Denopamine did not inhibit either particulate or supernatant phosphodiesterase activity of guinea pig heart ventricle except for a slight inhibition of the particulate enzyme at 10^{-3} M of denopamine, while inhibition of this enzyme by theophylline was apparent in both fractions (Table 4).

DISCUSSION

In the previous study, it was shown that plasma levels of c-AMP were elevated after administration of denopamine to the rat [28]. The present study demonstrated that the heart (Tables 1 and 2) and adipose tissues (Fig. 1) are among the organs that respond to denopamine by elevation of c-AMP levels.

Intracellular c-AMP levels are increased by either activation of adenylate cyclase or inhibition of phosphodiesterase or both [1]. The lack of inhibition of guinea pig heart phosphodiesterase at the denopamine concentration of 10^{-4} M (Table 4), and slight but significant activation of adenylate cyclase by denopamine (Fig. 4B) indicate that the elevation of cardiac c-AMP levels by denopamine (Tables 1 and 2) is mediated by activation of adenylate cyclase. With the fat cells membrane preparation, concentration-dependent activation of adenylate cyclase by denopamine is more apparent (Fig. 4A). Thus, the mechanism of the c-AMP-elevating effect of denopamine is different from that of amrinone, a cardiotonic drug whose c-AMP-elevating effect was ascribable to inhibition of phosphodiesterase [37].

The inhibition of denopamine-induced elevation of ventricular c-AMP levels in the perfused guinea pig heart by pretreatment with propranolol (Table 2) and similar antagonisms by propranolol and practolol of denopamine-induced c-AMP-elevation (Fig. 1C) and lipolysis (Fig. 1A) in the rat fat cells indicate that these effects by denopamine are mediated by stimulation of β-receptors. The observations that the maximal degrees of lipolysis-stimulating (Fig. 1) and cardiotonic (Table 2) actions of denopamine were almost the same as those by isoproterenol indicate that denopamine is a full agonist at least in these tissues.

In pharmacological studies, denopamine has been shown to have β_1-adrenoceptor agonistic properties with very weak vasodilating activity [27]. In biochemical studies, administration of denopamine to rats lowered blood glucose, a property never observed with other known β-agonists, such as isoproterenol and terbutaline, and other "β_1"-cardiotonic drugs such as prenalterol and dobutamine [28]. Like other β-agonists, denopamine elevated circulating concentrations of FFA, glycerol, insulin and c-AMP, but, unlike β_2-agonists, it did not elevate blood lactate levels [28]. Thus, denopamine exhibited unique metabolic properties, which may be ascribable to its β_1-selectivity. Beta$_1$-selectivity of denopamine was also observed in a ligand binding study [38].

In the present study, denopamine showed a high β_1-selectivity towards the β-receptor-adenylate cyclase system: i.e. significant activation of the system was observed only in the fat cells (Figs. 1 and 4A) and cardiac muscle (Tables 1 and 2), but not in the rat reticulocytes (Fig. 2) and diaphragm (Table 3). Prenalterol [19, 24] and dobutamine [20, 21] have been reported to have some affinity to β_2-receptors,

Table 4. Effects of denopamine on guinea-pig ventricular phosphodiesterase activities

Drug	Concentration (M)	Activity (nmoles/min/mg protein)	Inhibition (%)
(A) Particulate			
—		0.079, 0.079	
Denopamine	10^{-4}	0.085, 0.093	−13
	10^{-3}	0.067, 0.065	16
Theophylline	3×10^{-5}	0.072, 0.069	10
	10^{-4}	0.053, 0.051	34
(B) Supernatant			
—		1.50, 1.42	
Denopamine	10^{-4}	1.54, 1.42	−1
	10^{-3}	1.47, 1.48	−1
Theophylline	10^{-3}	0.58, 0.55	61

The 12,000 *g* particulate (100 μg protein/vessel) and supernatant (0.48 μg protein/vessel) fractions of guinea-pig ventricle were used for the assay of phosphodiesterase activity. See the text for detailed procedures. Values represent two duplicate observations.

but β_2-blocking activity of denopamine as estimated by the inhibition of isoproterenol-induced c-AMP elevation in the rat reticulocytes was very weak (Fig. 3).

Stimulation of β-receptors of skeletal muscle, a tissue with pure β_2-receptors [11, 12, 39], is known to increase lactate production [39]. Marked elevation of the c-AMP level of diaphragm by isoproterenol in the present study (Table 3) corresponds to the elevation of blood lactate observed in our previous study [28]. Similarly, the lack of changes in diaphragm c-AMP levels by denopamine (Table 3) is in agreement with the lack of elevation of blood lactate levels [28]. On the other hand, stimulation of lipolysis and elevation of the c-AMP level in the fat cells *in vitro* by denopamine and isoproterenol (Fig. 1) corresponded to the elevation of blood glycerol and FFA *in vivo* [28].

The elevation of tissue c-AMP levels is believed to mediate, *via* activation of protein kinase, numerous physiological or pharmacological responses. These responses, however, do not necessarily correlate with the degree of increase in cellular c-AMP levels. For instance, epinephrine and prostaglandin E_1 both elevated c-AMP levels and activated c-AMP-dependent protein kinase in perfused rat heart, while only epinephrine increased phosphorylase activity and contractile force [40]. A similar result was obtained with the perfused guinea-pig heart [41]. Inotropic action without a measurable change in c-AMP levels of cat papillary muscle was reported by Venter *et al.* who used isoproterenol bound to glass beads [42]. These authors proposed a "spare receptor theory" to explain this result [43]. It has also been reported that serotonin elevated c-AMP levels of fat cells, but it never stimulated lipolysis [44]. Discrepancies between the change in c-AMP levels and physiological responses were also observed in other tissues or cells [45–49]. Compartmentalization of c-AMP and/or protein kinase was proposed to explain these results [50].

In the present study, *in vitro* stimulation of lipolysis by denopamine was observed at low drug concentrations where there was no detectable changes in c-AMP, while the stimulation of lipolysis by isoproterenol was always accompanied with an increase in c-AMP (Fig. 1). In the perfused guinea-pig heart, the degree of c-AMP elevation by denopamine was considerably smaller than that by isoproternol, when the magnitudes of their cardiotonic action were nearly equal (Table 2). These observations may be explained either by the "compartment theory", which assumes a compartment of c-AMP more directly involved than the rest in the cardiotonic or lipolytic action, or by a hypothetical c-AMP-independent mechanism [51] through which denopamine may exert its pharmacological effects. Smaller activation of the c-AMP system by denopamine in comparison with isoproterenol was also reported by Bing *et al.*, who studied *in vitro* effects of the two drugs on sarcolemmal adenylate cyclase of dog heart [52].

A role for myocardial c-AMP in the genesis of ventricular arrhythmias has been proposed [53, 54], and, in fact, ventricular fibrillation in animal models of myocardial ischemia was prevalent at the time when myocardial c-AMP levels were elevated [55–57]. In coronary-ligated dogs, arrhythmogenicity by denopamine was weaker than by catecholamines [58]. Lower activation of the cardiac adenylate cyclase-c-AMP system by denopamine than by isoproterenol (Fig. 4B, Tables 1 and 2) may be responsible for the weak arrhythmogenicity of denopamine.

In conclusion, the selectively β_1-adrenergic cardiotonic drug denopamine stimulates the adenylate cyclase system of cardiac muscle and fat cells, whose β-receptors are predominantly of the β_1-subtype, but it did not affect the adenylate cyclase system linked to the β_2-receptors of rat diaphragm and reticulocytes. The adenylate cyclase activation by denopamine was much weaker than by isoproterenol, in the presence of similar degrees of cardiotonic or lipolytic action. Denopamine differed from the cardiotonic drugs dobutamine and prenalterol in its β_1-selectivity and β_2-antagonistic activity, respectively. All these properties may contribute to the characteristics of its cardiotonic [27] as well as metabolic [28] effects.

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