Biochemical Pharmacology, Vol. 37, No. 4, pp. 679–686, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00
Pergamon Journals Ltd.

EFFECT OF DENOPAMINE ON THE PHOSPHORYLATION OF CARDIAC MUSCLE PROTEINS IN THE PERFUSED GUINEA-PIG HEART

COMPARISON WITH ISOPROTERENOL

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(*Received* 10 *June* 1987; *accepted* 21 *August* 1987)

Abstract—Effects of the new selectively β_1-adrenergic cardiotonic drug denopamine (TA-064) on the phosphorylation of cardiac muscle proteins in the perfused guinea-pig heart were investigated in comparison with isoproterenol. Denopamine at 3×10^{-6} M and isoproterenol at 10^{-7} M were equipotent in their effects on the contractile force and + (dF/dt). Under these conditions, the increases in heart rate and tissue c-AMP levels by denopamine were significantly less than those by isoproterenol. Isoproterenol exerted a greater effect on $-(dF/dt)$ than on $+(dF/dt)$, whereas denopamine influenced both to the same extent. Denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M) both stimulated ^{32}P incorporation into the proteins of molecular weights of 150,000, 30,000, 19,000, 15,000 and 11,000 daltons. Among these proteins, the 30,000 and 11,000 dalton proteins, probably troponin-I and phospholamban, were phosphorylated to significantly lesser extents by denopamine than by isoproterenol.

The above differences in the effects on c-AMP levels and protein phosphorylation between denopamine and isoproterenol may be causally related to the differences in their pharmacological properties such as the weaker arrhythmogenicity and comparatively less marked relaxation effect of denopamine compared with isoproterenol in the presence of similar cardiotonic effects.

Accumulated evidence indicates that the cardiotonic action of β-adrenergic drugs is mediated by stimulation of β-receptor-linked adenylate cyclase followed by activation of c-AMP-dependent protein kinase that phosphorylates cardiac muscle proteins. Their cardiotonic effects are characterized by an enhancement of both contraction and relaxation rates and a decrease in the time required to reach the peak of tension. In cardiac muscles, at least three phosphoproteins are the candidate proteins functioning in the regulation of contraction: sarcolemmal membrane protein [1], phospholamban [2] and troponin-I [3].

Denopamine is a new β-adrenergic cardiotonic drug possessing positive inotropic action accompanied by relatively weak positive chronotropic action [4]. Thus, denopamine is assumed to exert the cardiotonic action through stimulation of β-receptor-linked adenylate cyclase followed by phosphorylation of some cardiac muscle proteins linked to cardiac functions. However, the degree of c-AMP elevation by denopamine has been reported to be considerably smaller than that by isoproterenol when the magnitudes of their cardiotonic action were nearly equal [5, 6].

Effects of the non-selective β-agonist isoproterenol on the phosphorylation of cardiac muscle proteins have been extensively investigated [7–10], but there are no reports of similar studies with selectively β_1-adrenergic agonists, because highly selective β_1-agonists have not been available. The cardiotonic agents dobutamine [11, 12] and prenalterol [13] have been claimed to be selectively β_1-adrenergic agonists. However, dobutamine has been reported to show a poor separation of β_1- versus β_2 selectivity [11] and prenalterol to have β_2-adrenergic antagonistic properties [14]. The new cardiotonic agent denopamine has been shown to be a selectively β_1-adrenergic agonist from pharmacological [4], ligand-binding [15] and biochemical [5, 6, 16] studies. In the present investigation, we studied effects of denopamine on the phosphorylation of cardiac muscle proteins in comparison with isoproterenol, using perfused guinea-pig hearts. The results showed that denopamine, like isoproterenol, stimulated phosphorylation of five cardiac muscle proteins, but the degrees of phosphorylation in troponin-I and phospholamban by denopamine were less marked than those by isoproterenol.

MATERIALS AND METHODS

Perfusion method. The heart was quickly isolated from stunned guinea pigs (280–350 g in body weight, Shizuoka Laboratory Animal Center, Slc: Hartley, Hamamatsu, Japan) and perfused by the Langendorff technique at a flow rate of 4 ml/min at 30°. The perfusate was constantly gassed with 95% O_2–5% CO_2 in the bubble trap. The heart was first perfused with a non-recirculating perfusion system to flush the coronary circulation free of blood during the first 15 min of perfusion. Then, by using a three-way valve, the heart was switched to an 18 ml recirculating perfusion system and further perfused for 40 min to stabilize the basal tension before perfusion with denopamine or isoproterenol. The perfusate,

consisting of 118.4 mM NaCl, 4.69 mM KCl, 1.18 mM $MgSO_4$, 2.52 mM $CaCl_2$, 10 mM glucose, 25 mM $NaHCO_3$ and 0.12 mM KH_2PO_4, was saturated with a mixed gas (95% O_2–5% CO_2) and adjusted to pH 7.4 before use. After the stabilization, 2 ml of a denopamine or isoproterenol solution in the perfusion medium (see below) was added in a final concentration of 10^{-7} M or 3×10^{-9} M, respectively, to the perfusate in the bubble trap whose volume was adjusted to 18 ml just before the addition of each drug. After cardiotonicity reached a plateau, a 30 times more concentrated solution of denopamine or isoproterenol (each 1 ml) was added into 9 ml of the perfusate in the bubble trap, whose volume was adjusted just prior to the second addition, giving a final concentration of 3×10^{-6} M or 10^{-7} M in the perfusate, respectively. Thus, pharmacological data for two different doses of each drug were obtained with each perfused guinea-pig heart. Denopamine and isoproterenol were dissolved in the perfusion medium containing 5.3 mM sodium pyrosulfite before use. Perfusion methods for the study of c-AMP levels or phosphorylation were the same as above except for the following two points; (1) the hearts perfused for the study of phosphorylation were quickly frozen by freeze-clamping with aluminum clamps precooled in liquid nitrogen when cardiotonicity reached a plateau, i.e. 52 or 55 sec after the administration of denopamine (3×10^{-6} M) or isoproterenol (10^{-7} M), respectively, and the hearts perfused for c-AMP assay were frozen at 40 sec after the administration of each drug; (2) in the study of phosphorylation, ^{32}P-H_3PO_4 (300 μCi in 18 ml of the perfusing medium to give a sp. ac. of 140 μCi/μmole P_i) was added to 18 ml of perfusate after non-recirculation perfusion and circulated through the heart for 40 min of the stabilization period to allow [γ-^{32}P]ATP to accumulate in cardiac cells as substrate for phosphorylation. Control hearts were treated with vehicle and frozen in the same time schedule as for the hearts treated with denopamine.

Pharmacological methods. Contractile force was measured isometrically with a strain gauge transducer (UL-10GR. Mimebea Co. Ltd., U.S.A.) connected to the apex via a thread and a carrier amplifier (RP-5, Nihon Kohden, Tokyo, Japan). The initial tension was 2 g. The differentiation of contraction, $+(dF/dt)$, and that of relaxation $-(dF/dt)$, were measured by an analogue differentiator (S-5151, Nihon Kohden) and recorded on a chart recorder (W1-640G-S, Nihon Kohden).

Preparation of samples and determination of ATP, c-AMP and [γ-^{32}P]ATP specific radioactivity. Each frozen heart was pulverized with a pestle and mortar precooled in liquid nitrogen. One portion (*ca.* 160 mg) of the pulverized heart was homogenized at 0° in 1 ml of homogenization medium [20 mM potassium fluoride, 10 mM ethylenediaminetetraacetic acid (EDTA), 2.5 M sucrose and 20 mM β-glycerol phosphate, pH 6.8] with a Polytron® homogenizer (model PT-10) at a speed of "6" for three 5-sec intervals 20 sec apart, filtered through gauze, and subjected to gel electrophoresis (see below).

For determination of [γ-^{32}P]ATP specific radioactivity, another portion (*ca.* 360 mg) of the pulverized heart was homogenized at 0° in 2 ml of 0.6 N perchloric acid containing 1 mM EDTA with a Polytron® and centrifuged. The supernatant was neutralized with 3.2 M potassium carbonate and centrifuged again to remove potassium perchlorate. The supernatant was used for determination of [γ-^{32}P]ATP specific radioactivity according to the method of Cogoli and Dobson [17] except that histone (Sigma, type II-AS) was used as substrate.

Tissue homogenates for c-AMP and ATP assay were prepared in the same way as for determination of [γ-^{32}P]ATP specific radioactivity. The proteins precipitated with 0.6 N perchloric acid were dissolved by boiling for 10 min in 5 ml of 2 N sodium hydroxide for determination of protein. All the assay procedures for c-AMP and ATP were carried out at 4°. c-AMP and ATP were determined by radioimmunoassay using the "YAMASA cyclic AMP Assay Kit®" and "ATP-test kit®", respectively. Protein was determined by the method of Lowry *et al.* [18] using bovine serum albumin as standard. Amounts of c-AMP and ATP were expressed as pmole/mg protein and μmole/mg protein, respectively.

SDS–polyacrylamide gel electrophoresis. SDS–polyacrylamide gel electrophoresis was performed using the buffer system of Laemmli [19] in 3.75% acrylamide stacking gel and 10–15% acrylamide gradient resolving gel (gel size; $14 \times 10 \times 0.2$ cm^2). The samples for gel electrophoresis were dissolved in a buffer containing 2% SDS, 10% glycerol, 0.035% bromophenol blue, 5% β-mercaptoethanol and 0.3 M tris (hydroxymethyl) aminomethane (Tris) (pH 6.8) at the concentration of 2 mg protein/ml. One aliquot (*ca.* 150 μl) was boiled for 3 min and another aliquot (*ca.* 50 μl) was held at room temperature for 5 min prior to electrophoresis. The boiled and unboiled samples from a set of 3 hearts, each treated with vehicle, denopamine or isoproterenol (each 80 μg protein), were placed on the same gel, and 35 mA was applied for about 4 hr. Electrophoresis was continued until the bromophenol blue tracing dye reached the gel bottom. Each gel contained the following molecular weight markers: myosin, 205,000; β-galactosidase, 116,000; phosphorylase b, 97,400; bovine serum albumin, 66,000; egg albumin, 45,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; β-lactoglobulin, 18,400; lysozyme, 14,300; and cytochrome *c*, 12,000. Protein bands on the gel were stained with 0.2% Coomassie Brilliant Blue R-250 in 50% methanol and 7% acetic acid. Background of the gel was destained in the solution of 25% methanol and 7% acetic acid with an ATTO Electronic Destaining Apparatus (model SJ-1060 DE, ATTO corporation, Tokyo, Japan). The gel was dried with an ATTO Gel Slab Dryer (model AE-3701) and autoradiographed in a cassette containing Kodak X-ray film (DEF-5) at −70° for about 10 days. The stained gel and the autoradiograph were scanned with an Automatic Computing Densitometer (model ACD-18, Gelman Sciences Inc, U.S.A) and the amount of protein and radioactivity of each band were expressed as heights of optical density peaks on a densitometer tracing. There were linear relationships between the peak heights and the levels of protein amount and radioactivity in the observed ranges.

Materials. Denopamine hydrochloride was synthesized at the Organic Chemistry Research Laboratory, Tanabe Seiyaku Co. Ltd. (Toda, Saitama, Japan). Isoproterenol hydrochloride (DL-form) was purchased from Nakarai Chemicals Ltd. (Kyoto, Japan), molecular markers for SDS-polyacrylamide gel electophoresis from Sigma Chemical Co. (St Louis, MO), ATP-test kit® from Boehringer Mannheim GmbH. (Mannheim, F.R.G.), [γ-^{32}P]ATP from New England Nuclear (Cambridge, MA), ^{32}P-H_3PO_4 from Japan Atomic Laboratory and "YAMASA cyclic AMP Assay Kit®" from Yamasa Shoyu Co. Ltd. (Choshi, Chiba, Japan). All other reagents were of the highest grade of purity available.

Statistical analysis. In the study of phosphorylation, five combinations of one control and two (isoproterenol and denopamine) experimental hearts were side-by-side carried through all procedures of homogenization, electrophoresis and analysis in a total of five experiments. In this manner, any variations in technique would influence both control and experimental members of each trio to the same extent. Therefore, randomized Block's method (Tukey's method) for paired samples was used for data presented in Fig. 5. One-way layout analysis of variance (Tukey's method) and unpaired Student's *t*-test were used for the data of ATP and c-AMP levels (Fig. 2) and for the pharmacological data (Fig. 1), respectively. Numerical data were expressed as means ± SE. A value of $P < 0.05$ was considered significant.

RESULTS

Effects of denopamine and isoproterenol on cardiotonicity of perfused guinea-pig hearts

When guinea-pig hearts were perfused by the Langendorff technique, both denopamine and isoproterenol increased cardiotonicity 30 sec after addition of each drug to the circulating perfusate. As shown in Fig. 1, cardiotonicity [contractile force and $+(dF/dt)$] induced by 10^{-7} M denopamine was of the same level as that by 3×10^{-9} M isoproterenol, but denopamine needed a longer time to reach a maximum tension than isoproterenol (denopamine: 163 ± 4 sec; isoproterenol: 91 ± 6 sec). On increasing the dose, 3×10^{-6} M denopamine and 10^{-7} M isoproterenol further increased the cardiotonicity to similar levels, and shortened the time required to reach a further elevated maximum tension (denopamine: 53 ± 2 sec, isoproterenol: 55 ± 2 sec). On the other hand, the ratio of $-(dF/dt)$ to $+(dF/dt)$ for 10^{-7} M isoproterenol was more than unity and significantly higher than for 3×10^{-6} M denopamine (Fig. 1). This means that isoproterenol exerts a greater effect on $-(dF/dt)$ than on $+(dF/dt)$, whereas denopamine shows similar effects on either parameter. The increase in heart rate by denopamine was significantly less than that by isoproterenol at both cardiotonically equipotent doses.

Effects of denopamine and isoproterenol on c-AMP and ATP levels

With an *in vivo* working heart preparation, it has been shown that administration of a bolus injection of epinephrine causes a near maximal elevation of myocardial c-AMP and activation of c-AMP dependent protein kinase prior to a peak of cardiotonic effects [20]. Therefore, c-AMP levels were measured 40 sec after the addition of each drug (about 10 sec before a maximum tension). ATP levels were also determined along with c-AMP assay. As shown in Fig. 2, the elevation of c-AMP levels by 3×10^{-6} M denopamine was significantly lower than that by

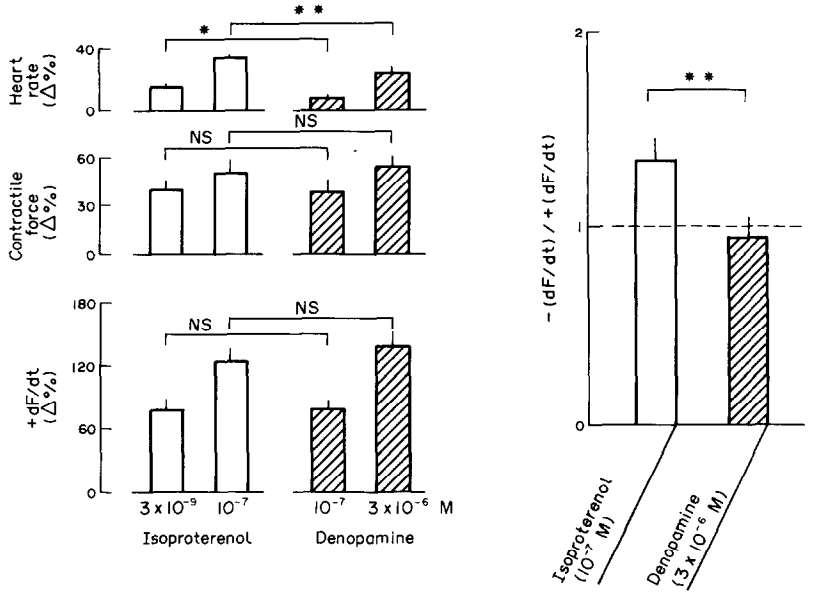

Fig. 1. Effects of denopamine and isoproterenol on heart rate, contractile force, $+(dF/dt)$ and the ratio of $-(dF/dt)$ to $+(dF/dt)$ in perfused guinea-pig hearts. Guinea-pig hearts were perfused by the Langendorff technique with isoproterenol (3×10^{-9} and 10^{-7} M; open column) or denopamine (10^{-7} and 3×10^{-6} M; hatched column) at 30°. The times to reach a maximum tension were given in Results. Each column and bar represent the mean and SE, respectively, of 8 hearts. Significant differences from corresponding isoproterenol values: * $P < 0.05$, ** $P < 0.01$, NS, not significant.

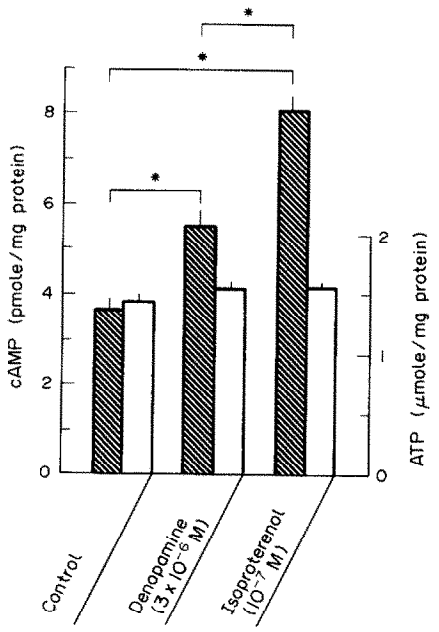

Fig. 2. Effects of denopamine and isoproterenol on ATP and c-AMP levels in perfused guinea-pig hearts. Guinea-pig hearts were perfused by the Langendorff technique with vehicle, denopamine (3×10^{-6} M) or isoproterenol (10^{-7} M) for 40 sec at 30° prior to freeze clamping. ATP (open column) and c-AMP levels (hatched column) were measured as described in Methods. Each column and bar represent the mean and SE, respectively, of 8 hearts. Significant differences from control or corresponding isoproterenol values: * $P < 0.01$.

10^{-7} M isoproterenol, while ATP levels were not influenced by either drug.

Effects of denopamine and isoproterenol on the phosphorylation of cardiac muscle proteins in perfused hearts

The patterns of the gel electrophoresis stained with Coomassie Blue showed no differences between the control and drug-treated hearts in the pattern of stained protein bands. However, when the patterns were compared between the two members of each pair of boiled and unboiled preparations, there appeared over 300,000-dalton proteins and the 20,000-dalton protein decreased in all the boiled samples. The densitometric tracings of these stained gels obtained with the heated samples from control and drug-treated hearts were superimposable to one another (data not shown). This was also true of unboiled samples, indicating that the two drugs did not affect the composition of cardiac muscle proteins.

A typical example of the autoradiogram of cardiac muscle proteins from one each of control and denopamine- and isoproterenol-treated hearts is shown in Fig. 3. Both adrenergic drugs stimulated ^{32}P incorporation into proteins having molecular weights of 150,000-, 30,000-, 19,000-, 15,000- and 11,000-daltons. Small amounts of the phosphorylated 11,000-dalton protein (arrow mark B) were detected in unboiled samples from the hearts treated with denopamine and isoproterenol. However, on boiling, these amounts increased with concomitant losses of the phosphorylated 27,000-dalton protein (arrow mark A). Neither the phosphorylated 11,000- nor the 27,000-dalton protein was detected in control samples. The results suggested that both denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M) stimulated phosphorylation of phospholamban (27,000-dalton protein), which would be completely converted to a dimer (11,000-dalton protein) on boiling [21–24].

The specific radioactivities of [γ-^{32}P]ATP (cpm/nmole) of the hearts treated with vehicle, denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M) were 1020 ± 94, 1141 ± 126 and 1084 ± 148, respectively, indicating that the two drugs did not affect the specific radioactivity of [γ-^{32}P]ATP. Figure 4 shows a typical set of densitometric scannings of an autoradiogram of boiled samples from the hearts treated with denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M). For comparison, after the optical density was corrected for the specific radioactivity of [γ-^{32}P]ATP of each heart, the tracings for the treated hearts were superimposed with that for the control heart. Fourteen radioactive peaks were recognized, and they were reproducible and easy to identify in all the densitometric tracings. Mean peak heights from the base line were compared among the three treatment groups in Fig. 5. Incorporation of ^{32}P was stimulated by both drugs in myocardial proteins having molecular weights of 150,000, 30,000, 19,000, 15,000 and 11,000 daltons. Denopamine, however, enhanced phosphorylation of the 30,000- and 11,000-dalton proteins, corresponding to troponin-I and phospholamban (dimer), less markedly than isoproterenol did.

DISCUSSION

In the guinea-pig hearts perfused with a small volume perfusate, the degree of myocardial c-AMP elevation by denopamine (3×10^{-6} M) was significantly lower than that by isoproterenol (10^{-7} M) in the presence of cardiotonic action of the same magnitude. The result agrees with the data obtained at a maximum tension of perfused guinea-pig hearts [5, 6]. 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 [25]. These observations with denopamine may be explained by the "compartment theory", which assumes a compartment of c-AMP more directly involved than the rest in cardiotonic action [26]. The time to get to a maximum cardiotonic action by 10^{-7} M denopamine was much longer than that by 3×10^{-9} M isoproterenol, though both drugs exerted similar cardiotonic actions at their peak stimulation. This observation might be related to the lower degree of c-AMP elevation by denopamine in comparison with isoproterenol.

A role for myocardial c-AMP in the genesis of ventricular arrhythmias has been proposed [27, 28]. In coronary ligated dogs, induction of arrhythmogenicity by denopamine was weaker than by catecholamines [29]. The lower degree of elevation of cardiac c-AMP levels by denopamine than by isoproterenol (Fig. 2) may also contribute to the weak arrhythmogenicity of denopamine.

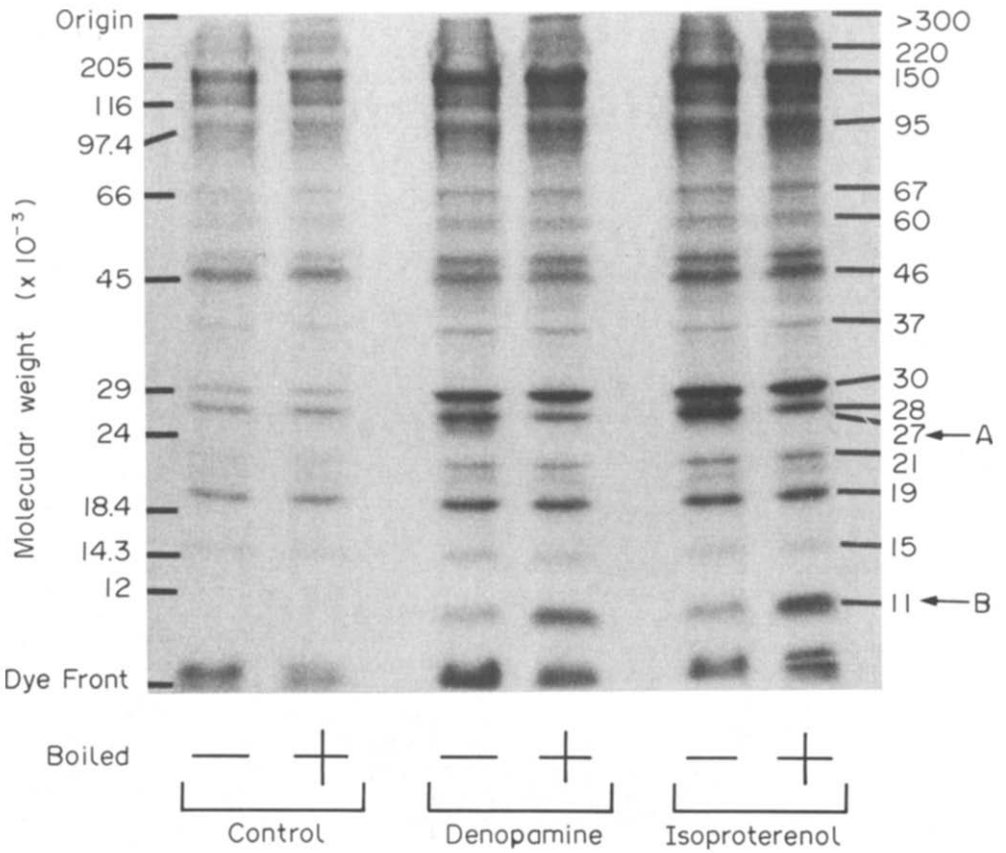

Fig. 3. Autoradiogram of an SDS–polyacrylamide gel electrophoresis of cardiac muscle proteins from the ^{32}P perfused guinea-pig hearts treated with vehicle, denopamine and isoproterenol. ^{32}P perfused hearts were treated with vehicle, denopamine (3×10^{-6} M) or isoproterenol (10^{-7} M) for 52 sec (for vehicle and denopamine) or 55 sec (for isoproterenol) at 30° prior to freeze clamping. Tissue samples were dissolved in a buffer (2% SDS, 10% glycerol, 0.035% bromophenol blue, 5% β-mercaptoethanol, 0.3 M Tris, pH 6.8), and an aliquot of the sample was boiled for 3 min (+) and another aliquot was kept at room temperature for 5 min (−) before gel electrophoresis. Each 40 μl aliquot containing 80 μg of protein was applied to the Laemmli 10–15% polyacrylamide gradient gel and was subjected to electrophoresis as described in Methods. The gels were stained with Coomassie Blue.

An autoradiogram of the SDS–polyacrylamide gel electrophoresis was developed after about 10 day exposure of the gel to Kodak X-ray film (DEF-5). The arrow marks A and B correspond to proteins having molecular weights of 27,000 and 11,000, respectively. Similar autoradiograms were obtained from five experiments.

According to Fenton and Dobson, who used ^{32}P perfused rat hearts (at 37°), isoproterenol (10^{-7} M) stimulated ^{32}P incorporation into cardiac muscle proteins having molecular weights of 155,000, 92,000, 30,000, 28,000, 22,000 and 20,000 daltons [30]. In the present study with perfused guinea-pig hearts (at 30°), ^{32}P incorporation into 150,000, 30,000, 19,000, 15,000 and 11,000 dalton proteins were significantly stimulated by isoproterenol (10^{-7} M) (Fig. 5). The discrepancies of phosphorylated proteins between these two studies may be ascribable to the differences in animal species or some experimental conditions (temperature during perfusion and conditions for gel electrophoresis, etc.).

The 150,000-dalton protein was tentatively identified as C-protein, a component of the thick filament of skeletal and cardiac muscles [31, 32]. Although the function of C-protein remains unknown, the protein has been suggested to regulate the thick filament length [33, 34], participate in thick filament structural support or regulate cross-bridge movement during contraction [31]. Recently, it has been shown that the C-protein of cardiac muscle was phosphorylated in response to β-adrenergic agonists [35] and its phosphorylation was correlated with the rate of relaxation in amphibian cardiac muscle [36].

The 30,000-dalton protein is known to be troponin-I, which is also phosphorylated by stimulation of β-adrenergic receptors [37]. When troponin-I is phosphorylated, Ca^{2+}-sensitivity of troponin-C is reduced, resulting in stimulation of the relaxation rate and shortening of the contractile phase [38].

According to Barany, both troponin-I and the 150,000-dalton protein are phosphorylated in parallel in the epinephrine-treated rat heart, suggesting that these two proteins are phosphorylated by the same protein kinase [7]. In the present study, however, the degrees of stimulation of phospho-

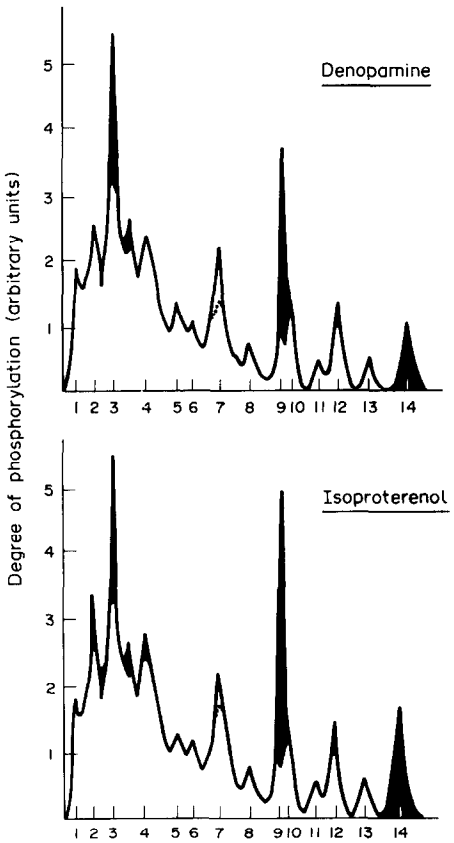

Fig. 4. Densitometric scanning of autoradiograms from SDS–polyacrylamide gel electrophoresis. Densitometric scanning of the autoradiogram shown in Fig. 3 was carried out, and those from the heart treated with denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M) were superimposed with that of control (– – –). Peak heights of the densitometric tracing of an autoradiograph from the gel electrophoresis of cardiac proteins were corrected for the specific radioactivity of ^{32}P-ATP of each heart.

rylation of troponin I by both drugs were more marked than those of the 150,000-dalton protein, especially with isoproterenol.

The 19,000-dalton protein has been reported to be myosin light chain by Barany and Barany [7]. However, results from three different laboratories [39–41] showed that exposure of the rabbit or rat heart to epinephrine or isoproterenol does not increase phosphorylation of this protein under conditions when positive inotropy is observed, suggesting that cardiac contractility may not be controlled by the phosphorylation of myosin light chain. In the present experiment, however, isoproterenol as well as denopamine stimulated phosphorylation of the 19,000-dalton protein (Fig. 5). According to England [9], the 20,000-dalton protein is myosin P light chain which can be phosphorylated by isoproterenol in perfused rat hearts [30]. The 19,000-dalton protein of our study might correspond to England's 20,000-dalton protein.

The 15,000-dalton protein is known to be a sarcolemmal protein, which is also phosphorylated, via activation of c-AMP-dependent protein kinase, by stimulation of β-adrenergic receptors [42, 43], and whose phosphorylation is involved in the increase in slow inward current [44].

Phospholamban, located in SR, is known to be also phosphorylated in response to β-adrenergic agonists and its tetramer (27,000-dalton) form is converted to dimers (11,000-dalton) when it is boiled prior to gel electrophoresis [21–24]. Phosphorylated phospholamban stimulates Ca^{2+} uptake into SR through activation of Ca^{2+}-ATPase, resulting in enhancement of relaxation in cardiac muscle cells [45].

A notable finding was that denopamine (3×10^{-6} M) and isoproterenol (10^{-7} M) stimulated ^{32}P incorporation into the same set of proteins in spite of lower degrees of c-AMP elevation compared with isoproterenol. However, the degrees of stimulation of ^{32}P incorporation into the 30,000-dalton (troponin-I) and 11,000-dalton (a dimer of phospholamban) proteins by denopamine were less marked than those by isoproterenol (Fig. 5). The higher degree of c-AMP elevation by isoproterenol than by denopamine might be related to the higher degrees of phosphorylation in these proteins by isoproterenol.

Sato *et al.* have reported that denopamine exerts almost equal effects on both $-(dF/dt)$ and $+(dF/dt)$ in isolated guinea-pig ventricular muscles, while isoproterenol showed greater effects on $-(dF/dt)$ [46]. We also obtained similar results. These observations may be related to the higher degrees of phosphorylation in troponin-I and phospholamban by isoproterenol than by denopamine. The phosphorylation of these proteins is known to be related to cardiac muscle relaxation [7]. Also, the lower degrees of phosphorylation in these two proteins by denopamine, as well as the lower degree of c-AMP elevation, might be responsible for the weaker arrhythmogenicity of denopamine.

The similar degrees of phosphorylation in the 15,000-dalton protein by the two drugs, although not substantial in comparison with the other phosphorylated proteins, may correspond to the equipotent cardiotonic effects of these drugs at the tested doses.

Phosphorylated phospholamban is known to stimulate the rate of relaxation through enhancement of Ca^{2+} uptake into cardiac SR and then increase contractility through acceleration of Ca^{2+} release from the SR in subsequent contractions [47–50]. However, the cardiotonic effects of 10^{-7} M isoproterenol and 3×10^{-6} M denopamine were nearly equal (Fig. 1), when larger amounts of phosphorylated phospholamban were observed with isoproterenol (Fig. 5).

In the case of isoproterenol, the above "indirect" stimulating effect of phosphorylated phospholamban on contractility might have been cancelled out by rapid abbreviation of systole, when the spontaneous transition from Ca^{2+} release to Ca^{2+} uptake by the SR was accelerated by the highly phosphorylated phospholamban, or by the excess relaxation caused by the concomitantly occurring highly phosphorylated troponin-I.

Cardiac muscle proteins involved in the propagation of cardiac contraction are known to be phosphorylated not only by c-AMP dependent protein kinase but also by Ca^{2+}-calmodulin dependent protein kinase or protein kinase C. For example,

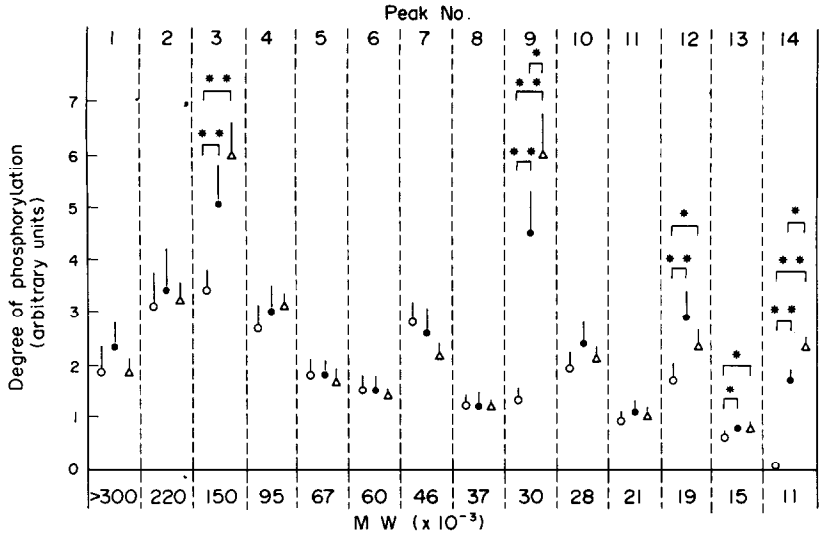

Fig. 5. Effects of denopamine and isoproterenol on the phosphorylation of cardiac muscle proteins in ^{32}P perfused guinea-pig hearts. ^{32}P perfused hearts were treated with vehicle (○), denopamine (●, 3×10^{-6} M) or isoproterenol (△, 10^{-7} M). Peak heights of the densitometric tracing of an autoradiograph from the gel electrophoresis of cardiac proteins were corrected for the specific radioactivity of ^{32}P-ATP of each heart. Values are means ± SE for observations from five experiments. Significant differences from control or corresponding isoproterenol values: $^{*}P < 0.05$, $^{**}P < 0.01$.

phospholamban is phosphorylated also by Ca^{2+}-calmodulin dependent protein kinase [51, 52] or protein kinase C [53, 54]. Recently, the 15,0000-dalton protein in canine cardiac sarcolemmal membranes has been reported to be phosphorylated by endogenous membrane-bound protein kinase C [55]. Whether denopamine can affect these protein kinase has not been examined. Further studies will be necessary to elucidate the exact mechanism of cardiotonic action of denopamine.

Acknowledgements—We would like to thank Dr Hiromichi Nakajima, Director of Biological Research Laboratory, Tanabe Seiyaku Co. Ltd., for encouragement throughout this study.

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