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Dopamine stimulation of cardiac β-adrenoceptors: the involvement of sympathetic amine transporters and the effect of SKF38393

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1 Mechanisms underlying β-adrenoceptor stimulation by dopamine were examined on guinea-pig Langendorff-perfused hearts and isolated cells from the right atrium, by using the chronotropic effects and the enhancement of L-type Ca^{2+} current ($I_{Ca,L}$) in the presence of prazosin as indicators of β-adrenoceptor stimulation. Dopamine-induced overflow of noradrenaline (NA) concentrations was measured by high-performance liquid chromatography.

2 Dopamine caused positive chronotropic effects with an EC_{50} of 2.5 μM and induced NA overflow with a similar EC_{50} (1.3 μM). The chronotropic effect of dopamine was abolished by bisoprolol (1 μM).

3 The effects of dopamine were maintained during prolonged application, whereas the effects of tyramine faded with time. Dopamine (3 μM) restored the chronotropic effects and the NA release suppressed by pretreatment with tyramine, suggesting a *de novo* synthesis of NA during the exposure to dopamine.

4 Dopamine (3 μM)-induced NA release was not affected by tetrodotoxin, ω-conotoxin, rauwolscine, ICI118551 or sulpiride, but was inhibited by desipramine, a NA uptake inhibitor (IC_{50} ~1 μM). It was also not affected by GBR12909 and bupropion, dopamine uptake inhibitors in the central nervous system.

5 SKF38393, a D_1 receptor partial agonist, potently inhibited the 3 μM dopamine-induced release of NA (IC_{50} ~0.1 μM). D_1 receptors are not involved in the DA-induced release of NA, since SCH23390 (3 μM), a potent D_1 antagonist, inhibited the NA release only slightly, and dihydrexidine (1 μM) and chloro-APB (1 μM), full D_1 agonists, caused no significant NA release.

6 SKF38393 inhibited tyramine-induced overflow of NA, and potentiated the field stimulation-induced NA release. SKF38393 and desipramine retarded the decay of the stimulation-induced tachycardia in a similar manner. These results indicate that SKF38393 is a potent monoamine transport inhibitor and a useful tool for the functional evaluation of indirectly-acting sympathomimetic agonists in the heart. In the presence of SKF38393 (10 μM), dopamine at 1 μM showed no chronotropic effect.

7 Voltage clamp experiments with isolated atrial cells revealed that dopamine is a weak partial agonist. The EC_{50} for $I_{Ca,L}$ stimulation by dopamine was high (13 μM). As a result, dopamine at 1 μM did not affect $I_{Ca,L}$. Bisoprolol abolished the stimulation of $I_{Ca,L}$ by dopamine (30 μM), and dihydrexidine (1 μM) did not affect $I_{Ca,L}$.

8 It was concluded that the cardiac effects of dopamine at clinically relevant concentrations (<1 μM) result almost exclusively from the indirect effect of β adrenoceptor stimulation, involving the release of NA from sympathetic nerve terminals. The roles of the direct stimulation of β adrenoceptors by dopamine at these concentrations and the stimulation of postjunctional D_1 receptors seem negligible. The desipramine- and SKF38393-sensitive monoamine transporter mediates the release of NA.

Keywords: Dopamine; noradrenaline; sympathetic nerve; chronotropic effect; β adrenoceptor; D_1 dopamine receptor; monoamine transporter; SKF38393; Ca^{2+} current

Introduction

Dopamine-specific receptors are classified into two major subtypes (D_1 and D_2), depending on their functional and pharmacological properties (Kebabian & Calne, 1979; Goldberg & Kohli, 1983). Their wide distribution (Goldberg, 1972; Lokhandwala & Barrett, 1982) indicates that dopamine is a neurotransmitter in the peripheral tissues as well as in the central nervous system (CNS). Clinically, dopamine has been widely used for the treatment of congestive heart failure or cardiovascular shock. It decreases the vascular resistance through D_1-mediated potentiation of adenylate cyclase in the vascular smooth muscles and D_2-mediated inhibition of noradrenaline (NA) release from sympathetic nerves (Lokhandwala & Barrett, 1982; Goldberg & Kohli, 1983). This inhibition of NA release was also observed in the heart (Fuder & Muscholl, 1978; Lokhandwala & Barrett, 1982; Rump *et al*., 1995). However, dopamine exerts stimulator effects on the heart: i.e., positive inotropic and chronotropic effects mediated mainly by β-adrenoceptors (Tsai *et al*., 1967; Goldberg, 1972; Mugelli *et al*., 1977). In addition to the direct stimulation of adrenoceptors, dopamine enhances NA release from the heart (indirect stimulation) (Goldberg, 1972), which seems to conflict with the D_2-mediated effect on the sympathetic nerve terminals.

The strongest evidence for the indirect stimulation by some sympathomimetic agonists is that their effects are attenuated by pretreatment with reserpine, a vesicular monoamine transport inhibitor. This is the case with dopamine. Thus, in most experimental studies, the functional evaluation of direct (and indirect) actions of dopamine has been based on its effects on reserpine-treated hearts (Tsai *et al*., 1967; Mugelli *et al*., 1977;

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Brodde *et al*., 1980). However, results thus obtained will vary according to the degree to which reserpine had depleted NA. In addition, reserpine pretreatment does not inhibit the release of NA from the cytosol of the nerves (Bönisch & Rodrigues-Pereira, 1983). The precise roles of the direct and indirect mechanisms in the effects of dopamine on the heart are unclear at present.

Indirectly-acting sympathomimetic amines are taken up into the nerve terminals by monoamine transporters, thereby extruding NA from the nerves (Trendelenburg *et al*., 1987). In addition, dopamine may augment the accumulation of NA in the cleft by occupying the transporters and inhibiting NA reuptake into the nerve terminals (Tsai *et al*., 1967). On the other hand, dopamine, as a neurotransmitter, may mediate NA release by stimulating some amine receptors on the nerve terminals, or, postjunctional D_1 receptors (Ozono *et al*., 1996) may contribute in part to the activation of adenylate cyclase in the myocardium. Dopamine transported into the nerve terminals may also act as an immediate substrate for NA synthesis and increase the NA storage.

Dopamine or dopamine receptor agonists administered orally are being used more widely for treatment of cardiovascular and neuropsychiatric disorders (Rajfer & Davis, 1990; Gilmore *et al*., 1995). To use these compounds efficiently and avoid adverse effects, it is important to clarify the precise roles of the individual mechanisms involved in the stimulation of the heart by dopamine. In this study, we aimed to elucidate the mechanism(s) that mediate the dopamine-induced stimulation of β adrenoceptors. We used Langendorff-perfused guinea-pig hearts to study the release of NA induced by dopamine and adrenoceptor stimulation. In addition, we evaluated the direct effect of dopamine with voltage clamp method on nerve-free, single right atrial cells. Some of the results have been published in a preliminary form (Habuchi *et al*., 1997).

Methods

Langendorff experiments

Guinea-pigs each weighing 250–300 g were anaesthetized with diethyl ether, then killed by cervical dislocation. The aorta was cannulated quickly and the heart perfused on a Langendorff apparatus with a constant pressure of 150 cmH_2O at 37°C. In some experiments, reserpine (3 mg kg^{-1}) was injected intraperitoneally twice, 24 h and 8 h before the commencement of the experiments. The perfusate was a phosphate buffered solution bubbled with 100% O_2. It contained (in mM): NaCl 142, KCl 5.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 0.33, Na_2HPO_4 2.24 and glucose 10 (pH = 7.4). The electrocardiogram was obtained through teflon-coated stainless steel wire electrodes plunged into the right ventricular outflow and the apex, and the heart rate (HR) was monitored with a beat-to-beat HR counter (Nihon Kohden AT601G, Tokyo, Japan). Measurements were commenced after an equilibrium period of 40–60 min. Duration of the perfusion with dopamine or other chemicals at each concentration was 7 min unless otherwise specified. At the end of each perfusion, 3 ml of the post-perfusion solution was collected downstream of the Langendorff apparatus. This sample of solution was acidified with 6 M HCl (0.06 ml), then stored at −30°C until measurements of the NA concentration were made. The NA concentration was measured as previously described (Yoshimura *et al*., 1993), by use of high-performance liquid chromatography (Catecholamine Analyzer HPC 8030, Tosoh, Tokyo). After an equilibrium period, the overflow NA concentration from the control hearts was less than 0.05 nM ($n = 102$). In a preliminary study, we measured the NA concentration in dopamine products obtained from several chemical and pharmaceutical companies, and found that all of these dopamine products had a small contamination of NA ranging from 0.01 to 0.07%. In this study, we used a consistent lot purchased from Sigma (St. Louis, MO, U.S.A.). High-performance liquid chromatography revealed that 10 μM dopamine solution prepared with this product contained 1.0 ± 0.04 nM of NA ($n = 6$). The NA concentrations shown in the text are the data after subtracting the contaminant NA concentration.

In some experiments, other stainless steel wire electrodes were placed on the right and left atria to apply field stimulation. Rectangular pulses of 3 ms duration and a strength 3 times larger than the threshold for atrial pacing (approx. 6 mA) were applied at 4 Hz for 3 min. In these field stimulation experiments, atropine was added to the perfusing solution at a concentration of 1 μM.

In both the Langendorff and voltage clamp experiments, we focused on the β-adrenoceptor stimulation and added prazosin at 1 μM to all of the solutions to avoid α_1-mediated effects.

Voltage clamp experiments

The isolation of single cells and the measurement of the membrane currents were performed as described previously from this laboratory (Habuchi *et al*., 1996). Briefly, guinea-pig isolated hearts mounted on the Langendorff apparatus were perfused with the Ca^{2+}-free phosphate-buffered solution for 10 min, and then the perfusate was switched to one containing 10 u ml^{-1} collagenase (Yakult, Tokyo, Japan) and 0.06 u ml^{-1} protease (Type XIV, Sigma). After the first digestion for 10 min, small pieces of the right atrium were stirred in 0.3 ml of the second enzyme solution in a small culture bottle. The second enzyme solution contained 1.3 u ml^{-1} collagenase (Type H, Sigma). The supernatant was collected every 5 min, and isolated cells were stored in the stock solution containing 0.1% bovine serum albumin (Fraction V, Sigma). The stock solution contained in mM K-glutamate 90, oxalate 10, KCl 25, KH_2PO_4 10, $MgSO_4$ 1, taurine 10, ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 0.5, N-2-hydroxyethyl-piperazine-N′-2-ethanesulphonic acid (HEPES) 5, and glucose 10 (pH = 7.2 adjusted by KOH).

L-type Ca^{2+} current ($I_{Ca,L}$) was used as an indicator for stimulation of β-adrenoceptors or adenylate cyclase. Experiments were carried out with the membrane-perforated method to minimize the run-down of $I_{Ca,L}$ and keep the intracellular metabolism or signal transduction intact (Horn & Marty, 1988). K^+ currents were eliminated by intracellular perfusion with Cs^+ and external application of Ba^{2+}. The pipette solution contained in mM: CsCl 140, NaCl 6 and HEPES 5 (pH = 7.2 adjusted by CsOH). Amphotericin B, an ionophore for monovalent cations, was dissolved into the pipette solution at a concentration of 0.3 mg ml^{-1} (Habuchi *et al*., 1996). The external perfusate was the same as that used for the Langendorff experiments except that $BaCl_2$ was added at 0.5 mM. The cells were clamped at a holding potential of −40 mV to inactivate the fast Na^+ and T-type Ca^{2+} currents. Test pulses were applied to 0 mV for 200 ms at 0.87 Hz. The voltage clamp amplifier was an Axopatch 1-D (Axon Instrument, Foster, CA, U.S.A.). After filtration at 2 Hz, the membrane currents were monitored on a digital oscilloscope (Nicolet 310C) with a sampling time of 0.2 ms. The digitized data were subsequently analysed on a computer (NEC 98, Tokyo). $I_{Ca,L}$ was measured as the difference between the inward peak and the current at the end of the test pulse.

Materials

Dopamine HCl, NA bitartrate, tetrodotoxin (TTX), bupropion and amphotericin B were purchased from Sigma. SKF38393 (1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine-7,8-diol), SCH23390 (**R**-3-methyl-7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine), ICI118551 (erythro-D,L-1-(methyl-indan-4-yloxy)-3-isopropylamino-butan-2-ol), GBR12909 (1-[2-{*bis*(4-fluorophenyl)methoxy}ethyl]-4-(3-phenylpropyl) piperazine), SKF82958 (chloro-APB (±) 6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) and rauwolscine were purchased from Research Biochemical International (Natick, MA U.S.A.).

ω-Conotoxin GVIA was from Calbiochem (La Jolla, CA, U.S.A.) and dihydrexidine from Tocris Cookson (Bristol, U.K.). SKF82526 (fenoldopam) and bisoprolol were provided from SK&F (King of Prussia, PA, U.S.A.) and Tanabe Seiyaku Co. (Osaka, Japan), respectively. All other chemicals were purchased from Wako Pure Chem. (Osaka, Japan). Stock solutions of dopamine and isoprenaline (Iso) were prepared on the day of each experiment (1–100 mM in distilled water). Prazosin was dissolved in methanol at a concentration of 2.5 mM. ω-Conotoxin and TTX were dissolved at 0.1 mM in distilled water and 10 mM acetate-Na (pH = 4.5), respectively (−30°C). All other chemicals were dissolved into the test solution immediately before use.

Statistics

Statistical analysis was based on Student's *t* test, and *P* values less than 0.05 were considered significant. Data are presented as means ± s.e.

Results

Indirect and direct stimulation of β_1-adrenoceptors by dopamine

Figure 1a shows dose-dependent chronotropic effects of dopamine, which were abolished by bisoprolol, a selective β_1-antagonist. According to the dose-response curves (Figure 1b), the maximal response to dopamine was comparable to that to a full agonist, Iso, although the EC_{50} for dopamine (2.5 μM) was one order higher than that for Iso (3.9 nM). Reserpine-treatment (see Methods) significantly attenuated the chronotropic responses to dopamine at concentrations between 1 and 30 μM (non-paired *t* test). We also examined the chronotropic effects of NA. NA at concentrations below 3 nM did not significantly increase HR, so that the contaminant NA in the dopamine product used must not have modified the chronotropic effects of dopamine in these experiments. The overflow NA values in the corresponding experiments are shown as dose-response curves in Figure 1c. The EC_{50} for the dopamine-induced NA release was 1.3 μM, which resembles that for the chronotropic effects. This dopamine-induced NA release was attenuated, but not abolished, by pretreatment with reserpine. Iso caused no overflow of NA.

Since the dopamine product contained contaminant NA, the dose-response data for overflow of NA might have been distorted by exogenous NA to some extent, especially when dopamine was perfused at high concentrations (10 and 30 μM). In most of the following Langendorff experiments, therefore, we used a concentration of 3 μM, at which dopamine exerts submaximal effects. Figure 2a shows that the chronotropic effects of dopamine correlated temporally with NA release, developing in response to 3 μM dopamine with a similar time constant around 3 min. These close relationships between the dopamine-induced chronotropic effects and NA overflow suggest an essential role of the sympathetic nerves in the β-adrenoceptor stimulation by dopamine. We then performed the same experiments with tyramine, an indirectly-acting sympathomimetic amine which does not have direct β agonistic action. The chronotropic response and NA overflow caused by tyramine (30 μM) faded with time (Figure 2b), reflecting depletion of NA in the storage (tachyphylaxis). When dopamine was applied after a 2 h treatment with tyramine, the initial responses to dopamine were significantly reduced, indicating that dopamine and tyramine extrude NA from the same storage site. Prolonged dopamine application (up to 1 h) gradually restored the responses. However, such an increment of the responses was absent when NA was depleted by reserpine (Figure 2a). These findings suggest that NA is supplied during exposure to dopamine, and that dopamine must be taken up into the vesicles before being released as NA.

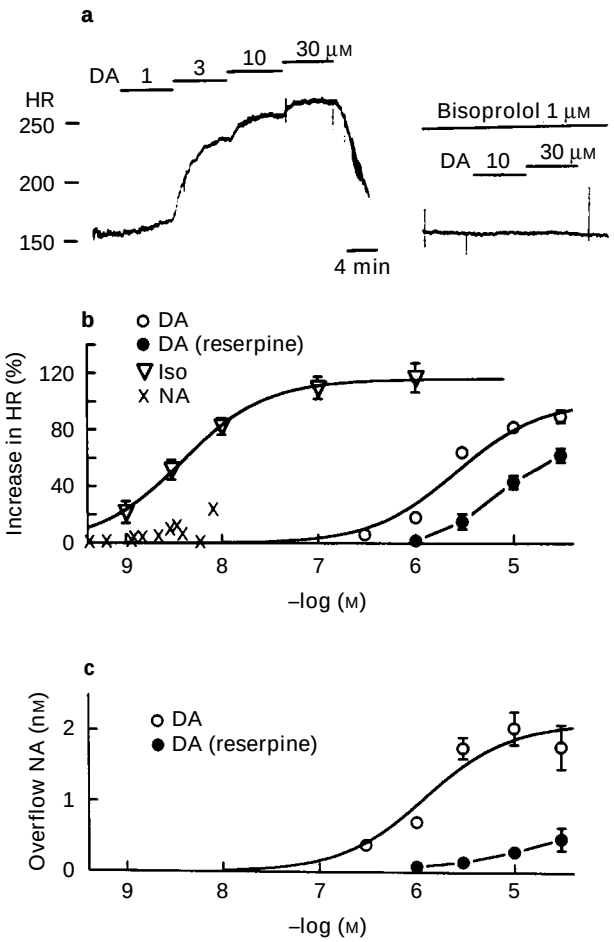

Figure 1 (a) Chronotropic effects of dopamine (DA). The traces show the responses of heart rate (HR) in beats min^{-1} of a guinea-pig Langendorff-perfused heart to dopamine during control (left) and during perfusion with bisoprolol at 1 μM (right). The concentrations of dopamine are indicated above the traces. (b) Dose-dependent effects of dopamine and isoprenaline (Iso). DA or Iso was applied in a cumulative manner at selected concentrations; $n = 8–18$ for DA, $n = 6$ for DA on reserpine-treated hearts, $n = 6–8$ for Iso. In five hearts, NA bitartrate was applied at concentrations below 10 nM, and the free NA concentration of the applied perfusate was also measured with high-performance liquid chromatography. The curves are the least squares fits to:

$$\text{Effect} = E_{max}/(1 + EC_{50}/D),$$

where E_{max} and D represent the maximal effect and the applied concentration, respectively. DA; $E_{max} = 99\%$ and $EC_{50} = 2.5$ μM. Iso; $E_{max} = 115\%$ and $EC_{50} = 0.0039$ μM. (c) Dose-response curve for the dopamine-induced overflow of NA. The measurements were made in corresponding experiments to those in (b). The fit to the control data (open circles) has an E_{max} of 2.1 nM and EC_{50} of 1.3 μM.

Transporter-mediated NA release by dopamine

The exocytotic NA release from the sympathetic nerves is triggered by activation of the TTX-sensitive Na^+ and ω-conotoxin-sensitive N-type Ca^{2+} channels (Hirning *et al*., 1988; De Luca *et al*., 1990; Vega *et al*., 1995), and is regulated by autoreceptors (Starke *et al*., 1977). On the other hand, the NA release induced by indirect sympathomimetic agonists involves monoamine transporters and is not mediated by nerve excitation (Trendelenburg *et al*., 1987). In our preparations, TTX (100 nM) and ω-conotoxin (100 nM) affected neither the dopamine-induced chronotropism nor the NA release (Figure 3a). We then examined the effects of the α_2 antagonist rauwolscine (1 μM, $n = 4$), the β_2 antagonist ICI118551 (1 μM, $n = 3$) and the D_2 antagonist sulpiride (100 μM, $n = 5$) on the 3 μM dopamine-induced NA overflow, and found that none of

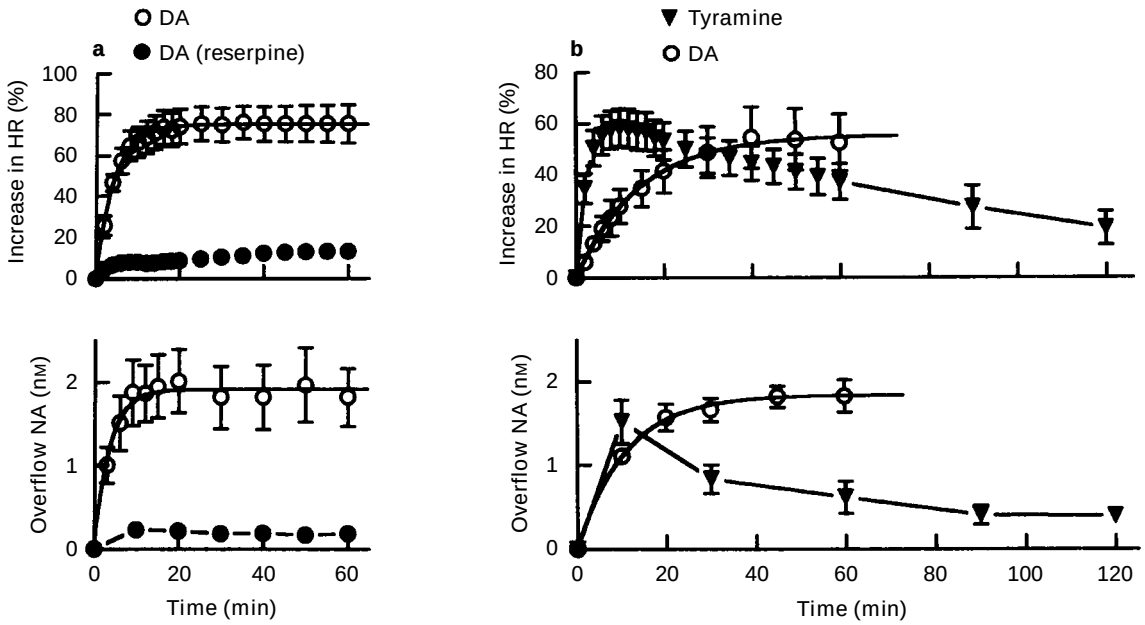

Figure 2 (a) Temporal changes in the HR and overflow of NA after exposure to dopamine (DA) 3 μM. Data were obtained from control hearts ($n=5$) and reserpine-treated hearts ($n=6$). The curve fitted to the control data has a time constant of 2.7 min for the chronotropic effect, and 3.3 min for the NA overflow. (b) Reversal by DA of tyramine-induced tachyphylaxis. Tyramine was applied at 30 μM for 2 h. After washout of tyramine, DA was applied at 3 μM for 1 h. Time constant = 14.6 min for the chronotropic effect, and 11.2 min for the NA overflow in response to DA.

them affected NA release significantly (Table 1). Although ICI118551 reduced the chronotropic effects of dopamine, at 1 μM it reduced 3 nM Iso-induced chronotropic effects similarly ($n=3$, data not shown). Therefore, the dopamine-induced NA release is independent of nerve excitation and presynaptic autoreceptor regulation.

Two major types of amine transporters have been identified. One type, involved in dopamine uptake in the CNS, is blocked by GBR12909 or bupropion (Andersen, 1989). The other type, which is engaged in NA uptake, is blocked by antidepressants. As shown in Figure 3b and Table 1, desipramine, but not GBR12909 or bupropion, inhibited the dopamine-induced NA release and decreased the HR. Desipramine at 0.1 μM enhanced NA overflow. At higher concentrations, it inhibited NA overflow with an IC_{50} around 1 μM (Figure 3c). However, potent direct actions of desipramine on the myocardium hindered us from examining the chronotropic effects of dopamine in the presence of desipramine. That is, desipramine at 1–10 μM produced a progressive slowing of HR, various degrees of atrioventricular block and widening of the QRS complex. Desipramine 10 μM arrested the spontaneous beating of the heart.

Blockade of dopamine-induced NA release by SKF38393

We previously showed that SKF83742, a D_1 antagonist, inhibits the dopamine-induced NA release, which suggests a possible involvement of D_1 receptors in NA release (Habuchi *et al*., 1997). In the present study, we tested various D_1-specific agents including SKF38393, SKF82526 (D_1 partial agonists), SCH23390 (a potent D_1 antagonist), chloro-APB and dihydrexidine (D_1 full agonists) (Lovenberg *et al*., 1989; O'Boyle *et al*., 1989; Gilmore *et al*., 1995). Table 2 shows that some benzazepine derivatives (SKF38393, SCH23390 and chloro-APB) significantly inhibited both the overflow of NA and chronotropic effects induced by 3 μM dopamine. SKF38393 exhibited the highest potency. Figure 4a indicates that SKF38393 inhibits the dopamine-induced NA release with an EC_{50} of ~0.1 μM. Unlike desipramine, SKF38393 at concentrations up to 10 μM did not affect the 3 nM Iso-stimulated HR significantly ($n=3$, not shown). Thus, SKF38393 has neither direct nor β blocking actions, and represents a useful tool for the functional evaluation of direct and indirect β-adrenoceptor agonism. In Figure 4b and c, SKF38393 was applied at a concentration of 10 μM and was found to induce a small amount of NA release (0.24 ± 0.03 nM, $n=6$) and an increase in basal HR (0–10%). It markedly suppressed the NA release in response to a subsequent application of dopamine; no significant increase in the NA overflow was observed at dopamine concentrations of 1 and 3 μM. The chronotropic effects of dopamine were also reduced correspondingly.

Figure 4d shows that SCH23390 (3 μM) caused a small rightward shift in the dose-response curve for dopamine-induced NA release. However, this concentration of SCH23390 is much higher than that needed for the D_1 receptor blockade ($K_i<1$ nM) (O'Boyle *et al*., 1989). We then examined the effects of the potent D_1 agonists dihydrexidine (1 μM, $n=4$) and chloro-APB (1 μM, $n=4$) in the absence of dopamine. They produced neither chronotropic effects nor a significant (>0.05 nM) NA overflow. Furthermore, SKF38393 at 3 μM potently inhibited the 30 μM tyramine-induced NA release from 1.37 ± 0.18 to 0.20 ± 0.08 nM ($n=4$). Altogether, the D_1 receptor is unlikely to be involved in dopamine-induced NA release and its inhibition by SKF38393.

Since the monoamine transporters are essential in the actions of indirectly-acting sympathomimetic amines, benzazepine derivatives may have blocked these transporters. If so, SKF38393 would enhance the effects of sympathetic nerve stimulation by augmenting the accumulation of NA in the junctional cleft. To test this hypothesis, we applied field stimulation to the atria (see Methods). Electrical stimulation evoked a tachycardia, which decayed quickly in a two-exponential function after the cessation of the stimulation (Figure 5a and b). Bisoprolol markedly attenuated this tachycardia, indicating that it was due to a sequence of β adrenoceptor stimulation as a result of the electrical excitation. SKF38393 (10 μM) augmented the post-stimulation tachycardia and abolished the faster component of the decay. In accordance with these changes in HR, the electrical stimulation caused an overflow of NA (1.85 ± 0.35 nM), which was potentiated to 2.79 ± 0.11 nM by SKF38393 at 10 μM ($n=5$, $P<0.05$ with paired t test). Desipramine (1 μM) also abolished the faster

component, similar to SKF38393, and slightly increased the overflow of NA from the control value of 1.67±0.13 to 1.81±0.11 nM ($n=4$, statistically not significant). To express the speed of the fast component of the HR decay, we measured the time required for one-third recovery to the basal HR ($t_{1/3}$).

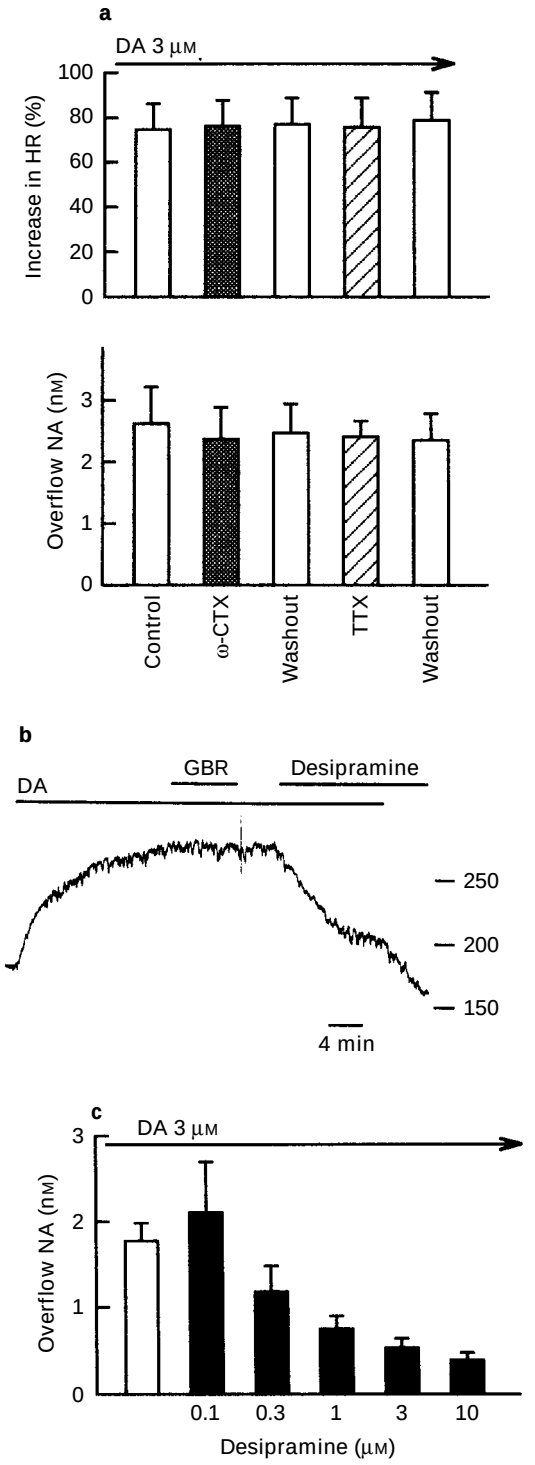

Figure 3 (a) Lack of effect of ω-conotoxin (ω-CTX) and tetrodotoxin (TTX) on the dopamine (DA)-induced chronotropic effect and NA overflow. The preparations ($n=4$) were first perfused with DA at 3 μM, which was followed by sequential applications of ω-CTX (30 nM) and TTX (100 nM), each for 7 min. (b) and (c) Inhibition of DA effects by desipramine. (b) The desipramine (1 μM) inhibition of 3 μM DA-induced chronotropic effect. Note that GBR12909 at 30 nM (marked as GBR) had no effect on the HR (shown as beats min^{-1}) In (c), desipramine was applied cumulatively in the presence of DA at 3 μM ($n=4$).

The arrows in Figure 5a illustrate a prolongation of the $t_{1/3}$ by SKF38393 from 0.68 to 2.42 min in the same preparation. In four preparations, field stimulation was applied sequentially during perfusion with desipramine and after addition of SKF38393. Subsequent application of SKF38393 in the presence of desipramine did not prolong $t_{1/3}$ significantly. A summary of the pooled data is shown in Figure 5c. These findings indicate that SKF38393 inhibits NA re-uptake by blocking the desipramine-sensitive monoamine transporter. The $t_{1/3}$, or the fast component of the HR decay, was not affected by dopamine at 1 μM.

Effects of dopamine on isolated heart cells

The aforementioned results suggest a minor role for the direct stimulation of β adrenoceptors in dopamine-induced heart stimulation. A straightforward method of evaluating the direct effect of dopamine is to examine it under completely denervated conditions in isolated single cells. In heart cells, $I_{Ca,L}$ has been used as a sensitive indicator for β adrenoceptor stimulation; and changes in this current are closely related to the positive inotropic and chronotropic effects of β agonists. Figure 6 shows the effects of dopamine at 3 μM and Iso at 3 nM on $I_{Ca,L}$ in the same cell. Although dopamine and Iso at these concentrations exerted a similar chronotropic effect in the whole heart, the responses of the $I_{Ca,L}$ were quite different. That is, 3 μM dopamine potentiated the $I_{Ca,L}$ only slightly, whereas 3 nM Iso doubled it. The stimulating response of $I_{Ca,L}$ to dopamine at 30 μM was completely eliminated by bisoprolol 1 μM ($n=5$, data not shown), and dihydrexidine (1 μM) did not increase $I_{Ca,L}$ at all ($n=5$, Figure 7a). In contrast to the potent inhibition of the dopamine-induced chronotropic effects, SKF38393 did not affect the dopamine-stimulated $I_{Ca,L}$ ($n=5$, Figure 7b). These findings indicate a weak stimulation of β-adrenoceptors by dopamine and argue against the involvement of D_1 receptors in the regulation of $I_{Ca,L}$ or adenylate cyclase in heart cells. The dose-response curves shown in Figure 7c indicate that Iso augmented $I_{Ca,L}$ with an EC_{50} similar to that for the chronotropic effects on the whole heart. As compared with Iso, dopamine is a weak partial agonist having a maximal response four times smaller than Iso. In addition, the EC_{50} for dopamine was as high as 13 μM.

Discussion

Direct and indirect stimulation of heart β adrenoceptors by dopamine

An important rationale for the acute therapeutic usage of dopamine is the resulting positive inotropic effect, which is mediated by β-adrenoceptors (Tsai *et al*., 1967; Goldberg, 1972; Mugelli *et al*., 1977). In this study, using the chronotropic effect as an indicator of β-adrenoceptor stimulation, we compared the relationship between the dopamine-induced β-adrenoceptor stimulation and overflow concentration of NA in the same heart preparations. Our results clearly showed that the positive chronotropic effects of dopamine are closely correlated with NA release (Figures 1 and 2). In addition, inhibition of NA release by benzazepine derivatives resulted in corresponding attenuation of the chronotropic effects. The roles of direct and indirect mechanisms can be separated under conditions where the NA release is completely abolished. We found that SKF38393 at 10 μM nearly completely abolished the NA release in response to dopamine 1 and 3 μM. Although 3 μM dopamine exerted submaximal chronotropic effects in the absence of SKF38393, this concentration of dopamine increased HR by only 8.0±1.5% in the presence of SKF38393 (Figure 4c). Voltage clamp experiments also revealed that dopamine is a weak partial β agonist with a high EC_{50}. Namely, dopamine potentiated $I_{Ca,L}$ with a threshold concentration of 3 μM, similar to the chronotropic effects of dopamine on the Langendorff preparations in the presence of

Table 1 Effects of autonomic blockers and monoamine transport inhibitors on 3 μM dopamine-induced chronotropic effects and overflow of NA

	Overflow of NA (nM)		*Increase in HR* (%)	
	DA	*DA + agent*	*DA*	*DA + agent*
Rauwolscine (1 μM, $n=4$)	2.39±0.37	2.68±0.44	60.6±4.7	63.3±4.2
ICI118551 (1 μM, $n=3$)	1.73±0.28	1.76±0.14	57.6±8.3	29.9±6.0*
Sulpiride (100 μM, $n=5$)	2.53±0.47	2.72±0.43	62.2±11.2	64.3±11.0
Desipramine (1 μM, $n=5$)	2.21±0.21	0.98±0.11*	68.2±9.1	24.3±4.2*
GBR12909 (30 nM, $n=6$)	1.99±0.20	1.96±0.18	74.8±10.4	71.8±12.6
Bupropion (1 μM, $n=4$)	1.78±0.19	1.69±0.15	59.6±8.3	58.2±8.4

The preparations were perfused with dopamine (DA) at 3 μM for 20–30 min, then the agent was added to the solution. The chronotropic effects are expressed as % increase in HR with respect to basal HR immediately before the application of dopamine. *Indicates that the value is significantly ($P<0.05$ with paired t test) different from that obtained during the control perfusion with dopamine.

Table 2 Effects of D_1-related agents on 3 μM dopamine-induced chronotropic effects and overflow of NA

	Overflow of NA (nM)		*Increase in HR* (%)	
	DA	*DA + agent*	*DA*	*DA + agent*
SKF38393 (3 μM, $n=3$)	2.10±0.19	0.48±0.06*	75.5±5.2	28.7±4.9*
SKF82526 (3 μM, $n=4$)	1.97±0.22	1.87±0.21	58.4±8.2	51.3±7.8*
SCH23390 (3 μM, $n=4$)	1.86±0.10	1.26±0.29*	63.5±5.0	35.7±3.2*
Chloro APB (3 μM, $n=4$)	2.26±0.17	1.18±0.20*	61.2±9.4	49.1±8.2*
Dihydrexidine (1 μM, $n=3$)	2.66±0.23	2.60±0.31	66.6±9.7	68.1±9.7

*Indicates that the value is significantly different from that obtained during the control perfusion with dopamine (DA).

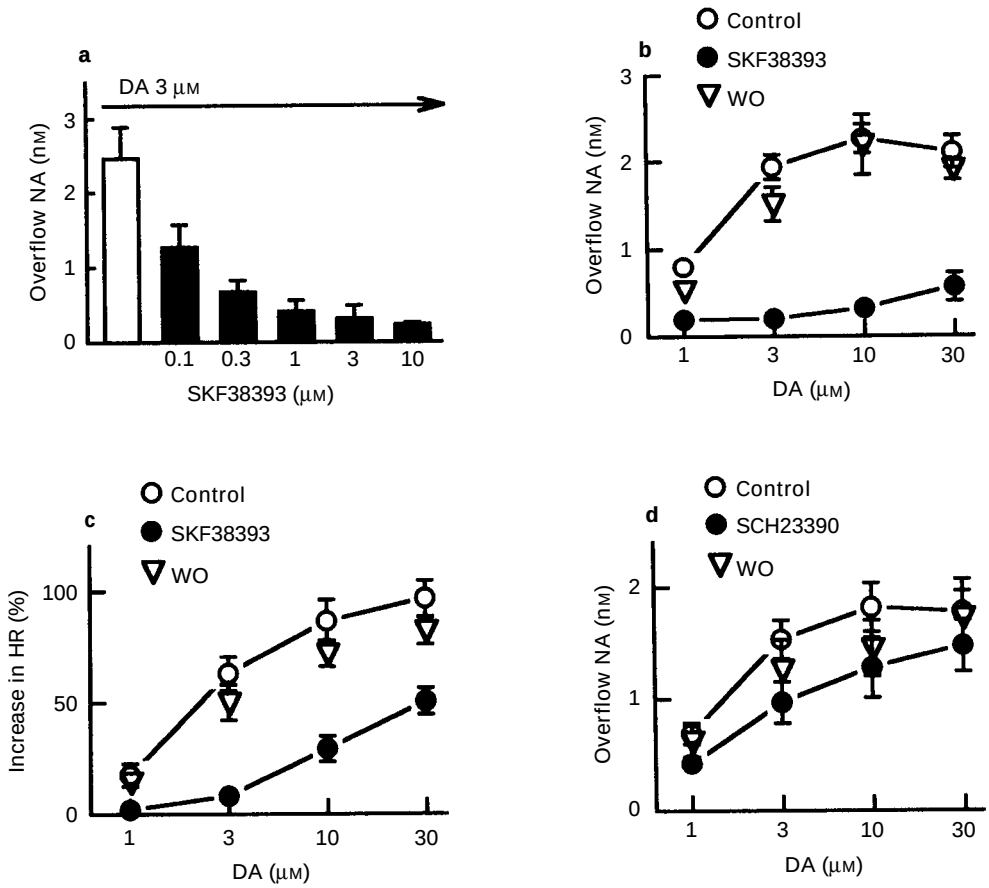

Figure 4 Inhibition of dopamine (DA)-induced effects by benzazepine compounds. (a) Inhibition of DA-induced NA release by SKF38393. SKF38393 was applied cumulatively in the presence of DA at 3 μM ($n=5$). (b) and (c) Effects of DA in the presence of SKF38393. The hearts were perfused with DA at concentrations ranging from 1 to 30 μM cumulatively during control, in the presence of SKF38393 at 10 μM, and after the washout (WO) of SKF38393 ($n=6$). SKF38393 alone increased the basal HR by 6.2±1.5% and caused an NA overflow of 0.24±0.03 nM. The % change in the HR (c) was measured with respect to the HR just before the application of DA. (d) Inhibition of DA-induced NA overflow by SCH23390. The experimental protocol was the same as that shown in (b); the concentration of SCH23390 used was 3 μM.

SKF38393. A resembling dose-dependence for $I_{Ca,L}$ stimulation by dopamine was recently found in rat cardiac cells by Zhao *et al.* (1977). In preliminary experiments, we did not observe a significant increase in $I_{Ca,L}$ in response to dopamine at 1 and 10 μM in rat ventricular cells (Habuchi *et al.*, 1995). Concomitant α_1-adrenoceptor stimulation by dopamine can mask the β-adrenoceptor-mediated effect (Boutjdir *et al.*, 1992), and the weak coupling between the dopamine-stimulated β adrenoceptors and the second messenger systems, as suggested from the partial agonism of dopamine, may have been disrupted by the intracellular dialysis or enzymatic procedure for cell isolation in our previous experiments.

All these findings strongly indicate that the dopamine-induced β-adrenoceptor stimulation is mostly mediated by NA released from the nerve terminals. The plasma concentration of free dopamine is 0.1–1 μM when injected therapeutically (Järnberg *et al.*, 1981). Thus, the dopamine-induced heart β-adrenoceptor stimulation during its therapeutic usage seems to be exclusively mediated by an indirect mechanism involving the sympathetic nerve terminals. Repeated or prolonged application of indirect sympathomimetic amines causes tachyphylaxis. Although dopamine exerts a potent indirect agonist effect, the effects of dopamine were sustained during the prolonged application, or dopamine reversed the tyramine-induced tachyphylaxis (Figure 2). It took 20–30 min for 3 μM dopamine to reverse the tyramine-induced tachyphylaxis. In rat hearts, radiolabelled dopamine has been found to be transformed to NA with a similar time course (Hellmann *et al.*, 1971). Since dopamine-β-hydroxylase is localized in the vesicles, the time-dependent potentiation of the effects of dopamine was not observed when NA was depleted by reserpine. Thus, the *de novo* synthesis of NA from dopamine in the vesicles is likely to contribute to maintenance of the indirect stimulation of adrenoceptors by dopamine.

In previous experimental studies, direct effects of dopamine on myocardial functions have been examined in reserpine-treated hearts (Tsai *et al.*, 1967; Mugelli *et al.*, 1977). However, our data revealed that pretreatment with reserpine (3 mg kg^{-1} twice per 24 h) does not necessarily abolish the dopamine-induced NA release. Potent chronotropic and inotropic responses of reserpine-treated hearts to dopamine in

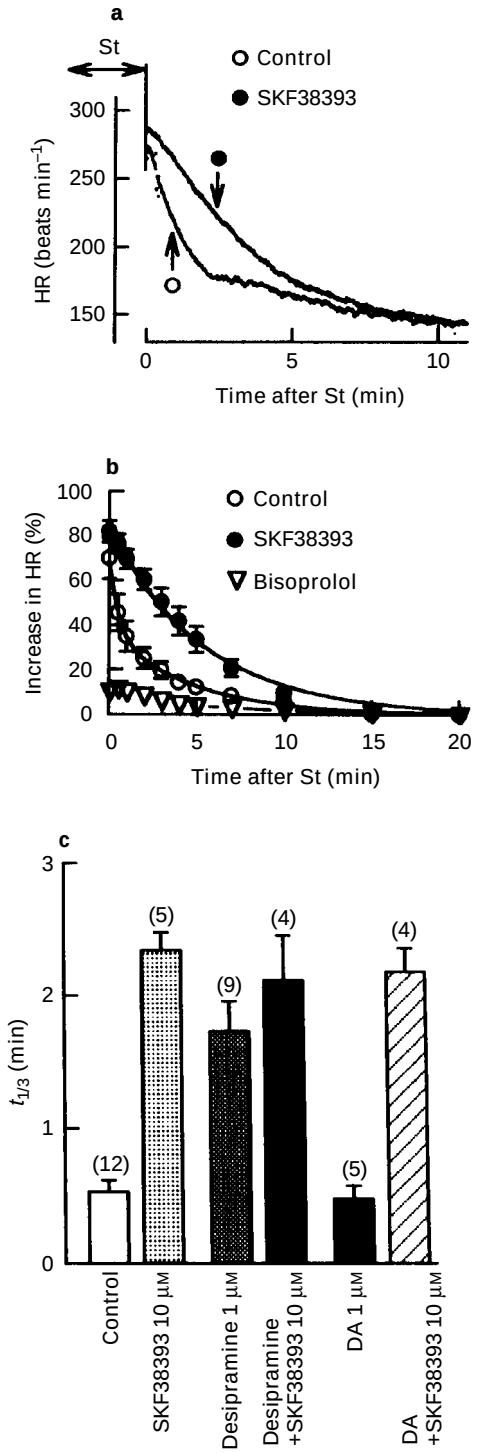

Figure 5 Potentiation of stimulation-induced tachycardia by SKF38393. (a) The records of HR following field stimulation (St). The stimulation was applied repeatedly during control and after exposure to 10 μM SKF38393. The arrows indicate the time required for withdrawal of 33% of the increased HR ($t_{1/3}$). The reference HR was measured 20 min after the cessation of the stimulation. The results of the same experiments are summarized in (b) ($n=5$). The decay of the HR was best described as a two-exponential function having time constants of 0.55 and 4.61 min during the control, whereas it could be expressed as a single-exponential function (time constant = 5.26 min) in the presence of SKF38393 (10 μM). Data from other hearts stimulated in the presence of bisoprolol (2 μM) ($n=3$) are also shown. (c) The $t_{1/3}$ under various conditions. The number of heart preparations is indicated in parentheses.

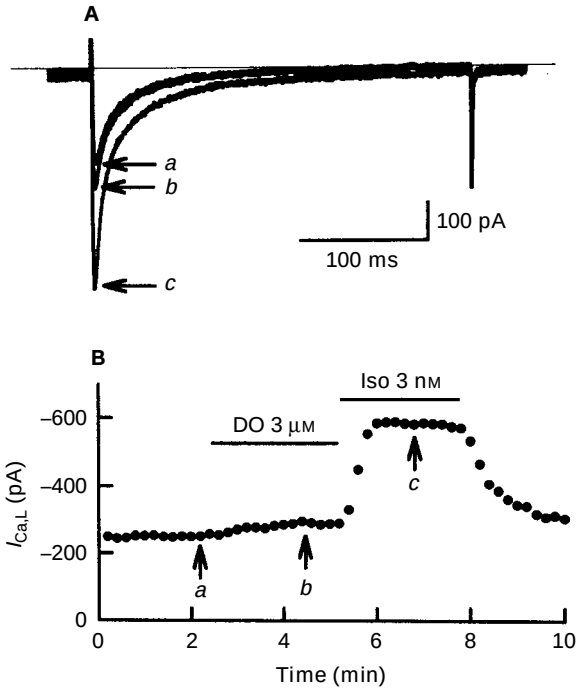

Figure 6 Effects of dopamine (DA) and isoprenaline (Iso) on the L-type Ca^{2+} current ($I_{Ca,L}$) in a single cell isolated from the right atrium. The cell was perfused with DA at 3 μM and Iso at 3 nM sequentially. (B) A temporal plot of the $I_{Ca,L}$ amplitude. The periods of perfusion with DA or Iso are indicated by the horizontal bars. The raw data obtained at the times indicated by arrows (a–c) appear in (A).

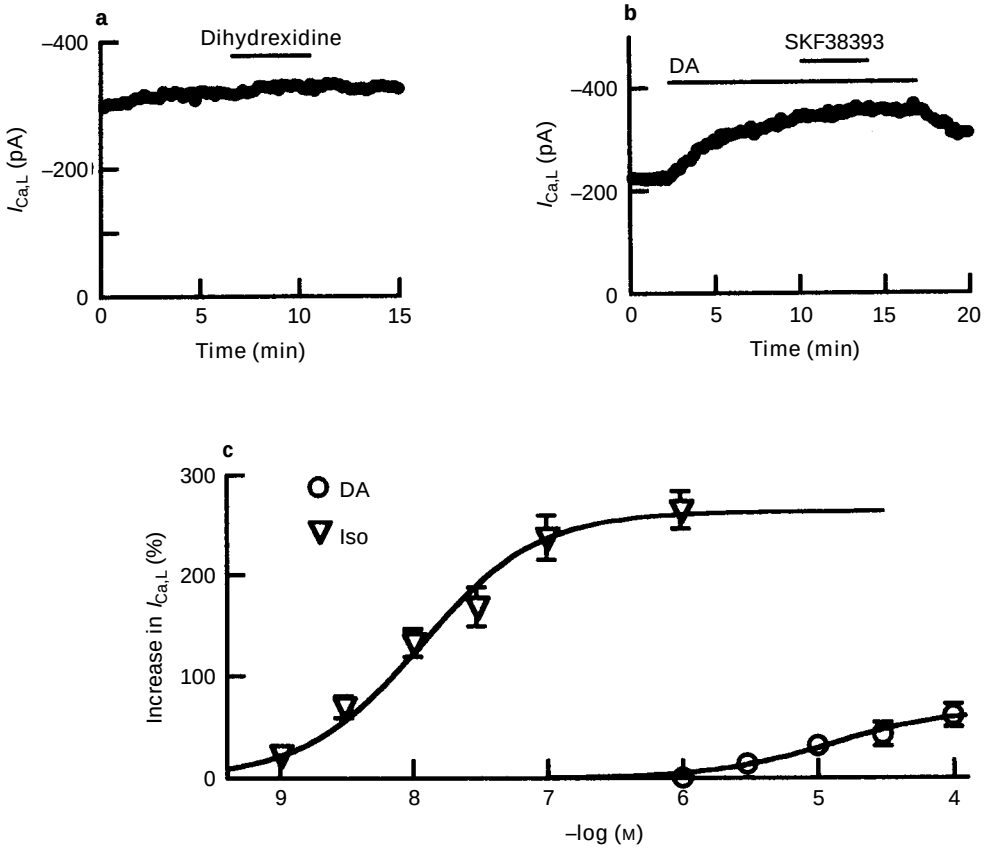

Figure 7 (a) Absence of effect of dihydrexidine on $I_{Ca,L}$. The concentration of dihydrexidine was 10 μM. (b) Absence of effect of SKF38393 on dopamine (DA)-stimulated $I_{Ca,L}$. The concentrations of DA and SKF38393 were 30 and 10 μM, respectively. (c) Dose-response curves for $I_{Ca,L}$ stimulation by DA and isoprenaline (Iso). One or two concentrations of the agonist were tested on each cell. DA; $E_{max}=63\%$ and $EC_{50}=12.7$ μM ($n=12-16$ for each symbol). Iso; $E_{max}=259\%$ and $EC_{50}=10.6$ nM ($n=8-12$).

those studies must have been due, at least in part, to a release of residual NA from the vesicles or from the cytosol. On the other hand, Brodde *et al.* (1980) demonstrated a predominant role of the indirect mechanism in the dopamine-induced β-adrenoceptor stimulation in rabbits, by showing that reserpine-treatment or cocaine markedly inhibited the dopamine-induced increase in the tissue cyclic AMP concentration. Using human heart muscles, Port *et al.* (1990) found that the maximal inotropic effect of dopamine in denervated (previously transplanted) hearts is one third of that in normal hearts. These findings favour our conclusion. It may be argued that α_1-adrenoceptor stimulation by dopamine or NA, which modifies the inotropic effects or intracellular cyclic AMP concentration, affected these results (Brodde *et al.*, 1980). Since we added prazosin to all the test solutions, the influence of α_1-adrenoceptor stimulation must be negligible in our data.

Ozono *et al.* (1996) demonstrated postjunctional D_1-receptors on myocardial sarcolemma of rats, the stimulation of which caused a small but significant increase in cyclic AMP concentration. However, contrasting radioligand binding and autoradiographic results were obtained by Amenta *et al.* (1993); they showed no specific binding of [^{3}H]-SCH23390 to human hearts. In the present study, the dopamine-induced chronotropic effect or potentiation of $I_{Ca,L}$ was abolished by bisoprolol. Dihydrexidine and chloro-APB failed to increase HR or $I_{Ca,L}$. Several previous experimental studies also indicated no significant role of D_1 receptors in the inotropic effects of dopamine on isolated cardiac muscles (Motomura *et al.*, 1978; Martinez-Mir *et al.*, 1987). Van Woerkens *et al.* (1991) found that the dopamine-induced increases in the HR and the rate of rise of left ventricular pressure were abolished by α- and β-adrenoceptor blockade *in situ*. It therefore seems that the role of D_1-mediated activation of adenylate cyclase is negligible in the heart.

Desipramine- and benzazepine-sensitive monoamine transporter in the heart

The role of the monoamine transporters in the actions of indirect agonists is of fundamental importance. They do not only take up the indirect agonists to replace NA in the storage sites; they also counter-transport the replaced NA out of the nerves (Trendelenburg *et al.*, 1987). In canine and baboon hearts, radiolabelled fluorodopamine was shown to be taken up by the desipramine-sensitive transporters (Goldstein *et al.*, 1990; Ding *et al.*, 1995). The presence of the GBR12909-sensitive transporters is still controversial in peripheral tissues, including the heart (Som *et al.*, 1994; Ding *et al.*, 1995). Our experimental results clearly indicate that the GBR12909-sensitive component must be very small if present in the heart. Although NA and dopamine share the same transporter, our finding that dopamine at 1 μM did not affect the fast decay of the electrical excitation-induced tachycardia indicates that the transporter involved takes up NA preferentially to dopamine. Inhibition of NA re-uptake has been proposed to contribute to the dopamine-induced adrenoceptor stimulation (Tsai *et al.*, 1967). The present data clearly argue against this hypothesis (at least at dopamine concentrations of <1 μM).

SKF38393 inhibited dopamine-induced NA release by blocking the transporter more potently than did desipramine. SKF38393 alone caused a small release of NA. We could not elucidate the mechanism of SKF38393-induced NA release in this study. Some molecules of SKF38393 may have been transported into the nerve terminals as a result of their high affinity to the transporter, and an increase in the intracellular

binding sites may have resulted in a countertransport of NA, similar to the case with indirect sympathomimetic agonists (Trendelenburg *et al*., 1987). Since some other benzazepine derivatives also inhibited dopamine-induced release of NA, the desipramine-sensitive transporters and D_1 receptors probably have some similarity in structure to bind with benzazepines. D_1 agonists and antagonists are now being widely used in numerous experimental studies to clarify the functional and anatomical evaluation of D_1 receptors. Our results show that some of the D_1 receptor-related compounds block the monoamine transporter, and may therefore exert some effects independent of D_1 receptors.

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