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Pharmacological evidence for α_{1D}-adrenoceptors in the rabbit ventricular myocardium: analysis with BMY 7378

Huang-Tian Yang & [1]Masao Endoh

Department of Pharmacology, Yamagata University School of Medicine, Yamagata 990-23, Japan

1 It was examined by means of BMY 7378, a selective antagonist of α_{1D}-adrenoceptors, whether α_{1D}-adrenoceptors contribute to the regulation of myocardial contractility and hydrolysis of phosphoinositide (PI) in rabbit ventricular muscle.

2 BMY 7378 had a biphasic antagonistic action on the positive inotropic effect (PIE) of phenylephrine depending on the concentration. BMY 7378 at 1–10 nM shifted the concentration–response curve (CRC) for the PIE of phenylephrine to the right and downward and at 100 nM to 1 μM it antagonized the PIE in a competitive manner, the slope of Schild plot being 0.93 and the pA_2 being 7.17 ± 0.09.

3 The inhibitory action of BMY 7378 at 1–10 nM is ascribed to the selective action on α_1-adrenoceptors because the PIE of neither isoprenaline nor endothelin-3 and angiotensin II was affected by this compound over this concentration range.

4 In the presence of 100 nM WB 4101, the antagonistic action of BMY 7378 at 1–10 nM remained unchanged but the antagonistic action of BMY 7378 at 100–300 nM disappeared. The antagonistic action of BMY 7378 at 1 nM was unaffected by 100 nM (+)-niguldipine.

5 Following pretreatment with chloroethylclonidine, BMY 7378 at 1 nM inhibited the maximal response to phenylephrine but the pD_2 value for phenylephrine was increased in the presence of BMY 7378. The CRC for phenylephrine was shifted to the left in the presence of 10–100 nM BMY 7378 but it was shifted to the right by BMY 7378 at 300 nM.

6 Stimulation of PI hydrolysis induced by phenylephrine was not affected by BMY 7378 up to 10 nM but it was reduced significantly by BMY 7378 at higher concentrations (100 nM to 1 μM).

7 BMY 7378 inhibited the [^{3}H]prazosin specific binding to the rabbit ventricular membrane fraction in a monophasic manner with a pK_i value of 7.53 ± 0.09.

8 The results indicate that in rabbit ventricular muscle, BMY 7378 at 1–10 nM suppressed the maximal response to phenylephrine (probably mediated by α_{1D}-adrenoceptors) and at 10–100 nM it inhibited the negative inotropic effect of phenylephrine, the mechanisms of which remain to be characterized. At higher concentrations (100 nM to 1 μM) BMY 7378 antagonized the functional and biochemical response via a presumed interaction mainly with the α_{1B}-adrenoceptor and partially with the α_{1A}-adrenoceptor.

Keywords: α_1-Adrenoceptors; positive inotropic effect; phosphoinositide hydrolysis; [^{3}H]prazosin binding; selective antagonists of α_1-adrenoceptors; rabbit ventricular myocardium

Introduction

Pharmacological studies have demonstrated the existence of three distinct α_1-adrenoceptors, α_{1A}-, α_{1B}- (Morrow & Greese, 1986; Han *et al*., 1987; Minneman, 1988) and, more recently, α_{1D}- (Saussy *et al*., 1994; Kenny *et al*., 1995; Piascik *et al*., 1995) subtypes in mammalian tissues. Furthermore, three distinct α_1-adrenoceptors have been cloned and designated as α_{1a}-, (historically termed α_{1c}-subtype; Schwinn *et al*., 1990, 1991), α_{1b}- (Cotecchia *et al*., 1988) and α_{1d}- (previously named α_{1a}, $\alpha_{1a/d}$ or α_{1d}-subtype; Lomasney *et al*., 1991; Perez *et al*., 1991; Schwinn & Lomasney, 1992) subtypes. It is now recognized that the cloned α_{1a}-, α_{1b} and α_{1d}-adrenoceptors correspond to the α_{1A}-, α_{1B} and α_{1D}-adrenoceptors, respectively (Hieble *et al*., 1995). The α_{1A}-adrenoceptors show a high affinity to the selective antagonists, such as WB 4101 and (+)-niguldipine. The α_{1B}-adrenoceptors are readily and irreversibly inactivated by the alkylating agent chloroethylclonidine (CEC) (Morrow & Greese, 1986; Han *et al*., 1987), while the α_{1D}-adrenoceptors have a high affinity for BMY 7378 and low affinity for (+)-niguldipine (for a review see Michel *et al*., 1995).

Current experimental evidence indicates that both α_{1A}- and α_{1B}-adrenoceptors are involved in the regulation of myocardial contractility in most mammalian species (Takanashi *et al*., 1991; Endoh *et al*., 1992a, b; Williamson *et al*., 1994). In the rabbit ventricular muscle, displacement of specific binding of [^{3}H]prazosin with the selective antagonist implies that approximately 60% of α_1-adrenoceptors belong to the CEC-sensitive α_{1B}-subtype, while the WB 4101- and (+)-niguldipine-sensitive α_{1A}-subtype may be about 10–30% of the total population of α_1-adrenoceptors (Takanashi *et al*., 1991; Endoh *et al*., 1992a, b). Recently, α_{1d}-adrenoceptor mRNA has been definitely detected in rabbit heart, indicating the potential expression of α_{1D}-adrenoceptors in cardiac muscle of this species (Suzuki *et al*., 1997), but the functional relevance of α_{1D}-adrenoceptors in regulation of myocardial contractility has not been determined.

It has been indicated that BMY 7378 has an approximately 100-fold higher selectivity of the α_{1D}- over the other two subtypes of α_1-adrenoceptors (Saussy *et al*., 1994; Goetz *et al*., 1995) and it has been proposed that this compound can be used to elucidate the specific role of α_{1D}-adrenoceptors in functional regulation (Kenny *et al*., 1995; Piascik *et al*., 1995). In the present study, we used BMY 7378 to elucidate whether α_{1D}-adrenoceptors contribute to the contractile regulation in adult rabbit ventricular muscle. Our data suggest that the BMY 7378-sensitive functional α_{1D}-adrenoceptors exist in this tissue, and they contribute to the α_1-adrenoceptor-mediated regulation of contractile force in the rabbit ventricle. A preliminary account of a part of the present results has been presented as an abstract (Yang & Endoh, 1996).

[1] Author for correspondence.

Methods

Measurement of inotropic effects

Male albino rabbits (1.9–2.2 kg) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.v.) and then two or three papillary muscles were excised from the right ventricle of each rabbit. Muscles were mounted in 20 ml organ baths that contained Krebs-Henseleit solution with 0.057 mM ascorbic acid and 0.027 mM EDTA·2Na. The solution was bubbled with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). The concentrations (mM) of the various constituents of the solution were as follows: Na^+, 142.9; K^+, 5.9; Mg^{2+}, 1.2; Ca^{2+}, 2.5; $H_2PO_4^-$, 1.2; HCO_3^-, 24.9; SO_4^{2-}, 1.2; Cl^-, 127.8 and glucose, 11.1. The muscles were stimulated electrically by square-wave pulses of 5 ms duration with a voltage about 20% above threshold at a frequency of 1 Hz. During the equilibration period (60 min) the muscles were initially stretched under a tension of 5 mN and the length was then adjusted to give 90% of the maximal developed tension.

In experiments a β-adrenoceptor antagonist timolol (1 μM) was included in the buffer to avoid any possible interference with β-adrenoceptor stimulation by phenylephrine or released noradrenaline. The concentration–response curve (CRC) for phenylephrine was determined for each preparation by cumulative addition of the drug. Each succeeding concentration was added only when the preparation had achieved a steady-state response to the previous concentration. After determination of the control CRC for phenylephrine, the drug was washed out for at least 120 min and then BMY 7378 (100 pM to 1 μM) was allowed to act for 30 min before the redetermination of CRC for phenylephrine. In some experiments, the first CRC for phenylephrine was determined in the presence of either 100 nM WB 4101 or (+)-niguldipine. After washout of the drugs, the preparations were incubated with either 100 nM WB 4101 or (+)-niguldipine that was applied 10 min prior to the addition of BMY 7378, the drugs were incubated together for 30 min, and then the second CRC for phenylephrine was determined in their presence. In another series of experiments, the preparations were treated with 10 μM CEC for 30 min followed by a 30 min washout period, and then the CRCs for phenylephrine were determined as described above in the absence or presence of BMY 7378. At the end of each experiment, the maximum contractile force was determined for each muscle by administration of isoprenaline (0.1–100 μM). The response to phenylephrine was expressed as a percentage of the maximal response to isoprenaline or of the maximal response to phenylephrine in the first CRC. Influence of BMY 7378 on the β-adrenoceptor-mediated positive inotropic effect (PIE) of isoprenaline was investigated in the same protocol used with phenylephrine to confirm the specificity of inhibitory action of BMY 7378 on the α_1-adrenoceptors. Influence of BMY 7378 on the PIE of other receptor agonists, endothelin-3 and angiotensin II, that stimulate the hydrolysis of phosphoinositide (PI) in the rabbit ventricular myocardium (Endoh *et al*., 1996; Ishihata & Endoh, 1993) was also examined. In this series of experiments, when the PIE for 30 nM endothelin-3 or 100 nM angiotensin II reached a steady level, 1 and 10 nM BMY 7378 were allowed to act for 20 min each in the presence of receptor agonists. This protocol was chosen because the CRC for endothelin-3 was not repeatable (Endoh *et al*., 1996) and the PIE of angiotensin II displayed a variation among individual preparations (Talkuder & Endoh, 1997).

Quantitation of [^{3}H]inositol monophosphate (IP_1)

The heart was quickly removed from rabbits and placed in Krebs-Henseleit solution, bubbled with 95% O_2 and 5% CO_2, at 37°C to wash out the blood. The experimental procedure was the same as that described previously (Yang & Endoh, 1994). Slices of ventricular muscle (0.5 mm thick) were prepared with a tissue slicer (Arthur H. Thomas Company, Philadelphia, PA, U.S.A.), weighed and equilibrated in Krebs-Henseleit solution for 30 min at 37°C. Then the slices were preincubated with 10 μCi ml^{-1} *myo*-[^{3}H]inositol in Krebs-Henseleit solution for 120 min. After the preincubation, the solution was changed to a fresh solution containing 5 mM *myo*-inositol and all the experiments were performed in the Li^+-containing solution. Timolol (1 μM) was added to the solution 20 min prior to addition of the agonist to avoid any interference with activation of β-adrenoceptors. BMY 7378 was allowed to act for 20 min before and during administration of 10 μM phenylephrine. Phenylephrine was allowed to act for 30 min and then the slices were quickly blotted and put into a mixture of chloroform, methanol and 12 N HCl (100:200:1 by volume) to terminate the reaction. After addition of 5 mM EDTA, the tissue was homogenized (Polytron PT-10; Kinematica, Lucerne, Switzerland). The tip of the homogenizer was rinsed with a mixture of chloroform, methanol, 12 N HCl and 5 mM EDTA (100:200:1:80 by volume), and the rinsing fluid was added to the original solution. Chloroform and 5 mM EDTA was added sequentially and the samples were centrifuged at 1400 *g* for 20 min to separate the aqueous and organic phases. An aliquot of the aqueous layer was applied to a column that contained a 50% slurry of AG1-X8 (anion-exchange resin; 100–200 mesh; formate form; Bio-Rad, Richmond, CA, U.S.A.). The column was washed first with 20 ml of distilled water and then glycerophosphoryl esters were eluted with 8 ml of a solution of 5 mM sodium tetraborate and 60 mM sodium formate (Berridge *et al*., 1983). Aliquots of the eluate were monitored for radioactivity in a scintillation mixture (ACS-II; Amersham, Arlington Heights, IL, U.S.A.) with a scintillation counter (TRI-CARB 1500, Packard, Downers Grove, IL, U.S.A.) at a counting efficiency of 66%. [^{3}H]IP_1, which has been shown to be a good indicator of stimulation of PI hydrolysis (Yang & Endoh, 1994), was collected and its radioactivity was quantitated.

[^{3}H]Prazosin binding assay

The binding assay with [^{3}H]prazosin was carried out as described in detail elsewhere (Hiramoto *et al*., 1988). In brief, pieces of right and left ventricular muscle, including free walls and septum, were excised from the heart of rabbits and homogenized in 10 volumes of ice-cold buffer (0.25 M sucrose containing 5 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.4) by means of Polytron (PT-10; Kinematica) three times for 15 s each at setting 7. Each homogenate was then centrifuged at 500 *g* for 15 min at 4°C. The supernatant was filtered through a single layer of cheesecloth and centrifuged at 50 000 *g* for 20 min at 4°C. The resulting pellet was washed twice with ice-cold incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) by repeated resuspension and recentrifugation. The final pellet was resuspended in ice-cold incubation buffer yielding a protein concentration of approximately 1 mg ml^{-1}.

To investigate the effect of BMY 7378 on the specific binding of [^{3}H]prazosin, the membrane fraction was incubated with [^{3}H]prazosin (1 nM) and various concentrations of BMY 7378 or incubation buffer for 20 min at 25°C and the reaction was terminated by addition of 2 ml of ice-cold incubation buffer. Then the mixture was rapidly filtered through a GF/C glass filter (Whatman International Ltd, Maidstone, U.K.) in a cell harvester (M-24R; Brandel, Gaithersburg, MD, U.S.A.). Each filter was washed rapidly with 12 ml (3×4 ml) of ice-cold incubation buffer. After the filter had been dried for 1 h at 90°C, radioactivity bound to the filter was quantitated. Nonspecific binding of [^{3}H]prazosin was defined as the binding detected in the presence of 10 μM unlabelled phentolamine. Specific binding of [^{3}H]prazosin was defined as the total radioactivity minus the radioactivity due to nonspecific binding. Each binding assay was carried out in duplicate. Protein was quantitated by the method of Lowry *et al*. (1951) with bovine serum albumin as standard.

Statistics

Experimental values are presented as means ± s.e.mean. The potency of the agonist was expressed as pD_2 ($-\log EC_{50}$) value. The potency of the antagonist was expressed as pA_2 value which was obtained from a plot of log [agonist DR-1] against log [antagonist concentration] where the slope was not different from unity (Arunlakshana & Schild, 1959). Significance of differences was estimated by a repeated measures analysis or one-way analysis of variance and/or by Student's *t* test where it is appropriate. A *P* value of less than 0.05 was considered to be significant. Data of receptor binding assay were analysed by use of the programme (Daiichi Pure Chem. Co. Ltd, Tokyo, Japan and modified by Tsuchihashi & Nagatomo, 1987) based on LIGAND programme (Munson & Rodbard, 1980).

Drugs

The drugs and reagents used were (−)-phenylephrine hydrochloride, (−)-isoprenaline hydrochloride, lithium chloride, *myo*-inositol and bovine serum albumin (Sigma, St.

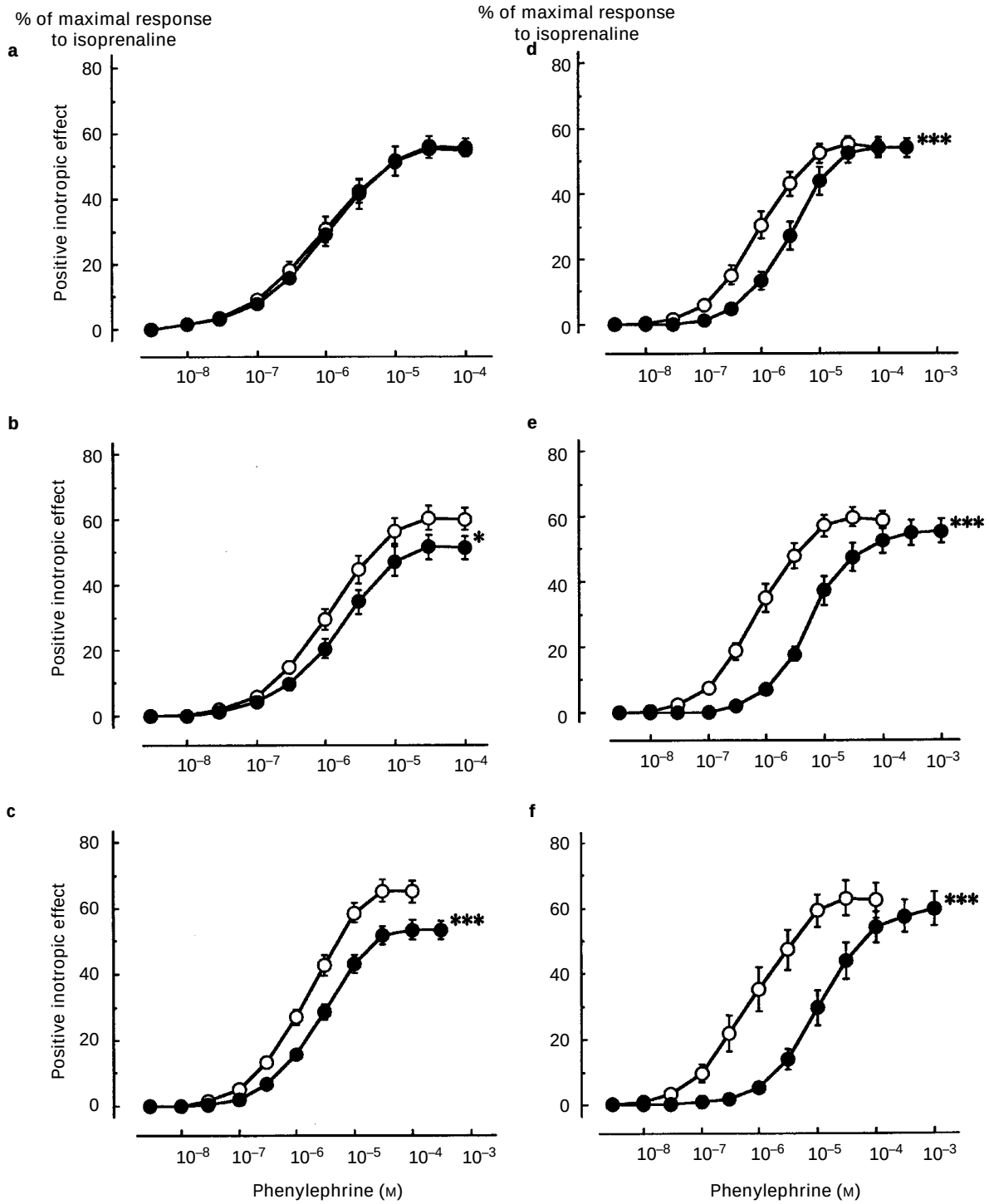

Figure 1 Effects of BMY 7378 on the concentration–response curve for the phenylephrine-induced positive inotropic effect mediated by α_1-adrenoceptors, in the presence of 1 μM timolol, in isolated rabbit papillary muscle. (○) Control, (●) in the presence of BMY 7378. BMY 7378 concentrations were 100 pM (a, $n=7$), 1 nM (b, $n=6$), 10 nM (c, $n=7$), 100 nM (d, $n=7$), 300 nM (e, $n=6$) and 1 μM (f, $n=6$), $^*P<0.05$ and $^{***}P<0.001$ vs the corresponding control groups estimated by a repeated measures analysis of variance.

Louis, MO, U.S.A.); (+)-niguldipine hydrochloride and ammonium formate (Wako Pure Chemicals Co., Osaka, Japan); BMY 7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride), WB 4101 (2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride), CEC dihydrochloride and (−)-timolol maleate (Research Biochemicals Inc. Natick, MA, U.S.A.); angiotensin II and endothelin-3 (Peptide Institute, Osaka, Japan); sodium pentobarbitone (Abbott Laboratories, North Chicago, IL, U.S.A.; *myo*-[2-^{3}H]inositol (specific activity, 86 Ci mmol^{-1}) and [7-methoxy-^{3}H]prazosin (specific activity 74 Ci mmol^{-1}; Amersham, Buckinghamshire, U.K.). The stock solution of isoprenaline was prepared in 0.1% ascorbic acid solution, diluted with 0.9% NaCl solution and kept ice-cold.

Results

Influence of BMY 7378 on the α_1-adrenoceptor-mediated PIE

Figure 1 shows the influence of BMY 7378, a selective antagonist of α_{1D}-adrenoceptors, on the PIE of phenylephrine in the presence of 1 μM timolol. The pD_2 values for phenylephrine in six different control groups were between 5.85 ± 0.04 to 6.13 ± 0.18, which did not show statistically significant difference among individual groups ($P > 0.05$). BMY 7378 up to 1 μM did not affect the basal force of contraction, but it inhibited the PIE of phenylephrine in different mode of antagonism depending on the concentration. BMY 7378 at 1–10 nM shifted the CRCs for phenylephrine to the right and downward as shown in Figure 1b and c; the extent of shift of the CRC for phenylephrine induced by BMY 7378 was almost similar at 1 and 10 nM. BMY 7378 at 100 nM to 1 μM shifted the CRCs for phenylephrine to the right without affecting the maximal response (Figure 1d, e and f). The slope of Schild plot was 0.93 when analysed at 100 nM to 1 μM BMY 7378, which was not significantly different from unity (Figure 2); pA_2 value was 7.17 ± 0.19.

To elucidate whether a non-specific depressant action of BMY 7378 contributes to the inhibition induced by the compound on the PIE of phenylephrine, we examined the influence of BMY 7378 on the PIE of isoprenaline, endothelin-3 and angiotensin II. BMY 7378 at 1 nM and 1 μM did not affect the CRC of isoprenaline (Figure 3). The PIEs of endothelin-3 at 30 nM and angiotensin II at 100 nM were not influenced by 1 and 10 nM BMY 7378 either, while the PIE of phenylephrine at 10 μM was significantly inhibited by $17.7 \pm 2.5\%$ and $27.2 \pm 5.0\%$ with BMY 7378 at 1 and 10 nM, respectively (Figure 4). The extents of the inhibition induced by BMY 7378 in this series were consistent with those ($16.3 \pm 4.4\%$ and $28.3 \pm 4.4\%$) observed in the previous series, in which BMY 7378 at 1 and 10 nM was administered prior to the determination of the second CRC for phenylephrine (Figure 1b and c).

Influence of BMY 7378 on the α_1-adrenoceptor-mediated PIE in the presence of WB 4101 and (+)-niguldipine

The antagonistic action of BMY 7378 at 1 and 10 nM was exerted in the presence of 100 nM WB 4101 (an antagonist of α_{1A}-adrenoceptors) and the inhibitory action of BMY 7378 was essentially the same as that in the absence of WB 4101: the maximal response to phenylephrine was significantly inhibited with little change in the pD_2 value for phenylephrine (Figure 5a and b; Table 1). By contrast, the effect of BMY 7378 at higher concentrations of 100–300 nM to induce the rightward shift of the CRC for phenylephrine disappeared in the presence of WB 4101 (Figure 5c and d).

As WB 4101 at 100 nM might elicit an antagonistic action on the effect mediated by α_{1D}-adrenoceptors (Hieble & Ruffolo, 1996), the effect of BMY 7378 was further examined in

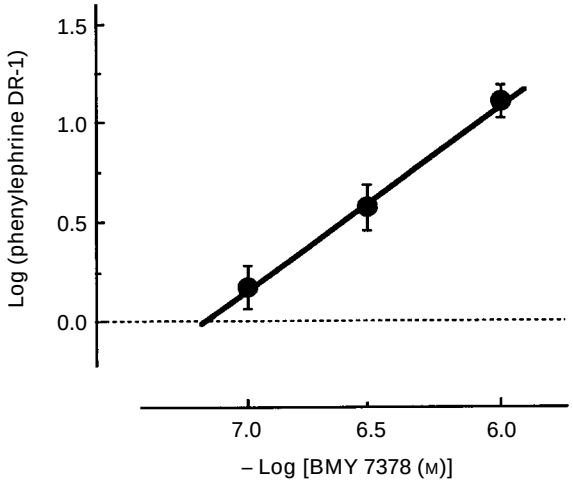

Figure 2 Schild plot of BMY 7378-induced antagonism against the effect of phenylephrine. Values presented are means ± s.e.mean. The data used were taken from Figure 1 and the slope of the regression line was calculated by the least-squares method over the concentration of 100 nM and 1 μM BMY 7378.

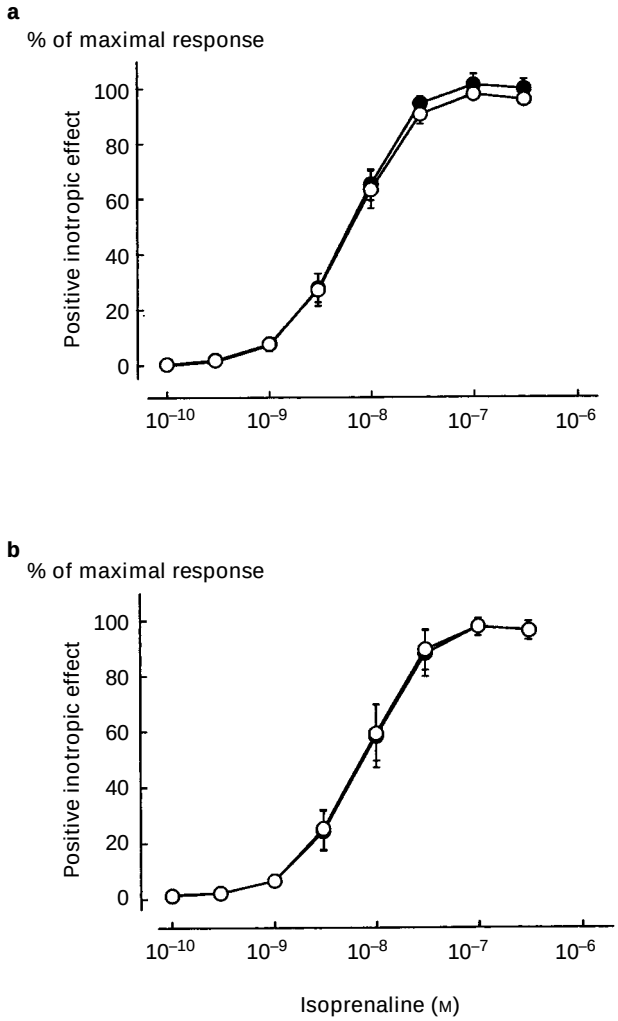

Figure 3 Influence of BMY 7378 on the positive inotropic effect of isoprenaline in rabbit isolated papillary muscle. (○) Control, (●) in the presence of BMY 7378; at 1 nM (a) and 1 μM (b), respectively ($n = 4$ each).

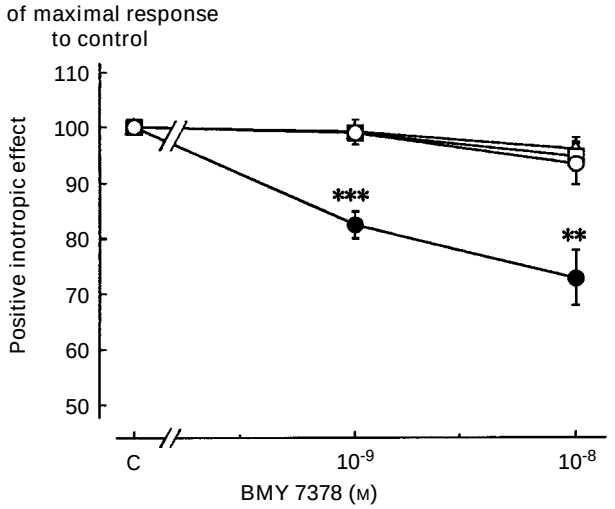

Figure 4 Influence of BMY 7378 (1 and 10 nM) on the positive inotropic effect of angiotensin II and endothelin-3 in isolated rabbit papillary muscle. For comparison, the influence of BMY 7378 on the positive inotropic effect of phenylephrine was examined in the same experimental protocol. Experiments were carried on in the presence of 1 μM timolol. (C) Control (100%); (△) 100 nM angiotensin II plus BMY 7378 (□) 30 nM endothelin-3 plus BMY 7378; (○) 10 μM phenylephrine without BMY 7378; (●) 10 μM phenylephrine plus BMY 7378; $n=5$ each. $^{**}P<0.01$ and $^{***}P<0.001$ vs the corresponding phenylephrine control.

Table 1 Influence of BMY 7378 on the positive inotropic effect (PIE) of phenylephrine in the isolated rabbit papillary muscle treated with WB 4101, (+)-niguldipine or CEC

		PIE of phenylephrine	
BMY 7378	n	*pD_2 value*	*Maximal response*
WB 4101 100 nM			% of ISO max
0	5	4.55±0.06	67.3±3.34
1 nM	5	4.47±0.09	51.0±5.31*
0	6	4.42±0.04	62.8±3.59
10 nM	6	4.38±0.06	49.6±4.55*
0	6	4.61±0.07	60.1±3.21
100 nM	6	4.56±0.10	61.0±2.10
0	5	4.74±0.03	58.6±6.69
300 nM	5	4.61±0.08	57.1±2.78
(+)-Niguldipine 100 nM			
0	5	5.63±0.08	48.6±3.16
1 nM	5	5.45±0.06	37.5±2.86*
Chloroethyclonidine 10 μM			% of control
0	5	4.96±0.12	100
1 nM	5	5.33±0.08*	62.5±5.14**
0	8	4.89±0.06	100
10 nM	8	5.22±0.04***	109.5±5.37
0	7	4.36±0.14	100
100 nM	7	4.83±0.10*	119.1±7.75*
0	6	5.34±0.09	100
300 nM	6	5.00±0.08*	95.3±11.9

ISO max: maximal response to isoprenaline; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs the corresponding control values.

the presence of (+)-niguldipine that has been reported to have a higher affinity to α_{1A}- than to α_{1D}-adrenoceptors (Michel *et al*., 1995). (+)-Niguldipine at 100 nM alone shifted the CRC or PIE for phenylephrine to the right and downward (Figure 6). In the presence of (+)-niguldipine, BMY 7378 at 1 nM shifted the CRC for phenylephrine downward without affecting the pD_2 value for phenylephrine (Figure 6; Table 1).

Effect of BMY 7378 on the α_1-adrenoceptor-mediated PIE in the papillary muscle pretreated with CEC

Pretreatment with 10 μM CEC did not alter the basal force of contraction, but it shifted markedly the CRC for phenylephrine to the right and downward. The maximal response to phenylephrine was decreased to 22.9±1.2% ($n=26$) of the maximal response to isoprenaline from a control value of 59.1±1.3% ($n=33$). In papillary muscles pretreated with 10 μM CEC, BMY 7378 at 1 nM inhibited significantly the maximal response to phenylephrine and increased pD_2 value for phenylephrine (Figure 7a; Table 1). BMY 7378 at 10 nM and 100 nM shifted significantly the CRC for phenylephrine to the left without affecting the maximal response (Figure 7b and c; Table 1) but at 300 nM it shifted the CRC for phenylephrine to the right (Figure 7d); however, the extent of this shift was much less than that with BMY 7378 alone (Figure 1e).

Influence of BMY 7378 on the α_1-adrenoceptor-mediated accumulation of [^{3}H]IP$_1$

Figure 8 shows the effect of BMY 7378 on the accumulation of [^{3}H]IP$_1$ in response to phenylephrine in the presence of 1 μM timolol. BMY 7378 from 100 pM to 1 μM did not affect significantly the basal accumulation of [^{3}H]IP$_1$. Thirty minutes after the addition of 10 μM phenylephrine, the accumulation of [^{3}H]IP$_1$ was increased to 166.2±4.2% of the basal level. This increase was not significantly affected by BMY 7378 up to a concentration of 10 nM but it was inhibited by BMY 7378 at 100 nM, 300 nM and 1 μM by 26.6±6.4%, 34.7±4.8% and 46.7±5.2%, respectively.

Effects of BMY 7378 on the [^{3}H]prazosin specific binding

BMY 7378 inhibited the [^{3}H]prazosin specific binding (1 nM) to membrane fractions of the rabbit ventricular myocardium in a concentration-dependent manner (Figure 9). Nonlinear regression analysis of the inhibition curve for BMY 7378 fitted best a one-site model. Hill plot analysis of the data indicated that the slope factor of BMY 7378 was 0.996±0.093; the pK_i value for BMY 7378 was 7.53±0.09.

Discussion

In rabbit isolated papillary muscle, the selective α_{1D}-adrenoceptor antagonist BMY 7378 inhibited the PIE of phenylephrine mediated by α_1-adrenoceptors with different modes of antagonism depending on the concentration. Based on these findings, we postulate that BMY 7378 might inhibit the PIE mediated by at least three subtypes of α_1-adrenoceptors that show different affinities and responses to BMY 7378. BMY 7378 over the concentration range (1 nM to 1 μM) examined in the present study had no effect on the basal force of contraction, an indication that BMY 7378 by itself does not cause a release of endogenous noradrenaline which could contribute to the positive (or negative) inotropic action.

α_1-Adrenoceptor subtype susceptible to low concentrations (1–10 nM) of BMY 7378

BMY 7378 at lower concentrations (1–10 nM) depressed significantly the maximal response with relatively small changes in pD_2 value for phenylephrine. This concentration range is consistent with the potency of BMY 7378 to antagonize the α_1-adrenoceptor-mediated contraction in rat aorta and iliac artery where α_{1D}-adrenoceptors play a dominant role in functional regulation (Kenny *et al*., 1995; Piascik *et al*., 1995). In these reports, BMY 7378 antagonized the noradrenaline-mediated contraction of rat aorta with a pK_B value of 8.3 (Kenny *et al*.,

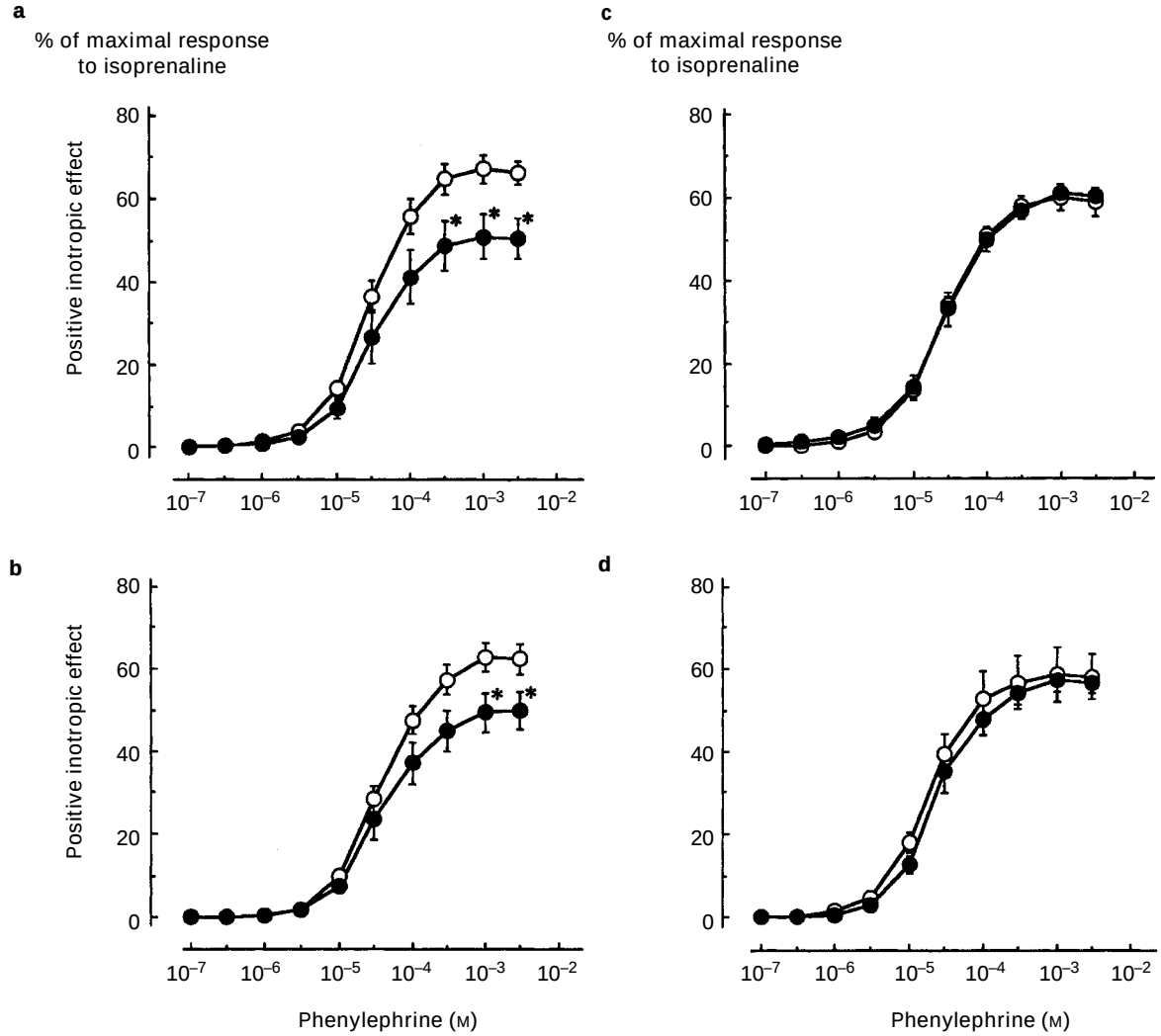

Figure 5 Effects of BMY 7378 on the α_1-adrenoceptor-mediated positive inotropic effect in the presence of WB 4101 and 1 μM timolol in isolated rabbit papillary muscle. The cumulative concentration–response curves for phenylephrine were determined in the presence of 100 nM WB 4101 alone (○) or of 100 nM WB 4101 plus BMY 7378 (●) at 1 nM (a, $n=5$), 10 nM (b, $n=6$), 100 nM (c, $n=6$) and 300 nM (d, $n=5$), $^*P<0.05$ vs corresponding control values.

1995) and antagonized the phenylephrine-mediated contraction with a K_i value of 0.9 nM in rat aorta and of 4.0 nM in iliac artery (Piascik *et al*., 1995). The inhibitory action of BMY 7378 at low concentrations (1–10 nM) is supposed to be selective for the α_1-adrenoceptor-mediated PIE because the basal force of contraction as well as the PIEs of isoprenaline (Figure 3), endothelin-3 and angiotensin II (Figure 4) was unaffected by BMY 7378 over this concentration range.

To elucidate whether α_{1A}- and/or α_{1B}-adrenoceptors contribute to the inhibition induced by BMY 7378 at 1–10 nM, we investigated the effect of the compound in the presence of known subtype selective adrenoceptor antagonists. The observations that an α_{1A}-adrenoceptor antagonist (+)-niguldipine, which has been reported to be able to differentiate α_{1A}- from α_{1D}-adrenoceptors (Michel *et al*., 1995), did not affect the inhibitory action of BMY 7378, indicate that the subtype of α_1-adrenoceptors other than α_{1A}-adrenoceptors remains to be antagonized by BMY 7378 at low concentrations (1–10 nM) in the presence of (+)-niguldipine. The inhibitory action of BMY 7378 at low concentrations was not influenced by WB 4101 that shows a selectivity for α_{1A}-adrenoceptors (Han *et al*., 1987; Minneman, 1988) either, which is inconsistent with the findings in non-cardiac tissues that WB 4010 at low nM concentrations inhibits also α_{1D}-adrenoceptors (Hieble & Ruffolo, 1996). The rabbit α_{1D}-adrenoceptor has now been cloned and shown to have an equivalent affinity for BMY 7378 and WB 4101 (Suzuki *et al*., 1997). It appears, therefore, that the pharmacological characteristics of the BMY 7378 sensitive α_1-adrenoceptor subtype in the rabbit ventricular muscle in the present study do not coincide with those of α_{1D}-adrenoceptors in non-cardiac tissue and the cloned α_{1D}-adrenoceptor of the rabbit in respect to susceptibility to WB 4101. It is likely that the PIE susceptible to BMY 7378 at low concentrations (1–10 nM) might be a consequence of interactions of multiple α_1-adrenoceptor subtypes that coexist in the rabbit ventricular myocardium (Endoh *et al*., 1992a; Yang & Endoh, 1994).

In the papillary muscle pretreated with CEC, in which the maximal response to phenylephrine was markedly attenuated and the pD_2 value for phenylephrine increased, the inhibitory action of BMY 7378 at 1 nM was still present (Figure 7a). These findings clearly show that α_1-adrenoceptors that are susceptible for BMY 7378 at low concentrations are not susceptible to the inhibitory action of CEC, an indication that they have pharmacological characteristics different from those of α_1-adrenoceptors. In rabbit ventricular muscle, 10 μM CEC decreased the Bmax determined by specific binding of [^{3}H]prazosin by 63% and the remaining fraction was inhibited by adrenaline (Takanashi *et al*., 1991), an indication that approximately 40% of α_1-adrenoceptors in the rabbit ventricular myocardium are resistant to alkylation induced by CEC. It has been shown that the sensitivity of α_{1D}-adrenoceptors in non-cardiac tissues towards alkylation by CEC is intermediate be-

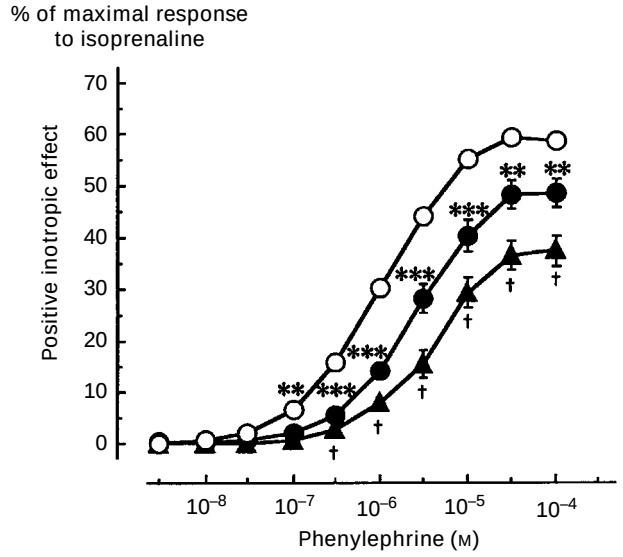

Figure 6 Effects of BMY 7378 on the α_1-adrenoceptor-mediated positive inotropic effect in the presence of (+)-niguldipine and 1 μM timolol in isolated rabbit papillary muscle. The cumulative concentration–response curves for phenylephrine were determined in the absence of α_1-adrenoceptor antagonists (○, $n=33$) or in the presence of 100 nM (+)-niguldipine alone (●, $n=5$) and of 100 nM (+)-niguldipine plus 1 nM BMY 7378 (▲, $n=5$). $^{**}P<0.01$ and $^{***}P<0.001$ vs the corresponding control values and $^{\dagger}P<0.05$ vs the corresponding values in the presence of (+)-niguldipine alone.

tween α_{1A}- and α_{1B}-adrenoceptors (Laz *et al*., 1994; Michel *et al*., 1995). While the findings that BMY 7378 has a high affinity to the subtype and that α_{1d}-adrenoceptor mRNA is definitely detected in rabbit cardiac muscle (Suzuki *et al*., 1997) are in accordance with the view that α_{1D}-adrenoceptors may play a role in producing the PIE that is antagonized by BMY 7378 at low concentrations (1–10 nM), circumstantial evidence does not allow us to characterize the BMY 7378 sensitive α_1-adrenoceptors. In the receptor binding assay, BMY 7378 scarcely displayed the specific [^{3}H]prazosin binding over the concentration range 1–10 nM. In addition, the accumulation of [^{3}H]IP$_1$ induced via α_1-adrenoceptors was not affected by BMY 7378 at 1–10 nM, suggesting that the α-adrenoceptors that are susceptible to low concentrations of BMY 7378 are not coupled to stimulation of the PI hydrolysis. It has been reported that α_{1D}-adrenoceptors (that are sensitive to BMY 7378) play a minor role in mediating the contractile response in adult rat atrial and ventricular muscles (Deng *et al*., 1996) and not contribute to inotropic response to sympathomimetic amines in neonatal rat myocardium (Deng & Varma, 1997). The identification of the BMY 7378 sensitive α_1-adrenoceptor subtype and its signal transduction mechanism in the rabbit ventricular myocardium remain yet to be fully characterized.

In the presence of WB 4101, the inhibitory action of BMY 7378 at 10 nM on the maximal response to phenylephrine was comparable to that of 1 nM (Figure 5a and b). The saturation of the inhibitory action of BMY 7378 at 10 nM and the attenuation at higher concentrations in the presence of WB 4101 are considered to involve a combina-

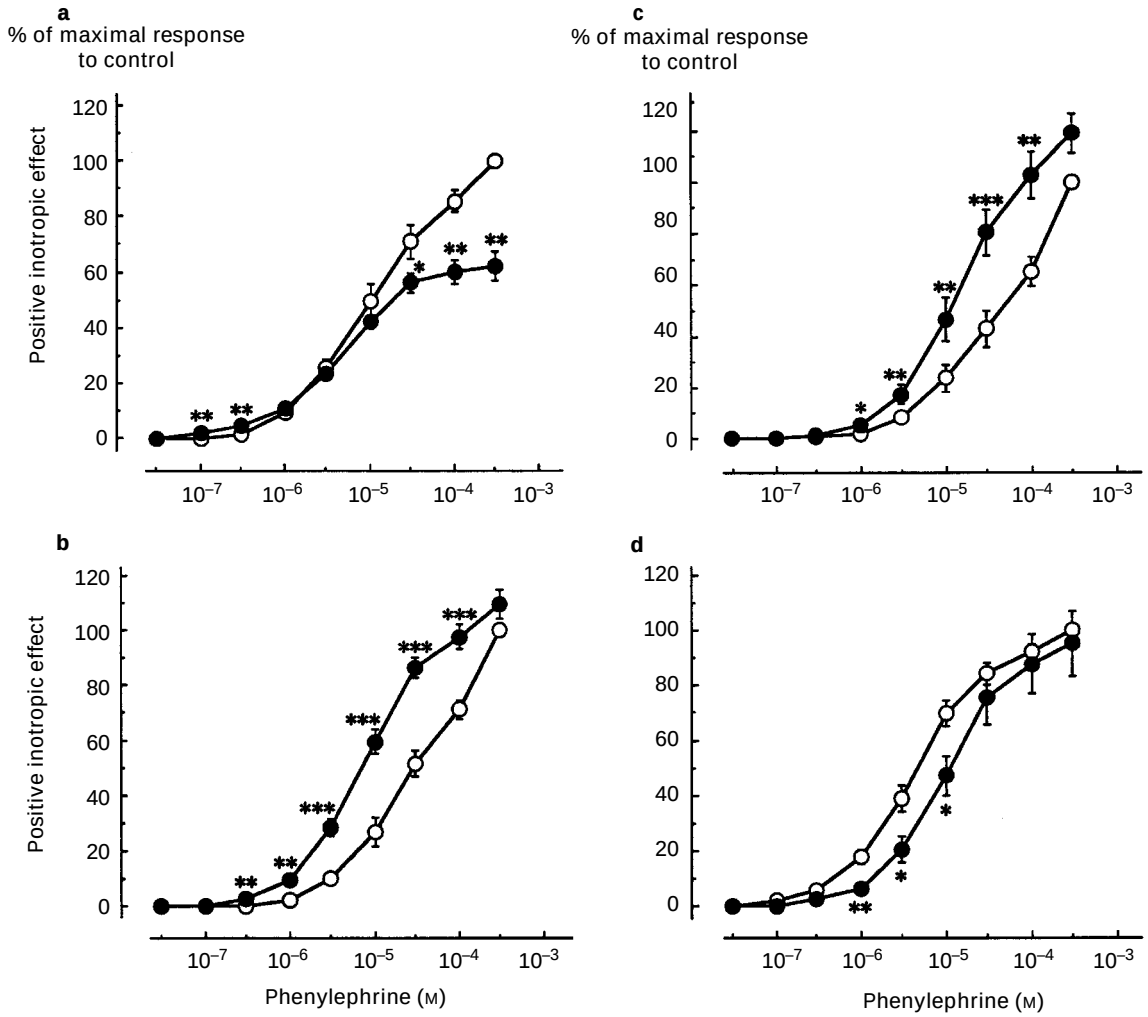

Figure 7 Effects of BMY 7378 on the positive inotropic effect of phenylephrine in papillary muscles pretreated with 10 μM CEC in the presence of 1 μM timolol. Control in the CEC-pretreated muscle (○); BMY 7378 (●) at 1 nM (a, $n=5$), 10 nM (b, $n=8$), 100 nM (c, $n=7$) and 300 nM (d, $n=6$) in the CEC-pretreated muscle, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs the corresponding control values.

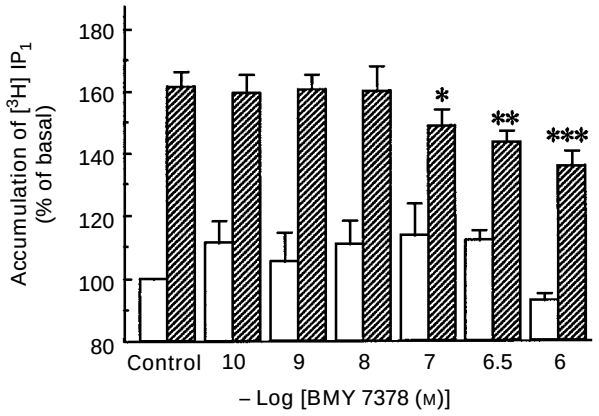

Figure 8 Influence of various concentrations of BMY 7378 on the accumulation of $[^3H]IP_1$ induced by phenylephrine 30 min after the addition in the presence of 1 μM timolol in slices of rabbit ventricular muscle. Values were determined after the addition of phenylephrine at 10 μM (▨) or saline (□). Data are expressed as a percentage of the corresponding basal levels that were determined simultaneously (mean ± s.e.mean, $n=6$ each). The mean radioactivity of control slices was 27.0 ± 3.7 dpm mg^{-1} of wet weight of the tissue. $^*P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$ vs the values with phenylephrine alone.

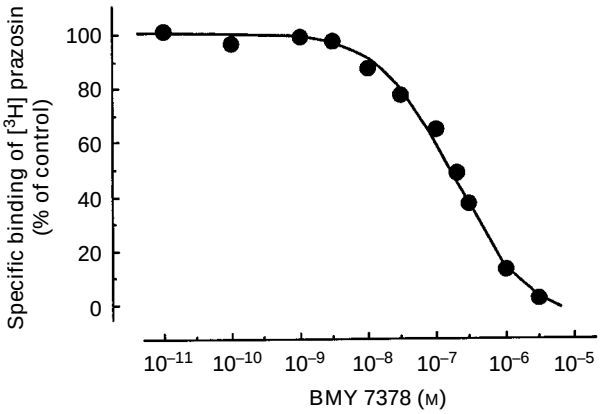

Figure 9 Concentration-dependent displacement of [^{3}H]prazosin (1 nM) specific binding by BMY 7378 in membrane fractions derived from rabbit ventricular myocardium. Values are presented as means ± s.e.mean of four experiments.

tion of at least the following two mechanisms: (1) BMY 7378 might inhibit the subtype of α_1-adrenoceptors that mediates a negative inotropic effect (NIE) and is less susceptible to the compound, and (2) the PIE that is mediated by α_{1B}-adrenoceptors and is least sensitive to the compound might interfere with the development of the NIE and this interference is more prominent under the blockade of α_{1A}-adrenoceptors by WB 4101. In the muscle pretreated with CEC, the inhibitory action of BMY 7378 at 10 nM disappeared and the CRC for phenylephrine shifted to the left, providing further support for the above postulate that the NIE mediated by α_1-adrenoceptors that are less susceptible to BMY 7378 may contribute to the decrease in the inhibitory action of BMY 7378 at low concentrations on the maximal response to phenylephrine.

α_1-Adrenoceptor subtype susceptible to high concentrations (100 nM to 1 μM) of BMY 7378

BMY 7378 at higher concentrations of 100 nM to 1 μM shifted the CRC for phenylephrine to the right in parallel without alteration of the maximal response. BMY 7378 has been shown to possess approximately 100-fold higher selectivity for α_{1D}-adrenoceptors than for other subtypes of α_1-adrenoceptors (Saussy *et al*., 1994; Goetz *et al*., 1995). The mode of antagonism induced by BMY 7378 on the PIE of phenylephrine was competitive with the pA_2 value of 7.17. Therefore the inhibitory action of BMY 7378 over this concentration range is likely to be due to a mixed effect on all the subtypes of α_1-adrenoceptors. By contrast to the inhibitory action of BMY 7378 at low concentrations, the action of the compound at high concentrations disappeared in the presence of WB 4101. This finding implies that α_{1A}-adrenoceptor subtype contributes to the PIE that is antagonized by high concentrations of BMY 7378, but it was not possible to characterize the pharmacological property of α_{1A}-adrenoceptors because of interference with the predominant effect mediated by α_{1B}-adrenoceptors in the rabbit ventricular myocardium (Endoh *et al*., 1992a; Yang & Endoh, 1994).

The inhibitory action of BMY 7378 over the concentration range 100 nM to 1 μM was inverted to the enhancement or became much weaker in the presence of CEC. In addition BMY 7378 over this concentration range inhibited the accumulation of $[^3H]IP_1$ induced by α_1-adrenoceptors in a concentration-dependent manner, which suggests that the inhibitory actions of BMY 7378 on the PIE and accumulation of $[^3H]IP_1$ induced by phenylephrine are exerted by activation of the same subtype of α_1-adrenoceptors. The observation that [^{3}H]prazosin specific binding was displaced by BMY 7378 with pK_i close to the pA_2 value, implies that the competitive antagonism of the PIE by BMY 7378 at 100 nM to 1 μM may be exerted by binding of the compound to the main population of α_1-adrenoceptors, i.e. α_{1B}-adrenoceptors in the rabbit ventricular myocardium (Takanashi *et al*., 1991; Yang & Endoh, 1994).

α_1-Adrenoceptor subtype that mediates the NIE

While BMY 7378 inhibited the maximal response to phenylephrine at low concentrations (1 – 10 nM), it produced a parallel shift of the CRC at higher concentrations (100 nM to 1 μM) (Figure 1). These findings indicate that the inhibitory action of BMY 7378 at low concentrations (1 – 10 nM) on the maximal response disappears when the concentration of the compound is increased. In the papillary muscle pretreated with CEC, the CRC for phenylephrine was shifted to the left, indicating that the subtype that mediates the NIE might be inhibited by increasing the concentration of BMY 7378. The inhibition of α_{1B}-adrenoceptors that play a dominant role in mediating the PIE in the rabbit ventricular myocardium (Takanashi *et al*., 1991; Yang & Endoh, 1994) unveiled the NIE, whereas in the absence of CEC the NIE might have been obscured by the simultaneous antagonism of BMY 7378 on both the α_{1B}-subtype that mediates PIE and the subtype that mediates the NIE. A question arises then which subtype mediates the NIE of α_1-adrenoceptors. From the previous discussion it is clear that α_{1B}-adrenoceptors are not responsible for the NIE. In the rabbit ventricular myocardium, methoxamine induced an inhibitory action on the PIE independent of the acceleration of PI hydrolysis via α_{1A}-adrenoceptors (Yang *et al*., 1996). However, as the depression of the maximal response to phenylephrine was induced by BMY 7378 in the presence of WB4101 or (+)-niguldipine, α_{1A}-adrenoceptors are not considered to mediate the NIE that was inhibited by BMY 7378. It is therefore postulated that the NIE may be also mediated by the α_1-adrenoceptor subtype that is relatively sensitive to BMY 7378, belonging to neither α_{1A}- nor α_{1B}-adrenoceptors, for elucidation of which further study is required.

In conclusion, it is revealed that α_1-adrenoceptor subtypes in rabbit ventricular myocardium are heterogeneous. BMY 7378 at low concentrations (1 – 10 nM) suppressed the maximal response to phenylephrine mediated by α_1-adrenoceptor sub-

type, and at 10 – 100 nM it antagonized the subtype that mediates the NIE. The inotropic effects induced by activation of these BMY 7378 sensitive and relatively sensitive α_1-adrenoceptor subtypes are not associated with stimulation of hydrolysis of PI and is mediated via an as yet uncharacterized mechanism. At higher concentrations (100 nM to 1 μM) BMY 7378 also antagonizes the PIE mediated by α_{1A}- and α_{1B}-adrenoceptors.

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