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α_1-Adrenoceptor subtypes involved in increased $^{86}Rb^+$ influx rate and inositol 1,4,5-trisphosphate mass in adult rat cardiomyocytes

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Abstract

The aim of the present study was to identify the receptor subtypes involved in the α_1-adrenoceptor-mediated increase in $^{86}Rb^+$ influx rate and in inositol 1,4,5-trisphosphate (IP_3) accumulation in isolated ventricular cardiomyocytes from adult rat heart, in order to identify a possible response pattern compatible with a causative relationship. Subtype-selective receptor antagonists used were: 5-methylurapidil (α_{1A}), WB 4101 {2([2,6-dimethoxyphenoxy-ethyl]aminomethyl)-1,4-benzodioxane} (α_{1A}), chloroethylclonidine (α_{1B}) and BMY 7378 {8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione} (α_{1D}). The basal $^{86}Rb^+$ influx rate was 0.22 ± 0.01 ml/g protein $\times$ min. At 15 min, 5×10^{-5} mol/l noradrenaline in the presence of 3×10^{-5} mol/l timolol increased the $^{86}Rb^+$ influx rate by $33 \pm 1\%$. This response was not affected by either chloroethylclonidine or BMY 7378 at concentrations up to 10^{-5} mol/l. 5-Methylurapidil dose dependently inhibited the response to 5×10^{-5} mol/l noradrenaline with a $-\log IC_{50}$ value of 5.27 ± 0.12 and 5.61 ± 0.27 in the presence and absence of 10^{-5} mol/l chloroethylclonidine, respectively. WB 4101 in the presence of 10^{-5} mol/l chloroethylclonidine dose dependently inhibited the response to 5×10^{-5} mol/l noradrenaline with a $-\log IC_{50}$ value of 6.10 ± 0.14. Noradrenaline in the presence of 10^{-5} mol/l chloroethylclonidine dose dependently increased the $^{86}Rb^+$ uptake rate with a $-\log EC_{50}$ value of 6.19 ± 0.35. The basal IP_3 level was 2.15 ± 0.19 pmol/mg protein. Incubation with 10^{-5} mol/l noradrenaline for 2 min increased this by $65 \pm 7\%$ of control levels. 10^{-5} mol/l chloroethylclonidine and 10^{-4} mol/l 5-methylurapidil reduced the response to $27 \pm 6\%$ and $18 \pm 9\%$ of control level, respectively. BMY 7378 dose dependently inhibited the IP_3 response at relatively high concentrations, and it was completely eliminated at 10^{-5} mol/l BMY 7378. The combination of chloroethylclonidine and 5-methylurapidil or 3×10^{-6} mol/l prazosin alone completely abolished the hormone-induced effect. We conclude that whereas the α_1-adrenoceptor-stimulated increase in $^{86}Rb^+$ influx rate is mediated via the α_{1A}-adrenoceptor subtype only, both α_{1A}- and α_{1B}-adrenoceptor subtypes are involved in the increase in IP_3 mass. Furthermore, a contribution from the α_{1D}-adrenoceptor in the IP_3 response cannot be excluded. Thus there does not appear to be a simple causative relationship between an increase in $^{86}Rb^+$ influx rate and an increase in IP_3.

Keywords: α_1-Adrenoceptor, subtype; Chloroethylclonidine; 5-Methylurapidil; WB 4101; BMY 7378; $^{86}Rb^+$, influx rate; Inositol 1,4,5-trisphosphate

1. Introduction

The functional existence of α_1-adrenoceptors in the mammalian myocardium is well established (e.g., Terzic et al., 1993). Pharmacologically, three subtypes of α_1-adrenoceptors, α_{1A}, α_{1B} and α_{1D}, have been identified and characterized, corresponding to three α_1-adrenoceptor subtypes, α_{1a}, α_{1b} and α_{1d}, that have been identified in rat tissues using molecular cloning techniques (Bylund et al., 1994; Hieble et al., 1995; Saussy et al., 1996). In binding studies both α_{1A}- and α_{1B}-adrenoceptors were present in an approximate 20:80% ratio in rat heart (Hanft and Gross, 1989; Michel et al., 1994). A study using reverse transcription and a competitive polymerase chain reaction detected mRNA for all three cloned subtypes of α_1-adrenoceptores in rat heart, although α_{1d}-adrenoceptor mRNA was present only at a low level (Scofield et al., 1995). Recently, Deng et al. (1996b) reported binding sites with high affinity for the α_{1D}-adrenoceptor subtype-selective antagonist BMY 7378 in rat heart and suggested a role, although minor, for the α_{1D}-adrenoceptor in the inotropic response to α_1-adrenoceptor agonists.

Studies using radioactive isotopes or measuring ionic concentrations have demonstrated both increased $^{86}Rb^+$ influx (Hanem et al., 1994; Viko et al., 1995, 1996) and increased K^+ influx (Ellingsen et al., 1987; Hanem et al.,

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1992; Viko et al., 1996) in the myocardium after α_1-adrenoceptor stimulation. It is well established that stimulation of myocardial α_1-adrenoceptors increases the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C, yielding the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (De Jonge et al., 1995). Whereas the physiological importance of IP_3 in the heart remains unclear, diacylglycerol activates protein kinase C, which has been implicated in the α_1-adrenergic inotropic effect (Terzic et al., 1993) and in the regulation of cardiac growth (Sugden and Bogoyevitch, 1995).

The aim of the present study was to examine the contribution of the different receptor subtypes both in the α_1-adrenoceptor-stimulated increase in $^{86}Rb^+$ influx rate and the increase in IP_3 content, in order to investigate whether the increase in IP_3 followed a pattern compatible with a second messenger function for the increase in $^{86}Rb^+$ influx rate. The α_{1A}-adrenoceptor subtype-selective reversible antagonists WB 4101 and 5-methylurapidil, the α_{1B}-adrenoceptor subtype-selective irreversible antagonist chloroethylclonidine (Minneman and Esbenshade, 1994), and the α_{1D}-adrenoceptor subtype-selective reversible antagonist BMY 7378 (Saussy et al., 1996) were used as experimental tools. We present results showing that the increase in $^{86}Rb^+$ influx rate and the IP_3 response in part were elicited by different α_1-adrenoceptor subtypes.

2. Materials and methods

2.1. Isolation and preparation of cardiomyocytes

Ventricular cardiomyocytes were isolated from the adult rat heart as described elsewhere (Viko et al., 1995). The cells isolated from one heart were suspended in 25 ml of a minimum essential medium (JOKLIK-MEM, composed of 5.3 mmol/l KCl, 1 mmol/l $MgCl_2$, 112 mmol/l NaCl, 8.5 mmol/l NaH_2PO_4, 11.1 mmol/l D-glucose and amino acids and vitamins) supplemented with 24 mmol/l $NaHCO_3$, 0.6 mmol/l $MgSO_4$, 1 mmol/l DL-carnitine, 10 mmol/l creatine and 20 mmol/l taurine, 0.5 mmol/l $CaCl_2$, 0.1 μmol/l insulin, 0.1 nmol/l triiodothyronine, 0.1% fatty acid-free bovine serum albumin, 67 mg/l penicillin and 0.1 g/l streptomycin and kept overnight at 37°C in a cell culture flask filled with 95% O_2/5% CO_2 and sealed. The cells were kept overnight because this drastically reduced the variability of the $^{86}Rb^+$ influx rate measured, possibly because of repair of cell surface damage during the isolation procedure. On the following day, the cells were sedimented twice for 10 min through 3–4 cm of the same resuspension solution, except that penicillin and streptomycin were omitted and the concentration of fatty acid-free bovine serum albumin was 1.0%. Finally, the cells were suspended in the same solution, but with a concentration of fatty acid-free serum albumin of 0.1%.

The animals were kept and handled according to institutional rules approved by The National Committee of Rules for Experimental Animals.

2.2. Measurement of $^{86}Rb^+$ influx rate

1 ml samples of the cell suspension, corresponding to 1.09 ± 0.06 mg protein ($n = 67$), were incubated in 25 ml Erlenmeyer flasks in a Grant shaking water bath set at 100 strokes/min and 37°C together with the appropriate receptor ligands and radiolabelled rubidium. The $^{86}Rb^+$ radioactivity was $2.69 \pm 0.10 \times 10^5$ counts per min (cpm)/incubate ($n = 56$). Both basal and stimulated $^{86}Rb^+$ influx rate increased linearly up to 20 min (data not shown). The $^{86}Rb^+$ influx rate was determined by incubating cell samples with $^{86}Rb^+$ for 15 min. The $^{86}Rb^+$ influx rate was expressed in a normalized way as cpm_{cells}/g protein $\times$ $cpm_{incubate} \times$ min. As the incubations were performed in 1 ml, this gives the clearance dimension of ml/g protein $\times$ min.

5×10^{-5} mol/l noradrenaline was used as a maximal agonist concentration. Increasing the noradrenaline concentration further did not increase the response, but rather tended to diminish it. β-Adrenoceptor stimulation was found to increase the $^{86}Rb^+$ influx rate in cardiomyocytes to a similar extent as α_1-adrenoceptor stimulation (Viko et al., 1996), and 3×10^{-5} mol/l timolol was added in order to prevent it. This concentration of timolol eliminated the β-adrenoceptor increase in $^{86}Rb^+$ influx rate completely (data not shown). 10^{-5} mol/l ascorbate was added to all incubates where noradrenaline was present. This concentration of ascorbate did not affect the basal $^{86}Rb^+$ influx rate (data not shown). Increasing concentrations of the respective receptor subtype-selective antagonists were used to obtain graded inhibition of the agonist-induced responses. The cells were preincubated in the presence of the respective antagonists for either 15 min (5-methylurapidil, WB 4101 and BMY 7378) or 30 min (with chloroethylclonidine present). As both chloroethylclonidine and WB 4101 slightly reduced the basal $^{86}Rb^+$ influx rate, up to about 10% for the highest concentrations used (data not shown), all antagonists were present in the same concentration both in the control and the stimulated samples. In order to investigate whether the removal of chloroethylclonidine after 30 min pretreatment had any effect on the $^{86}Rb^+$ influx rate, the whole cell population was divided in two portions and incubated for 30 min in the presence of either chloroethylclonidine or vehicle. The chloroethylclonidine/vehicle containing buffer (24 ml) was replaced by sedimentation of the cells for 10 min and resuspension in 30 ml incubation buffer (without any additions). The procedure was repeated three times before final incubation as described below.

After 15 min, 4 ml of a stopping and washing solution containing 87 mmol/l $MgCl_2$, 10 mmol/l KCl, 10 mmol/l RbCl and 0.1% bovine serum albumin fraction V, pH 7.2,

was added to the incubation flasks. The cells were separated from the medium by filtration through Whatman GF/A glass fibre filters using a vacuum of 17 kPa. The filters were then washed with 5×4 ml of the same solution. The whole separation procedure was complete within 10 s. The filters were then placed in counting vials, 0.1 ml of 0.5 M HCl and 10 ml H_2O added, and shaken for 30 min at about 150 strokes/min. The radioactivity was determined by Cerenkov radiation in a Packard liquid scintillation spectrometer, model 1900 TR. The binding of radioactivity to the filters was always low and stable, $19 \pm 1\%$ ($n = 33$) of mean value for the control $^{86}Rb^+$ cell uptake at 15 min in each sample.

2.3. IP_3 mass assay

1 ml samples of the cell suspension, corresponding to 1.27 ± 0.07 mg protein ($n = 25$) were incubated as above together with the appropriate receptor ligands. 10^{-5} mol/l noradrenaline was used as a maximal agonist concentration. 3×10^{-5} mol/l timolol was included in order to prevent β-adrenoceptor stimulation. The cells were preincubated in the presence of the respective antagonists for either 15 min (5-methylurapidil, WB 4101 and BMY 7378) or 30 min (with chloroethylclonidine present). All antagonists were present in the same concentration both in the control and the stimulated samples. The cells were stimulated by noradrenaline for 120 s (noradrenaline results in essentially constant levels of IP_3 between 10 s and 120 s after addition, (Viko et al., submitted)). The reaction was stopped by the addition of perchloric acid to a final concentration of 0.8 mol/l. The samples were left on ice for 30 min before centrifugation to remove precipitated protein. The supernatant was neutralized with 4 mol/l KOH containing 0.06 mol/l HEPES and 0.06 mol/l EDTA, and precipitated potassium perchlorate was removed by centrifugation. The IP_3 content of the supernatant was determined by a radioligand receptor binding assay, essentially as described by Challiss et al. (1990), with the IP_3 binding protein prepared from bovine adrenal cortex.

2.4. Calculations and statistics

Dose-inhibition and dose-response curves were constructed according to Ariëns and Simonis (1964), by estimating centiles (IC_{10} to IC_{100} and EC_{10} to EC_{100}) for each single experiment and calculating the corresponding means. The percentage increase in $^{86}Rb^+$ influx rate over the basal level was taken as the ligand-induced effect. Maximal response was determined in each single experiment. The data are presented as fractional plots, i.e. the graded responses in the presence or absence of antagonists were expressed in percentage of maximal agonist-induced effect (100%) of each experiment. The affinity constants (pK_i) of the competitive antagonists for the α_1-adrenoceptor subtypes were calculated according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973) by using the observed IC_{50} value. Chloroethylclonidine is a weak competitive antagonist on α_{1A}-adrenoceptors (Minneman and Esbenshade, 1994). When it was present, a correction (which was small) for a second antagonist was performed when calculating (by the Cheng-Prusoff equation) the pK_i for 5-methylurapidil and WB 4101. The affinity constant pK_i of chloroethylclonidine for the α_{1A}-adrenoceptor subtype was calculated according to the Schild equation (Arunlakshana and Schild, 1959). The significance levels of differences were calculated according to Student's *t*-test. When comparing more than two groups, the results were analyzed by one-way analysis of variance with Tukey's method for confidence intervals for all pairwise differences between level means. All statistical calculations were done using MINITAB PC Version, Release 8 (Minitab, State College, PA, USA). A significance level of $P \leq 0.05$ was considered to reflect statistically significant differences. All values are given as mean ± S.E.M. The value from each single experiment was calculated from 4–8 parallels. Each experiment was repeated 4–8 times.

2.5. Protein determination

Cell protein was determined according to Lowry et al. (1951) using bovine serum albumin as standard.

2.6. Chemicals and materials

D-[Inositol-1-^{3}H(N)]1,4,5-trisphosphate, specific activity 777 GBq/mmol, and ^{86}RbCl in 0.5 mol/l HCl, specific activity about 100 MBq/mg at time of dispatch, were both from Du Pont. JOKLIK-MEM and Penstrep (penicillin-streptomycin) were both from Gibco BRL. BMY 7378 {8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione} dihydrochloride and chloroethyl clonidinedihydrochloride, were both from Research Biochemicals International. Ascorbic acid, bovine serum albumin (essentially fatty acid-free), bovine serum albumin fraction V, DL-carnitine hydrochloride, creatine hydrate, insulin, L-noradrenaline (norepinephrine) D-bitartrate, prazosin hydrochloride, taurine, timolol maleate, triiodo-D-thyronine, trypsin, and WB 4101 {2([2,6-dimethoxyphenoxy-ethyl]aminomethyl)-1,4-benzodioxane} hydrochloride, were all from Sigma. Collagenase was from Wortingthon Biochemical. 5-Methylurapidil was a gift from Byk Gulden Lomberg through Pharmacia (Norway). All other chemicals were of analytical grade.

3. Results

3.1. Rb^+ influx rate

The basal $^{86}Rb^+$ influx rate was 0.22 ± 0.01 ml/g protein × min ($n = 67$). Maximal α_1-adrenoceptor stimula-

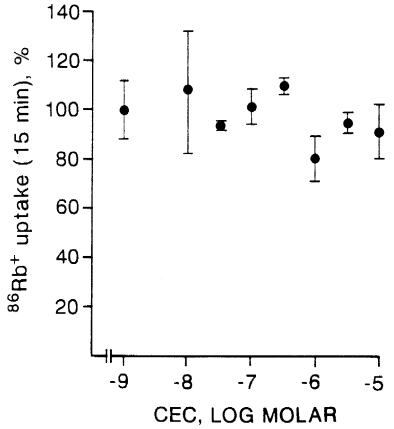

Fig. 1. Effect of increasing concentrations of chloroethylclonidine (CEC) on the α_1-adrenoceptor-mediated increase in $^{86}Rb^+$ uptake rate in isolated ventricular cardiomyocytes from adult rat heart. Cardiomyocytes were preincubated with chloroethylclonidine for 30 min before stimulation for 15 min with 5×10^{-5} mol/l noradrenaline in the presence of 3×10^{-5} mol/l timolol. The maximal increase in $^{86}Rb^+$ uptake rate above basal level in the absence of chloroethylclonidine is expressed as 100%. $n=14$.

tion, achieved by 5×10^{-5} mol/l noradrenaline, increased the influx rate by $33\pm1\%$ ($P<0.00005$, $n=67$). Similar values have previously been reported (Viko et al., 1995, 1996). 3 ± 10^{-6} mol/l prazosin eliminated the α_1-adrenoceptor-mediated effect on the $^{86}Rb^+$ influx rate completely (data not shown). There was no statistically significant effect of 30 min of chloroethylclonidine pretreatment on the α_1-adrenergic response to noradrenaline at concentrations up to 10^{-5} mol/l (Fig. 1). The maximal agonist-induced increase was $29\pm2\%$ and $24\pm2\%$ without and with 10^{-5} mol/l chloroethylclonidine pretreatment, respectively ($n=4$); pretreatment with chloroethylclonidine and subsequent removal did not affect the response.

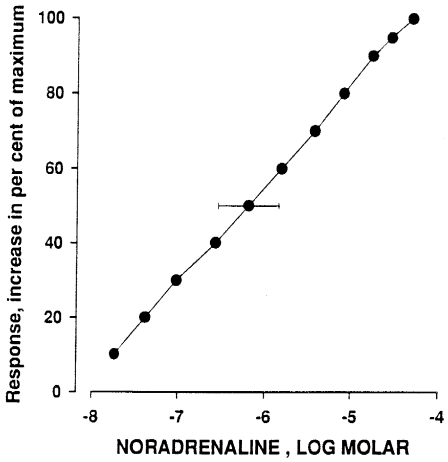

Fig. 2. Normalized dose-response curve for the α_1-adrenoceptor-mediated increase in $^{86}Rb^+$ uptake rate achieved by increasing concentrations of noradrenaline in the presence of 10^{-5} mol/l chloroethylclonidine. The dose-response curve was constructed according to Ariëns and Simonis (1964), by estimating centiles (IC_{10} to IC_{100}) of the maximal effect obtained for each single experiment and calculating the corresponding means. $n=7$.

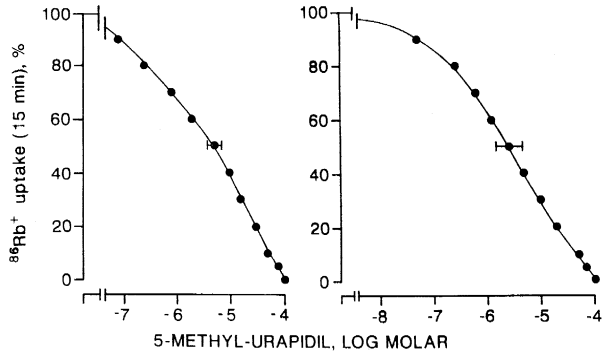

Fig. 3. Normalized dose-inhibition curves for the effect of increasing concentrations of 5-methylurapidil in the presence (left) or absence (right) of 10^{-5} mol/l chloroethylclonidine on the α_1-adrenoceptor-mediated increase in $^{86}Rb^+$ uptake rate. The dose-inhibition curves are constructed according to Ariëns and Simonis (1964), by expressing the maximal increase in $^{86}Rb^+$ uptake rate above basal level as 100%. $n=6$.

Fig. 2 shows that increasing concentrations of noradrenaline in the presence of 10^{-5} mol/l chloroethylclonidine dose dependently increased the $^{86}Rb^+$ influx rate with an $-\log EC_{50}$ value of 6.19 ± 0.35.

Fig. 3 shows the effect of preincubation with increasing concentrations of 5-methylurapidil in the presence (left) and absence (right) of 10^{-5} mol/l chloroethylclonidine, respectively. During both experimental conditions 5-methylurapidil dose dependently inhibited the α_1-adrenergic response with $-\log IC_{50}$ values of 5.27 ± 0.12 and 5.61 ± 0.27, respectively. Thus, the presence of chloroethylclonidine did not significantly affect the inhibitory potency of 5-methylurapidil. The corresponding calculated affinity constants pK_i of 5-methylurapidil were 7.89 ± 0.23 and 8.37 ± 0.28, respectively. The increase in $^{86}Rb^+$ influx rate was completely inhibited at 10^{-4} mol/l 5-methylurapidil. Preincubation with WB 4101 in the presence of 10^{-5} mol/l chloroethylclonidine dose dependently inhibited the α_1-adrenergic response with a $-\log IC_{50}$ value of

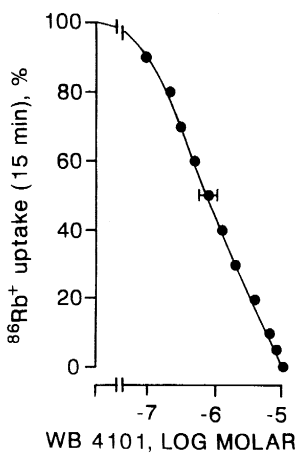

Fig. 4. Normalized dose-inhibition curve for the effect of increasing concentrations of WB 4101 in the presence of 10^{-5} mol/l chloroethylclonidine on the α_1-adrenoceptor-mediated effect on the increase in $^{86}Rb^+$ uptake rate. The inhibition curve was calculated as described in the legend to Fig. 3. $n=8$.

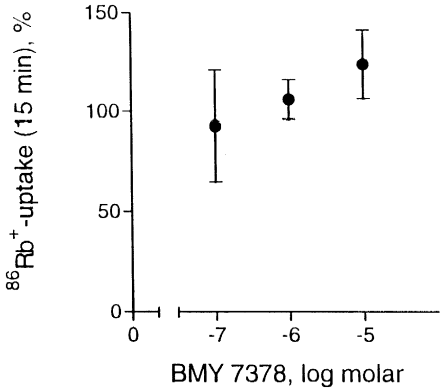

Fig. 5. The effect of increasing concentrations of BMY 7378 on the α_1-adrenoceptor-mediated increase in $^{86}Rb^{+}$ uptake rate. The maximal increase in $^{86}Rb^{+}$ uptake rate in the absence of BMY 7378 was expressed as 100%. $n = 6$.

6.10 ± 0.14 (Fig. 4). The corresponding calculated affinity constant pK_i of WB 4101 was 8.85 ± 0.14. The increase in $^{86}Rb^{+}$ influx rate was completely inhibited at 10^{-5} mol/l WB 4101.

Fig. 5 shows the effect of increasing concentrations of the α_{1D}-adrenoceptor subtype-selective antagonist BMY 7378 on the α_1-adrenergic response to noradrenaline. There was no statistically significant effect of BMY 7378 at concentrations up to 10^{-5} mol/l.

3.2. IP_3 response

The basal IP_3 level was 2.15 ± 0.19 pmol/mg protein ($n = 25$). α_1-Adrenoceptor stimulation increased the IP_3 level by $65 \pm 7\%$ compared to control level (Fig. 6) ($P < 0.00005$, $n = 16$). 10^{-5} mol/l chloroethylclonidine and

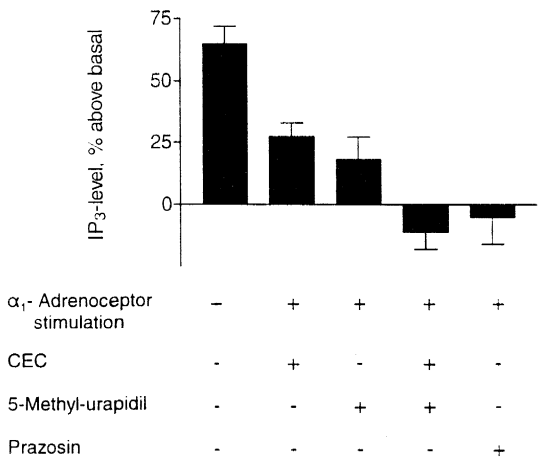

Fig. 6. Effect of exposure for 2 min to 10^{-5} mol/l noradrenaline in the presence of 3×10^{-5} mol/l timolol on the IP_3 level of isolated cardiomyocytes in the absence and presence of α_1-adrenoceptor antagonists ($n = 16$). The concentrations of antagonists were 10^{-5} mol/l chloroethylclonidine (CEC) ($n = 9$), 10^{-4} mol/l 5-methylurapidil ($n = 9$), a combination of chloroethylclonidine and 5-methylurapidil ($n = 7$), or 3×10^{-6} mol/l prazosin ($n = 6$).

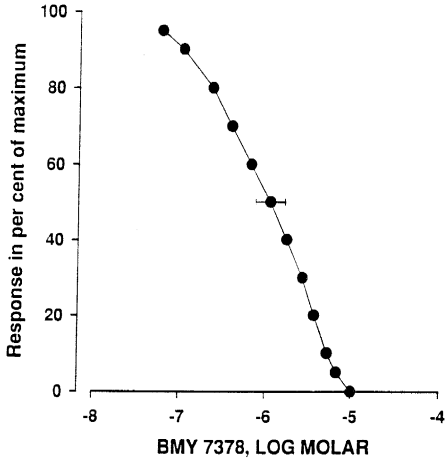

Fig. 7. Normalized dose-inhibition curve for the effect of increasing concentrations of BMY 7378 on the α_1-adrenoceptor-mediated effect on the increase in IP_3 level. The inhibition curve was constructed as described in the legend to Fig. 3, with the maximal increase in IP_3, obtained in the absence of BMY 7378 expressed as 100%. $n = 9$.

10^{-4} mol/l 5-methylurapidil significantly reduced this response to $27 \pm 6\%$ ($n = 9$) and $18 \pm 9\%$ ($n = 9$) above control level, respectively ($P < 0.0005$, one-way analysis of variance with Tukey's method for confidence intervals for all pairwise differences between level means). No significant increase in the IP_3 levels were obtained with α_1-adrenoceptor stimulation in the presence of either a combination of 10^{-5} mol/l chloroethylclonidine and 10^{-4} mol/l 5-methylurapidil ($n = 7$) or 3×10^{-6} mol/l prazosin alone ($n = 6$). Fig. 7 shows the effect of increasing concentrations of the α_{1D}-adrenoceptor subtype-selective antagonist BMY 7378 on the α_1-adrenoceptor-stimulated increase in IP_3 mass. The α_1-adrenoceptor-stimulated increase in the IP_3 level in this series was $100 \pm 11\%$ compared to control level ($n = 9$). BMY 7378 dose dependently inhibited the IP_3 response, with a $-\log IC_{50}$ value of 5.95 ± 0.17. The response was completely eliminated at 10^{-5} mol/l BMY 7378.

4. Discussion

In the present study we have compared the effects of α_1-adrenoceptor subtype-selective antagonists on the noradrenaline-stimulated increase in $^{86}Rb^{+}$ influx rate and IP_3 accumulation in cardiomyocytes. The results show that whereas the increase in $^{86}Rb^{+}$ influx rate was inhibited by α_{1A}-adrenoceptor subtype-selective antagonists only, the IP_3 response was partly inhibited by α_{1A}- or α_{1B}-adrenoceptor subtype-selective antagonist and completely inhibited by the α_{1D}-adrenoceptor subtype-selective antagonist BMY 7378 at relatively high concentrations. These results argue against a simple causative relationship between IP_3 accumulation and stimulation of $^{86}Rb^{+}$ influx rate.

Chloroethylclonidine has similar binding affinities for α_{1A}- and α_{1B}-adrenoceptors, but alkylates and inactivates

the α_{1B}-adrenoceptor selectively (Minneman and Esbenshade, 1994). Pretreatment with chloroethylclonidine did not affect the hormone-induced increase in $^{86}Rb^+$ influx rate, indicating that the involvement of α_{1B}-adrenoceptors in the response can be excluded. The lack of influence of chloroethylclonidine pretreatment and subsequent washout on the maximal effect of noradrenaline indicates that any competitive effect of chloroethylclonidine on the α_{1A}-adrenoceptors also can be ignored in this situation. The potency of noradrenaline in the presence of chloroethylclonidine ($-\log EC_{50} = 6.19 \pm 0.35$) was only slightly lower than the value obtained in the absence of chloroethylclonidine, which is 7.05 ± 0.40 (Viko et al., 1996). This is compatible with a low-affinity reversible binding of chloroethylclonidine to the α_{1A}-adrenoceptors, and also explains why the maximal responses were not reduced by chloroethylclonidine.

The selectivity of 5-methylurapidil of α_{1A}- over α_{1B}-adrenoceptors is 50- to 100-fold, whereas the selectivity of WB 4101 of α_{1A}- over α_{1B}-adrenoceptors is less, 10- to 20-fold (Minneman and Esbenshade, 1994). The inhibitory effect of both 5-methylurapidil and WB 4101 in the presence of chloroethylclonidine on the response must be due to the effect of the antagonists on the α_{1A}-adrenoceptors. The effect of 5-methylurapidil was not significantly different in the presence or absence of chloroethylclonidine, indicating that the α_{1B}- adrenoceptors do not have a regulatory influence on the α_{1A}-adrenoceptor-mediated effects. The calculated affinity constants of the functional α_{1A}-adrenoceptor antagonists corresponded well with the values previously reported in the literature (Bylund et al., 1994; Minneman and Esbenshade, 1994). The relatively shallow shape of the dose-inhibition curve of 5-methylurapidil (Fig. 3) could indicate two binding sites and the involvement of more than one α_1-adrenoceptor subtype in the α_1-adrenoceptor-stimulated increase in $^{86}Rb^+$ influx rate. However, the lack of effect of chloroethylclonidine and BMY 7378 strongly argues against this.

Because the functional significance of α_{1D}-adrenoceptors in the myocardium is not well established, the effect of BMY 7378 was also tested. The selectivity of BMY 7378 for α_{1D}- versus α_{1A}- and α_{1B}-adrenoceptor subtypes is 100-fold or more (Saussy et al., 1996). No statistically significant effect of this compound on the α_1-adrenoceptor-stimulated increase in $^{86}Rb^+$ influx rate could be detected.

The mechanism involved in the increased $^{86}Rb^+$ influx rate is not know. Several $^{86}Rb^+$ translocation mechanisms (e.g., the Na^+/K^+ATPase and the $Na^+/K^+/2Cl^-$ co-transporter) exist in the heart sarcolemma, and it should be noted that the Na^+/K^+ATPase pump current has been shown to be stimulated by α_{1B}-adrenoceptors but not by α_{1A}-adrenoceptors (Williamson et al., 1993).

Compared to previously published data on inositol phosphate accumulation after α_1-adrenoceptor stimulation in cardiomyocytes (Terzic et al., 1993), the increase in IP_3 mass after α_1-adrenoceptor stimulation was moderate, with no more than up to 2-fold increase. Most studies on phospholipase C activation after α_1-adrenoceptor stimulation have been performed by measuring radiolabelled inositol phosphates in the presence of millimolar concentrations of Li^+, which inhibits inositol phosphatases (Berridge et al., 1982). The present investigation measures IP_3 mass, which is the resultant of the production and breakdown of IP_3. This could explain the discrepancy between the present results and those generally reported.

The results with α_1-adrenoceptor subtype-selective antagonists on the noradrenaline-induced IP_3 accumulation are not easily quantitatively interpreted, due to the relative selectivity of the antagonists. However, neither chloroethylclonidine nor 5-methylurapidil alone completely inhibited the response, whereas a combination of the two did. Thus the present results indicate that both α_{1A}- and α_{1B}-adrenoceptors are involved in the response. Furthermore, the partial inhibition of IP_3 accumulation by BMY 7378 at relatively low concentrations, as well as a complete lack of effect of BMY 7378 on the α_{1A}-adrenoceptor-mediated stimulation of $^{86}Rb^+$ influx, indicate that α_{1D}-adrenoceptors contribute to the IP_3 response as well. The complete inhibition of the increase in IP_3 mass at the higher concentrations of BMY 7378 is likely due to an effect of the antagonist on more than one α_1-adrenoceptor subtype. The calculated $-\log IC_{50}$ value for BMY 7378 was 5.95 ± 0.17 (Fig. 7). The pK_i value for binding of BMY 7378 to the rat α_{1d}-, the rat α_{1a}- and the hamster α_{1b}-adrenoceptors (all cloned) is 8.16 ± 0.06, 6.54 ± 0.08 and $6.24 \pm .0.03$, respectively (Saussy et al., 1996). Our data suggest that α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors may all contribute to the noradrenaline-stimulated IP_3 accumulation, but the relative contribution from each receptor subtype cannot be accurately determinated from these experiments.

α_1-Adrenoceptor stimulation of phosphatidylinositol 4,5-bisphosphate breakdown has previously been reported to be mediated via both α_{1A}- and α_{1B}-adrenoceptor subtypes in adult rat myocardium (Lazou et al., 1994; Deng et al., 1996a) and rabbit ventricular myocardium (Yang and Endoh, 1994), and via the α_{1A}-adrenoceptor subtype in cultured neonatal rat ventricular myocytes (Knowlton et al., 1993; Deng et al., 1996a). Differences between neonatal and adult myocardium could be explained by ontogenic differences (Deng et al., 1996a). A concentration-dependent inhibitory effect of BMY 7378 on α_1-adrenoceptor-stimulated increase in radiolabelled inositol phosphates in rat myocardium similar to the present results has recently been reported (Deng et al., 1996b).

In conclusion, the increase in $^{86}Rb^+$ influx rate and in IP_3 mass content involves partly different α_1-adrenoceptors. Whereas the α_1-adrenoceptor-mediated increase in $^{86}Rb^+$ influx rate is mediated via the α_{1A}-adrenoceptor subtype only, the α_{1A}-, the α_{1B}- and possibly the α_{1D}-adrenoceptor subtypes are involved in the increase in IP_3 level. Thus, a simple causative relationship does not exist

between the IP_3 response and the increase in $^{86}Rb^+$ influx rate.

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