α₁D-Adrenoceptors Do Not Contribute to Phosphoinositide Hydrolysis in Adult Rat Cardiac Myocytes

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We have used the α₁D-adrenoceptor selective antagonist, BMY 7378, to investigate the presence of α₁D-adrenoceptor subtype in adult rat heart by radioligand binding assays. We also determined the role of this subtype in stimulating phosphoinositide (PI) hydrolysis in adult rat cardiac myocytes. BMY 7378 inhibited [³H]prazosin binding to cardiac membranes in a biphasic mode with a pKᵢ of 9.19 ± 0.26 for high affinity sites and 6.64 ± 0.09 for low affinity sites. The inhibition of the adrenaline-induced stimulation of PI hydrolysis by BMY 7378 fitted a one-site model and the calculated pKᵢ value (6.92 ± 0.28) was consistent with the involvement of α₁A and α₁B adrenoceptors. In addition, BMY 7378, at concentrations up to 100 nM, did not significantly affect the concentration-response curves for the adrenaline-induced stimulation of PI hydrolysis. Taken together, these data suggest that α₁D-adrenoceptors are expressed in adult rat heart but this subtype is not involved in the adrenaline-induced stimulation of PI hydrolysis.

Key Words: α₁D-adrenoceptor subtype; phosphoinositide hydrolysis; BMY 7378; adult rat cardiac myocytes.
couple preferentially to phosphtidylinositol turnover whereas the \( \alpha_{1D} \) subtype activates the mitogen activated protein kinase cascade (10). In contrast, studies with expressed cloned \( \alpha_1 \)-adrenoceptor subtypes have shown that all three subtypes are capable of activating PI hydrolysis in response to phenylephrine although with a different efficiency (19–22). In addition, all three subtypes not only couple to phospholipase C activation but also to activation of phospholipase A2 and phospholipase D and can elevate intracellular Ca\(^{2+} \).

\( [3H] \)Prasozin (sp act 79 Ci/mmol) was from New England Nuclear and \( [3H] \)inositol (sp act 18 Ci/mmol) was from Amerchol. BMY 7378 (8\( \cdot \)2-[4(2-methoxyphenyl)-1-piperazinyl]ethyl)-8-azaspir[4,5] decane-7,9-dione dihydrochloride was from Research Biochemicals International (Natick, MA). Materials. 1- Adrenoceptor mediated responses in various tissues as well as those of the cloned \( \alpha_1 \)-adrenoceptors, has not allowed assessing any conclusive signaling differences among distinct \( \alpha_1 \)-adrenoceptor subtypes. Thus, the roles of individual \( \alpha_1 \)-adrenoceptor subtypes in mediating specific physiological effects need to be investigated further.

The present study was designed to investigate the potential presence of \( \alpha_{1D} \)-adrenoceptor protein in adult rat heart by radioligand binding assays. Furthermore, we have used the \( \alpha_{1D} \)-adrenoceptor selective antagonist BMY 7378 in order to elucidate the role of \( \alpha_{1D} \)-subtype in stimulating PI hydrolysis in adult rat cardiac myocytes. Our data suggest that although \( \alpha_{1D} \)-adrenoceptors are expressed in rat heart, they do not contribute to PI hydrolysis.

**MATERIALS AND METHODS**

Materials. Collagenase (Worthington Type 1) was from Lorne Diagnostics (Twyford, UK). BMY 7378 (8\( \cdot \)2-[4(2-methoxyphenyl)-1-piperazinyl]ethyl)-8-azaspir[4,5] decane-7,9-dione dihydrochloride was from Research Biochemicals International (Natick, MA). \( [3H] \)Prasozin (sp act 79 Ci/mmol) was from New England Nuclear (Boston, MA). \( [3H] \)Inositol (sp act 18 Ci/mmol) was from Amercham Pharmacia Biotech (Merck, Helsas, Gyifa, Greece) G/F filters were from Whatman (Kent, UK). Adrenaline, phenolamine, propranolol, and bovine serum albumin (fraction V) were from Sigma-Aldrich (Deisenhofen, Germany). General laboratory chemicals were from Merck (Darmstadt, Germany).

Animals. Male Wistar rats (250–300 g) were used. All animals received humane care in accordance to the Guidelines for the Care and Use of Laboratory Animals published by the Greek Government (160/1991) based on EU regulations (86/609).

Membrane preparation. Rats were anesthetized with sodium pentobarbitone. Hearts were excised and perfused retrogradely with Krebs–Henseleit buffer for 5 min to remove blood. Atria were removed and ventricles were homogenized at 4°C in 9 vol of buffer A (50 mM Tris, 100 mM NaCl, and 2 mM EDTA, adjusted to pH 7.4 with HCl) using a Polytron homogenizer. Homogenates were centrifuged at 20,000g for 20 min at 4°C. Pellets were dispersed in the same volume of buffer B (50 mM Tris and 1 mM EDTA adjusted to pH 7.4) as used for the initial homogenization using a ground glass homogenizer. The suspensions were incubated at 37°C for 10 min. The centrifugation, resuspension, and incubation steps were repeated two more times. After a final centrifugation, the pellets were resuspended in buffer B at the appropriate tissue concentration. Protein concentration was determined by the method of Bradford (23).

Radioligand binding. Radioligand binding using \( [3H] \)prazosin as the ligand was performed as previously described (12). Briefly, aliquots of the membrane suspensions were incubated with \( [3H] \)prazosin in a total volume of 2 ml buffer B at 37°C for 45 min in the presence or absence of competing drugs. The incubation was terminated by rapid vacuum filtration over Whatman GF/C filters and each filter was washed with 15 ml ice-cold buffer B. Filters were immersed in 1 ml of double distilled water in scintillation vials and counted in Fluoran HV (BDH) in a LKB/Wallac scintillation counter. Nonspecific binding was determined in the presence of 10 \( \mu \)M phentolamine.

To determine the affinity (Kd) and the maximal binding capacity (Bmax) of \( [3H] \)prazosin to cardiac \( \alpha_1 \)-adrenoceptors, saturation curves were constructed by incubating membranes with increasing concentrations of \( [3H] \)prazosin (0.05–0.5 nM) and the data were analyzed by the method of Scatchard. Competition binding experiments were carried out at 0.2 nM \( [3H] \)prazosin with increasing concentrations (10\( -11 \)–10\( -6 \) M) of the \( \alpha_1D \)-adrenoceptor specific antagonist BMY 7378.

Preparation of ventricular cardiac myocytes. Ventricular myocytes were isolated by collagenase digestion of hearts as previously described (12). Freshly isolated cells from a single heart were washed twice with collagenase-free Krebs–Henseleit medium (hereafter referred to as incubation medium) containing 25 mM NaHCO\(_3\), 4.7 mM KCl, 118.5 mM NaCl, 1.2 mM MgSO\(_4\)7H\(_2\)O, 1.2 mM KH\(_2\)PO\(_4\), 2% bovine serum albumin (BSA), 10 mM glucose and 0.1 mM added Ca\(^{2+}\) (as CaCl\(_2\)), equilibrated with 95% O\(_2\)/5% CO\(_2\). They were finally resuspended in 10 ml of incubation medium, in which added Ca\(^{2+}\) was increased to 1 mM. After isolation and resuspension, 70–90% of the myocytes were rod shaped and quiescent.

Agonist-stimulated phosphoinositide (PI) hydrolysis. Cardiac myocytes were preincubated with myo-[\( ^{3H} \)]inositol for 60 min at 37°C. At the end of the incubation period, they were allowed to settle, the supernatant medium was discarded, and the myocytes were washed three times with fresh incubation medium. Finally, the cells were resuspended in 10 ml incubation medium containing 1 mM added Ca\(^{2+}\) and 10 mM LiCl for 15 min before experiments were initiated.

Cardiac myocytes (0.5 ml) were incubated at 37°C in siliconized stoppered glass tubes. Adrenaline and BMY 7378 were added, and the cells were incubated for 30 min more. In each set a control was included (without adrenaline added). At the end of the incubation period the cells were centrifuged in an Eppendorf centrifuge for 1–2 s. The medium was discarded and 1 ml of 0.8 M HClO\(_4\) was added. Precipitated protein was removed by bench-centrifugation and the supernatant fractions were neutralized with 0.8 M KOH/10 mM Tris. Precipitated KClO\(_4\) was removed by bench centrifugation and the supernatant fractions were retained. Competition experiments were carried out in the presence of 1 \( \mu \)M adrenaline over a concentration range of 0.01 pM to 50 \( \mu \)M BMY 7378. Incubations were carried out both in the absence and presence of \( \alpha_1 \)-propranolol, which, when present, was at a concentration 20-fold higher than the concentration of adrenaline. Concentration–response curves for adrenaline were determined in the presence of 100 nM, 1 \( \mu \)M, and 10 \( \mu \)M BMY 7378.

Pooled [\( ^{3H} \)]inositol mono-, bis-, and tris-phosphates were separated essentially as described in (12).

Data analyses. The results are presented as means \( \pm \) SE of n experiments. Curve fitting was performed using Prism program (GraphPad Software, San Diego, CA.). Saturation binding experiments were analyzed by fitting rectangular hyperbolic functions to the experimental data to determine the number of binding sites (Bmax) and their affinity for the radioligand (Kd). Competition binding data were analyzed using either one- or two-site models. A two-site fit was accepted only if it was statistically better compared with one-site model as assessed by the use of F test (P < 0.05). IC\(_{50}\) values from competition experiments were converted to K values using the Cheng-Prusoff equation (24) after ensuring that the Hill
FIG. 1. Competition between $[^3H]$prazosin and BMY 7378 for binding to membrane preparations of adult rat heart. Membrane fractions of heart were prepared and incubated with various concentrations of BMY 7378 in the presence of 0.2 nM $[^3H]$prazosin as described under Materials and Methods. $[3H]$Prazosin binding is expressed as a percentage of binding in the absence of BMY 7378 following subtraction of blanks. A two-site curve fit was fitted separately. $p_i$ values using a functional equivalent of the Cheng-Prusoff equation (25). Alternatively, 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 (26). Observation of curves for BMY 7378 best fitted a two-site model than a one-site model ($P < 0.001$); $pK_i$ of BMY 7378 was $9.19 \pm 0.26$ and $6.64 \pm 0.09$ for high and low affinity binding, respectively, whereas the percentage of high affinity sites was $28 \pm 2\%$. By comparison with the values obtained for cloned $\alpha_1$-adrenoceptor subtypes, the high affinity binding sites obtained for BMY 7378 were consistent with the presence of $\alpha_{1D}$-adrenoceptor subtype whereas the low affinity sites were consistent with the presence of $\alpha_{1A}$ or $\alpha_{1B}$-adrenoceptor subtypes (Table I).

### Results

#### Expression of $\alpha_{1D}$-Adrenoceptor Subtype in Adult Rat Heart

In preliminary experiments to confirm previous work (11, 12), the binding of $[^3H]$prazosin to cardiac membrane preparations was studied. Scatchard plots (results not shown) and yielded a $K_d$ of $0.194 \pm 0.062$ nM ($pK_a 9.71 \pm 0.095$) and a $B_{max}$ of $23.4 \pm 11.2$ fmol/mg protein.

The $\alpha_{1D}$-adrenoceptor population was characterized in competition experiments using $[^3H]$prazosin binding and the $\alpha_{1D}$-selective adrenergic antagonist BMY 7378 (Fig. 1). Nonlinear regression analysis of the inhibition curves for BMY 7378 best fitted a two-site model than a one-site model ($P < 0.001$); $pK_i$ of BMY 7378 was $9.19 \pm 0.26$ and $6.64 \pm 0.09$ for high and low affinity binding, respectively, whereas the percentage of high affinity sites was $28 \pm 2\%$. By comparison with the values obtained for cloned $\alpha_1$-adrenoceptor subtypes, the high affinity binding sites obtained for BMY 7378 were consistent with the presence of $\alpha_{1D}$-adrenoceptor subtype whereas the low affinity sites were consistent with the presence of $\alpha_{1A}$ or $\alpha_{1B}$-adrenoceptor subtypes (Table I).

### Effects of BMY 7378 on the Adrenaline-Induced PI Hydrolysis

The ability of the $\alpha_{1D}$-adrenoceptor subtype to activate PI hydrolysis was examined in isolated adult rat cardiac myocytes. We normally did not discriminate among the production of inositol monophosphate, inositol bisphosphate and inositol trisphosphate. Adrenaline was chosen as an agonist because it gives the largest relative stimulation of PI hydrolysis (approximately sevenfold vs control) compared with noradrenaline and phenylephrine (12).

In the first series of experiments, cardiac myocytes were incubated with various concentrations of BMY 7378 in the presence of 1 $\mu$M of adrenaline. Nonlinear regression analysis of the inhibition of adrenaline-stimulated PI hydrolysis fitted best a one-site model.

### Table I

Competition for $[^3H]$Prazosin Binding Sites in Membranes Prepared from Adult Rat Hearts and Comparison with Binding Affinities for the Cloned $\alpha_1$-Adrenoceptor Subtypes

<table>
<thead>
<tr>
<th>Competitor</th>
<th>One-site fit</th>
<th>Two-site fit</th>
<th>Percentage $R_H$</th>
<th>$pK_i$ $\alpha_{1A}$</th>
<th>$pK_i$ $\alpha_{1B}$</th>
<th>$pK_i$ $\alpha_{1D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>6.29 ± 0.06 $^a$</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6.06 ± 0.07 $^b$</td>
<td>6.03 ± 0.04 $^b$</td>
</tr>
<tr>
<td>5' MU</td>
<td>—</td>
<td>8.96 ± 0.50 $^a$</td>
<td>7.05 ± 0.13 $^a$</td>
<td>23 ± 6 $^a$</td>
<td>8.63 ± 0.32 $^c$</td>
<td>6.97 ± 0.50 $^c$</td>
</tr>
<tr>
<td>BMY 7378</td>
<td>—</td>
<td>9.19 ± 0.26</td>
<td>6.64 ± 0.09</td>
<td>28 ± 2</td>
<td>6.57 ± 0.02 $^b$</td>
<td>6.77 ± 0.03 $^b$</td>
</tr>
<tr>
<td>Prazosin</td>
<td>9.71 ± 0.09</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>9.52 ± 0.38 $^b$</td>
<td>9.79 ± 0.38 $^b$</td>
</tr>
</tbody>
</table>

Note: Competition curves were performed on membranes prepared from adult rat ventricles as described under Materials and Methods. Data were best fitted to either one-site or two-site model. Values are the means ± SE from six separate experiments, where each competition curve was fitted separately. $pK_i$ is the negative log of $K_i$, $K_m$ and $K_u$ are high and low affinity constants, respectively. $R_H$, high affinity binding sites; 5' MU, 5' methylurapidil. Affinity constants for the cloned mammalian $\alpha_1$-adrenoceptors are shown for comparison and they were obtained from the following reports (16) and (28). Affinity constants for adrenaline and 5' methylurapidil were obtained from (12).
(Fig. 2). The calculated affinity constant for BMY 7378, pK_b 6.92 ± 0.28, was similar to that observed for the low affinity sites in the radioligand binding studies (Table I), suggesting the relative absence of a α_{1D}-adrenoceptor subtype coupled to phospholipase C in cardiac myocytes. The same experiments were performed in the presence of propranolol with similar results (pK_b 7.02 ± 0.12). In addition, the inhibitory action of BMY 7378 was essentially the same (pK_b 6.60 ± 0.19) in the presence of the α_{1A}-adrenoceptor antagonist 5'-methylurapidil (results not shown).

In a second series of experiments, concentration-response curves for adrenaline-induced PI hydrolysis were compared in the absence and presence of 100 nM, 1 μM, and 10 μM BMY 7378 (Fig. 3A). The inhibition by BMY of the stimulation of PI hydrolysis by adrenaline fitted a single site curve. pEC_{50} values for adrenaline were 6.14 ± 0.13 in the absence of BMY 7378, 5.96 ± 0.12 at 100 nM, 5.85 ± 0.08 at 1 μM, and 5.07 ± 0.05 at 10 μM BMY 7378. The maximal response to adrenaline was significantly inhibited by approximately 22 and 30% in the presence of 1 and 10 μM BMY 7378, respectively. At these concentrations of BMY 7378, a significant decrease in the pEC_{50} of adrenaline (P < 0.001 relative to control) was also observed. The slope of Schild plot (Fig. 3B) was 0.85 ± 0.11 when analyzed at 100 nM to 1 μM BMY 7378, which is not significantly different from unity; the yielded pA_2 value was 6.31 ± 0.28, which is very close to the pK_b value obtained from the inhibition curves (Fig. 2). Both these values correlated poorly with binding pK_i values of BMY 7378 for the cloned α_{1D}-adrenoceptor subtype. The potency of BMY 7378 for inhibiting

Inositol phosphate responses to adrenaline in adult rat cardiac myocytes correlated well with binding pK_i values for cloned α_{1A}- and α_{1B}-adrenoceptor subtypes (Table I).

DISCUSSION

Receptor cloning experiments have clearly established the existence of at least three subtypes of α_{1}-adrenoceptors (2, 27, 28). The specific tissue distribution of all three subtypes at the mRNA level has been elucidated in rats (9, 29–31). For the α_{1A}- and α_{1B}-adrenoceptors, the tissue distribution has been also widely investigated at the protein level in radioligand binding studies (11–13, 32, 33). These studies have demonstrated two binding sites in adult rat heart although the exact identity of the subtypes corresponding to these binding sites was unclear due to the lack of suitable antagonists for the α_{1D}-adrenoceptor subtype. The present study was designed to investigate directly the potential presence of α_{1D}-adrenoceptor protein in rat heart by radioligand binding. Furthermore, another objective of the study was to examine whether α_{1D}-adrenoceptors contribute to α_{1}-adrenergic induced
PI hydrolysis. For this purpose we have used the α1D-adrenoceptor selective antagonist, BMY 7378. BMY 7378 has been shown to be 100-fold selective for α1D over α1A- and α1B-adrenoceptors (34, 35).

Inhibition curves for the α1D-selective antagonist, BMY 7378, were best fitted to a two-site model, where the high affinity representing the α1D-adrenoceptors, comprised approximately 28% of total binding sites (Fig. 1). In previous studies, using 5’-methylurapidil an α1A-adrenoceptor antagonist and CEC which alkylates α1B- and α1D-adrenoceptors with almost the same potency (36), the proportions of α1A- and α1D-adrenoceptor subtypes were found to be between 20–25 and 75–80%, respectively (12–14). Taken together, these data suggest that the proportion of α1A-, α1B-, and α1D-adrenoceptors in adult rat ventricles is approximately 20, 50, and 30% respectively. The above results are consistent with a recent study (17) where a similar proportion of α1-adrenoceptor subtypes in rat heart was reported. However, other investigators, using radioligand binding assays, have reported that α1D-adrenoceptors are not readily detectable in rat heart because BMY 7378 showed steep and monophasic competition curves with a low affinity (15, 16).

The coexistence of all three α1-adrenoceptors subtypes in rat heart makes it difficult to determine the role of each subtype. In the original studies leading to the concept of α1-adrenoceptor subtype heterogeneity, it had been postulated that α1A- and α1B-adrenoceptors could be distinguished based on their differential signaling mechanisms (6). Whereas this may be true in some model organisms, more recent data argue against this concept (3, 5). In previous studies in adult rat cardiac myocytes, it was demonstrated that both α1A- and α1B-adrenoceptor subtypes are involved in the α1-adrenergic induced stimulation of PI hydrolysis (12, 14). In this study, we used the α1D-selective antagonist, BMY 7378, in order to evaluate the role of α1D-adrenoceptor subtype in this response. BMY 7378 inhibited the adrenaline induced PI hydrolysis with low affinity constant indicative of α1A- or α1B-adrenoceptor response. There was no evidence for any high affinity constants suggestive of α1D-adrenoceptor subtype involvement (Fig. 2). In addition, the concentration–response curves for adrenaline-induced PI hydrolysis were not affected by BMY at 100 nM–1 μM and the pA2 value calculated from ScHild plot did not correlate with its high affinity pKt to cardiac membranes (Fig. 3, Table I). These data suggest that α1-adrenoceptors that are susceptible to low concentrations of BMY 7378 are not coupled to the stimulation of PI hydrolysis and the latter is exerted via interactions with the low affinity receptors, α1A and α1B. It is of interest that in studies with cloned α1-adrenoceptors, all three subtypes were capable of activating PI turnover. However, differences seem to exist in the effectiveness with which such receptors activate this signaling pathway, suggesting variations in intrinsic activities among receptor subtypes (21, 22, 37). In general, it has been observed that the α1A subtype is more effective than the α1B and that the α1D is usually the less effective of the three.

The physiological relevance of α1-adrenoceptor subtype remains unclear. This could involve the mediation of different functions within a tissue, activation under different conditions and/or different regulation patterns. In this respect, some data indicate that in cultured neonatal rat cardiomyocytes, the hypertrophic response may be mediated by α1D-adrenoceptors (38), whereas the inotropic response in adult rat heart muscle strips occurs mostly via α1B-adrenoceptors (39). Furthermore, in another study, it was shown that the α1A-adrenoceptor subtype couples preferentially to the PI hydrolysis pathway whereas the α1B subtype couples preferentially to the mitogen activated protein kinase pathway (10). Functional correlates of α1-adrenoceptor have not unequivocally been identified. It has been reported that α1D-adrenoceptors play a minor role in mediating the contractile response in adult rat atrial and ventricular muscles (15) and do not contribute to inotropic response to catecholamines in neonatal rat myocardium (40). The only function that has been attributed to α1D-adrenoceptors in rat is the contraction of vascular smooth muscle of the aorta (15, 35, 41) but even this has been controversial (42).

In conclusion, the present study demonstrates that, even though the α1D-adrenoceptor subtype is expressed in the adult rat heart, it does not appear to be coupled to the hydrolysis of PI. The functional role of α1D-adrenoceptor in adult rat heart remains to be determined.

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