Archives of Biochemistry and Biophysics
Vol. 392, No. 1, August 1, pp. 117–122, 2001
doi:10.1006/abbi.2001.2424, available online at http://www.idealibrary.com on IDEAL®

α_{1D}-Adrenoceptors Do Not Contribute to Phosphoinositide Hydrolysis in Adult Rat Cardiac Myocytes

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Received March 12, 2001; published online July 9, 2001

We have used the α_{1D}-adrenoceptor selective antagonist, BMY 7378, to investigate the presence of α_{1D}-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 [^{3}H]prazosin binding to cardiac membranes in a biphasic mode with a pK_i 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 pK_b value (6.92 ± 0.28) was consistent with the involvement of α_{1A} and α_{1B} 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 α_{1D}-adrenoceptors are expressed in adult rat heart but this subtype is not involved in the adrenaline-induced stimulation of PI hydrolysis.

***Key Words:* α_{1D}-adrenoceptor subtype; phosphoinositide hydrolysis; BMY 7378; adult rat cardiac myocytes.**

In the heart, activation of α_1-adrenoceptors has a number of physiological effects. These include rapid regulation of contractile activity through changes in chronotropy and inotropy and long-term maintenance of cardiac function through regulation of gene expression and cell growth (1–3).

α_1-Adrenergic receptors belong to the larger family of $G_{q/11}$-protein coupled receptors, which initiate signals by activating phospholipase C-dependent hydrolysis of membrane phosphoinositides (PI) in almost all tissues where this effect has been examined (3–5). Recent studies have shown that other signaling pathways can been activated upon α_1-adrenoceptor stimulation such as Ca^{2+} influx through voltage-dependent and independent Ca^{2+} channels, arachidonic acid release, and phospholipase D activation (2–5).

Pharmacologically distinct α_1-adrenergic receptor subtypes have been described and molecular cloning and expression of the cDNA for three α_1-subtypes, namely α_{1A}, α_{1B}, and α_{1D}, have been reported (2, 6, 7). At the RNA level, all three subtypes appear to be present in the heart (7–10). At the protein level, both the α_{1A}- and α_{1B}-adrenoceptor subtypes have been reported to be present in cardiac tissue, using selective receptor antagonists (11–14). However, there is a controversy concerning the existence of a α_{1D}-adrenoceptor binding site and its functional role. It has been argued that α_{1D}-adrenoceptors are not expressed at the protein level in the myocardium and in many other rat tissues where their mRNA has been described abundantly and they cannot be detected by competition radioligand binding studies (10, 15, 16). On the other hand, a recent study demonstrated the existence of α_{1D}-adrenoceptors at the mRNA and protein level in rat heart but their functional importance in mediating the inotropic response to noradrenaline remained unclear (17). However, α_{1D}-adrenoceptors have been detected in rabbit myocardium and have been found to contribute to the α_1-adrenoceptor mediated regulation of contractile force in this tissue (18).

The physiological rationale for multiple α_1-adrenoceptor subtypes is largely unclear. One possibility is that the subtypes couple to intracellular signaling pathways in qualitatively and/or quantitatively distinct manners. To this end, it has been demonstrated that in neonatal cardiac myocytes α_{1A}-adrenoceptors

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0003-9861/01 $35.00

couple preferentially to phosphatidylinositol turnover whereas the α_{1B} subtype activates the mitogen activated protein kinase cascade (10). In contrast, studies with expressed cloned α_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 A_2 and phospholipase D and can elevate intracellular Ca^{2+} concentration (22). However, the comparison among different α_1-adrenoceptor mediated responses in various tissues as well as those of the cloned α_1-adrenoceptors, has not allowed assessing any conclusive signaling differences among distinct α_1-adrenoceptor subtypes. Thus, the roles of individual α_1-adrenoceptor subtypes in mediating specific physiological effects need to be investigated further.

The present study was designed to investigate the potential presence of α_{1D}-adrenoceptor protein in adult rat heart by radioligand binding assays. Furthermore, we have used the α_{1D}-adrenoceptor selective antagonist BMY 7378 in order to elucidate the role of α_{1D}-subtype in stimulating PI hydrolysis in adult rat cardiac myocytes. Our data suggest that although α_{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[2-[4(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5] decane-7,9-dione dihydrochloride) was from Research Biochemicals International (Natick, MA). [^{3}H]Prasozin (sp act 79 Ci/mmol) was from New England Nuclear (Boston, MA), *myo*-[^{3}H]inositol (sp act 18 Ci/mmol) was from Amersham Pharmacia Biotech (Merck Hellas, Glyfada, Greece) GF/C filters were from Whatman (Kent, UK). Adrenaline, phentolamine, 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 anesthesized 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 20,000*g* 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 [^{3}H]prazosin as the ligand was performed as previously described (12). Briefly, aliquots of the membrane suspensions were incubated with [^{3}H]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 μM phentolamine.

To determine the affinity (K_d) and the maximal binding capacity (B_{max}) of [^{3}H]prazosin to cardiac α_1-adrenoceptors, saturation curves were constructed by incubating membranes with increasing concentrations of [^{3}H]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 [^{3}H]prazosin with increasing concentrations (10^{-11}–10^{-4} M) of the α_{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 $MgSO_4$ $7H_2O$, 1.2 mM KH_2PO_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*-[^{3}H] 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 μM adrenaline over a concentration range of 0.01 pM to 50 μM BMY 7378. Incubations were carried out both in the absence and presence of DL-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 μM, and 10 μM BMY 7378.

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

Data analyses. The results are presented as means ± 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 (B_{max}) and their affinity for the radioligand (K_d). 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_i values using the Cheng–Prusoff equation (24) after ensuring that the Hill

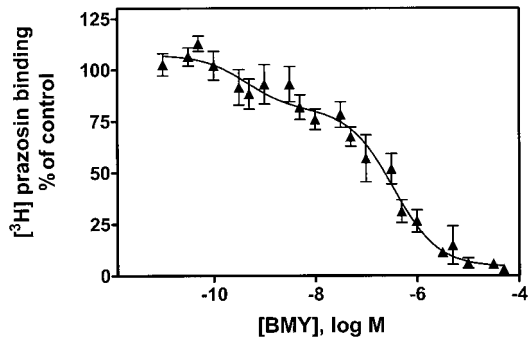

FIG. 1. Competition between [^{3}H]prazosin and BMY 7378 for binding to membrane preparations of adult rat heart. Membrane fractions of heart were prepared and incubated with various concentrations of BMY 7378 in the presence of 0.2 nM [^{3}H]prazosin as described under Materials and Methods. [^{3}H]Prazosin binding is expressed as a percentage of binding in the absence of BMY 7378 following subtraction of blanks. A two-site curve fit ($r^2 = 0.995$) was significantly better than one-site curve ($P < 0.001$). Data are the mean ± SE of six separate membrane preparations, where assays were performed in duplicate. Where no error bars are shown, their size is smaller than the size of the symbol.

coefficient (n_H) was not different from unity. IC_{50} values from functional inhibition were converted to K_b 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). Concentration–response data were fitted by nonlinear regression to a four parameter logistic equation. The Instat program (Graphpad Software) was used for all statistical calculations and $P < 0.05$ was considered significant.

RESULTS

Expression of α_{1D}-Adrenoceptor Subtype in Adult Rat Heart

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

The α_{1D}-adrenoceptor population was characterized in competition experiments using [^{3}H]prazosin binding and the α_{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 ± 0.26 and 6.64 ± 0.09 for high and low affinity binding, respectively, whereas the percentage of high affinity sites was 28 ± 2%. By comparison with the values obtained for cloned α_1-adrenoceptor subtypes, the high affinity binding sites obtained for BMY 7378 were consistent with the presence of α_{1D}-adrenoceptor subtype whereas the low affinity sites were consistent with the presence of α_{1A}- or α_{1B}-adrenoceptor subtypes (Table I).

Effects of BMY 7378 on the Adrenaline-Induced PI Hydrolysis

The ability of the α_{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 μM of adrenaline. Nonlinear regression analysis of the inhibition of adrenaline-stimulated PI hydrolysis fitted best a one-site model

TABLE I

Competition for [^{3}H]Prazosin Binding Sites in Membranes Prepared from Adult Rat Hearts and Comparison with Binding Affinities for the Cloned α_1-Adrenoceptor Subtypes

	Adult rat heart				Cloned α_1-adrenoceptors		
	One-site fit	Two-site fit			pK_i		
Competitor	pK_i	pK_{iH}	pK_{iL}	Percentage R_H	α_{1a}	α_{1b}	α_{1d}
Adrenaline	6.29 ± 0.06[a]	—	—	—	6.06 ± 0.07[b]	6.03 ± 0.04[b]	7.45 ± 0.03[b]
5′MU	—	8.96 ± 0.50[a]	7.05 ± 0.13[a]	23 ± 6[a]	8.63 ± 0.32[c]	6.97 ± 0.50[c]	7.31 ± 0.66[c]
BMY 7378	—	9.19 ± 0.26	6.64 ± 0.09	28 ± 2	6.57 ± 0.02[b]	6.77 ± 0.03[b]	8.94 ± 0.05[b]
Prazosin	9.71 ± 0.09	—	—	—	9.52 ± 0.38[c]	9.79 ± 0.38[c]	9.63 ± 0.40[c]

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_{iH} and K_{iL} are high and low affinity constants, respectively. R_H, high affinity binding sites; 5′MU, 5′ methylurapidil. Affinity constants for the cloned mammalian α_1-adrenoceptors are shown for comparison and they were obtained from the following reports [b](16) and [c](28). Affinity constants for adrenaline and 5′ methylurapidil were obtained from [a](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).

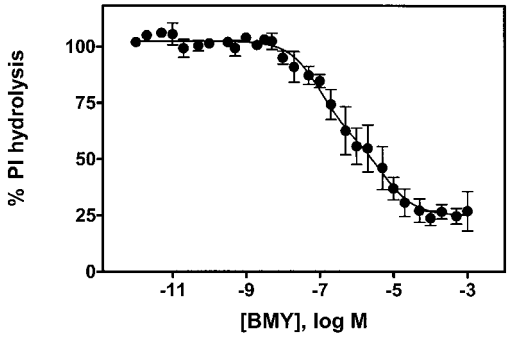

FIG. 2. Inhibition by BMY 7378 of the adrenaline-stimulated PI hydrolysis in adult rat cardiac myocytes. Cardiac myocytes were prepared and their PI pools were prelabeled as described under Materials and Methods. Cells were incubated with various concentrations of BMY 7378 in the presence of 1 μM of adrenaline for 30 min and [^{3}H]inositol phosphates were isolated as described under Materials and Methods. The rates of PI hydrolysis are expressed as a percentage of the rate of PI hydrolysis in the presence of adrenaline and in the absence of BMY 7378. Data are the mean ± SE of six different experiments. Where no error bars are shown, their size is smaller than the size of the symbol. A two-site curve fit was not significantly better than the one-site curve fir shown ($r^2 = 0.996$).

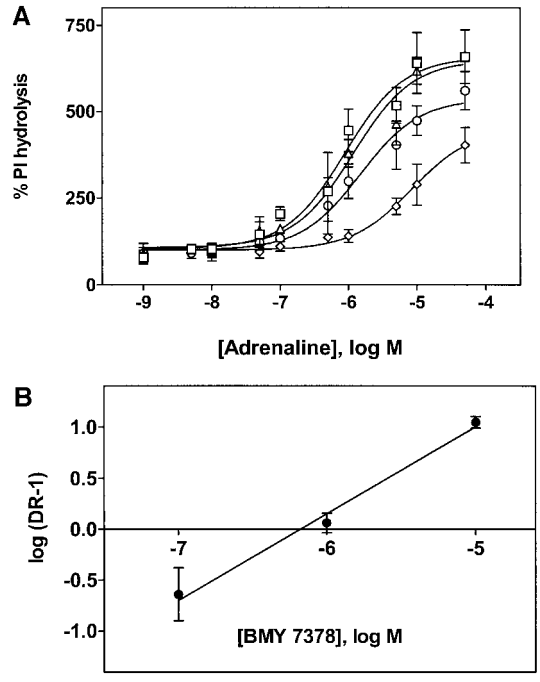

FIG. 3. (A) Effects of BMY 7378 on the concentration–response curves for the adrenaline-induced PI hydrolysis in adult rat cardiac myocytes. Control (□) and in the presence of 100 nM (△), 1 μM (○), and 10 μM (◇) BMY 7378. Values are means ± SE from four different experiments. Where no error bars are shown, their size is smaller than the size of the symbol. (B) Schild plot of BMY 7378-induced antagonism against the effect of adrenaline. Values presented are means ± SE. The data used were taken from (A) and the slope of the regression line was calculated by the least-square method.

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 α_{1D}-adrenoceptor selective antagonist, BMY 7378. BMY 7378 has been shown to be 100-fold selective for α_{1D} over α_{1A}- and α_{1B}-adrenoceptors (34, 35).

Inhibition curves for the α_{1D}-selective antagonist, BMY 7378, were best fitted to a two-site model, where the high affinity representing the α_{1D}-adrenoceptors, comprised approximately 28% of total binding sites (Fig. 1). In previous studies, using 5′-methylurapidil an α_{1A}-adrenoceptor antagonist and CEC which alkylates α_{1B}- and α_{1D}-adrenoceptors with almost the same potency (36), the proportions of α_{1A}- and α_{1B}-adrenoceptor subtypes were found to be between 20–25 and 75–80%, respectively (12–14). Taken together, these data suggest that the proportion of α_{1A}-, α_{1B}-, and α_{1D}-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 α_1-adrenoceptor subtypes in rat heart was reported. However, other investigators, using radioligand binding assays, have reported that α_{1D}-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 α_1-adrenoceptors subtypes in rat heart makes it difficult to determine the role of each subtype. In the original studies leading to the concept of α_1-adrenoceptor subtype heterogeneity, it had been postulated that α_{1A}- and α_{1B}-adrenoceptors could be distinguished based on their differential signaling mechanisms (6). Whereas this may be true in some model organisms, more recent data argues against this concept (3, 5). In previous studies in adult rat cardiac myocytes, it was demonstrated that both α_{1A}- and α_{1B}-adrenoceptor subtypes are involved in the α_1-adrenergic induced stimulation of PI hydrolysis (12, 14). In this study, we used the α_{1D}-selective antagonist, BMY 7378, in order to evaluate the role of α_{1D}-adrenoceptor subtype in this response. BMY 7378 inhibited the adrenaline induced PI hydrolysis with low affinity constant indicative of α_{1A}- or α_{1B}-adrenoceptor response. There was no evidence for any high affinity constants suggestive of α_{1D}-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 pA_2 value calculated from Schild plot did not correlate with its high affinity pK_i to cardiac membranes (Fig. 3, Table I). These data suggest that α_1-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, α_{1A} and α_{1B}. It is of interest that in studies with cloned α_1-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 α_{1A} subtype is more effective than the α_{1B} and that the α_{1D} is usually the less effective of the three.

The physiological relevance of α_1-adrenoceptor subtypes 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 α_{1A}-adrenoceptors (38), whereas the inotropic response in adult rat heart muscle strips occurs mostly via α_{1B}-adrenoceptors (39). Furthermore, in another study, it was shown that the α_{1A}-adrenoceptor subtype couples preferentially to the PI hydrolysis pathway whereas the α_{1B} subtype couples preferentially to the mitogen activated protein kinase pathway (10). Functional correlates of α_{1D}-adrenoceptor have not unequivocally been identified. It has been reported that α_{1D}-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 α_{1D}-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 α_{1D}-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 α_{1D}-adrenoceptor in adult rat heart remains to be determined.

ACKNOWLEDGMENT

The work was supported in part by a grant from the European Union (Grant BMH4-CT96-0287).

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