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Identification of α_1-Adrenergic Receptors and Their Involvement in Phosphoinositide Hydrolysis in the Frog Heart

ANTIGONE LAZOU,[1*] CATHERINE GAITANAKI,[2] SPIROS VAXEVANELLIS,[1] AND ANASTASIA PEHTELIDOU[1]
[1]*Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece*
[2]*Department of Animal and Human Physiology, School of Biology, University of Athens, Athens 15784, Greece*

ABSTRACT The aim of this study was to characterize α_1-adrenergic receptors in frog heart and to examine their related signal transduction pathway. α_1-Adrenergic binding sites were studied in purified heart membranes using the specific α_1-adrenergic antagonist [^{3}H]prazosin. Analysis of the binding data indicated one class of binding sites displaying a K_d of 4.19 $\pm$ 0.56 nM and a B_{max} of 14.66 $\pm$ 1.61 fmol/mg original wet weight. Adrenaline, noradrenaline, or phenylephrine, in the presence of propranolol, competed with [^{3}H]prazosin binding with a similar potency and a K_i value of about 10 μM. The kinetics of adrenaline binding was closely related to its biological effect. Adrenaline concentration dependently increased the production of inositol phosphates in the heart in the presence or absence of propranolol. Maximal stimulation was about 8.5-fold, and the half-maximum effective concentration was 30 and 21 μM in the absence and presence of propranolol, respectively. These data clearly show that α_1-adrenergic receptors are coupled to the phosphoinositide hydrolysis in frog heart. To our knowledge, this is the first direct evidence supporting the presence of functional α_1-adrenergic receptors in the frog heart. *J. Exp. Zool. 293:99–105, 2002.*

Noradrenaline and adrenaline play important roles as neurotransmitters and hormones throughout the body. The adrenoceptors through which these compounds act are subdivided into three families (α_1, α_2, β) based on their pharmacology, structure, and signaling mechanisms (Hieble et al., '95). Within each family subtypes exist that have been defined structurally by molecular cloning of their respective genes and pharmacologically by the interactions of subtype-selective agonists and antagonists with these receptors. Most of these studies have been carried out using mammalian adrenoceptors, and there is relatively little information available on adrenoceptors in amphibians. The presence of these subtypes of adrenoceptors and their role in lower vertebrate heart activity have been the subject of debate in the past, particularly with reference to their functional and morphological relationships (Ask, '83; Chiu and Sham, '85; Sham et al., '87).

In mammalian cardiac cells, responses to α_1-adrenergic stimuli include rapid changes in contractility, in electrophysiological properties, or in metabolic responses (Terzic et al., '93; Li et al., '97; Varma and Deng, 2000). α_1-Adrenergic stimulation may also have longtime effects on cardiac structure and function, since exposure to α_1-adrenergic agonists leads to activation of growth-related gene expression (Knowlton et al., '93; Sugden and Clerk, '98). The effects of α-adrenergic stimulation on the amphibian heart are far less understood and are still a matter of controversy. Whereas some authors have failed to find an α-adrenoceptor-mediated inotropic response (Morad et al., '78; Soustre and Rakotonirina, '81), others suggest that α-adrenergic receptors may participate in the positive inotropic action of sympathomimetic amines (Buckley and Jordan, '70; Niedergerke and Page, '77; Petroff et al., '94). It has also been questioned whether cardiac α-

*Correspondence to: Dr. Antigone Lazou, Laboratory of Animal Physiology, Department of Zoology, School of Biology, Aristotle University of Thessaloniki, Thessaloniki 54006, Greece. E-mail: lazou@bio.auth.gr
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adrenoceptors, if present, serve any functional role amongst the lower vertebrates (Benfey, '82; Ask, '83). In addition, temperature-dependent effects of adrenaline and other sympathomimetics have been reported for the frog myocardium, suggesting a conversion of the adrenoceptor form from a β-type at high to a α-type at low temperatures (Kunos and Nickerson, '76; Chiu and Chu, '89). However, this has been questioned in a more recent study (Herman et al., '96).

α_1-Adrenergic receptors belong to the larger family of $G_{q/11}$-protein-coupled receptors that initiate signals by activating phospholipase C-dependent hydrolysis of membrane phosphoinositides, thus leading to production of $Ins(1,4,5)P_3$ and diacylglycerol. The former regulates intracellular Ca^{2+} movements into a variety of mammalian tissues, whereas the latter is the physiological activator of protein kinase C (Berridge, '93; Zhong and Minneman, '99). Other signaling pathways have also been shown to be activated by α_1-adrenergic receptors, among which is Ca^{2+} influx through voltage-dependent and -independent Ca^{2+} channels, arachidonic acid release, and phospholipase D activation; however, these may vary depending on the cell type (Suzuki et al., '90; Xing and Insel, '96; Noguera et al., '97; Ruan et al., '98; Zhang et al., '98). In rat tissues, molecular cloning and pharmacological studies have revealed the existence of three α_1-adrenergic receptor subtypes, namely, α_{1A}, α_{1B}, and α_{1D} (Stewart et al., '94; Graham et al., '96). However, the existence of α_1-adrenoceptors in the heart of lower vertebrates has not been clearly demonstrated, and there has been no evidence for their coupling to signal transduction pathways.

The aim of the present study was to directly assess the expression of α_1-adrenoceptors in the frog heart by radioligand binding experiments and furthermore to determine whether these receptors are involved in the response of the heart to adrenergic stimulation.

MATERIALS AND METHODS

Materials

[^{3}H]prasozin (specific activity 79 Ci/mmol) was from New England Nuclear (Boston, MA), and *myo*-[^{3}H]inositol (specific activity 18 Ci/mmol) was from Amersham Pharmacia Biotech (Merck Hellas, Glyfada, Greece). GF/C filters were from Whatman (Kent, UK). Adrenaline, noradrenaline, phenylephrine, and prazosin were from Sigma-Aldrich (Deisenhofen, Germany). General laboratory chemicals were from Merck (Darmstadt, Germany).

Animals

Male frogs (*Rana ridibunda*) weighing 100–120 g were caught in the vicinity of Thessaloniki, Greece, and were supplied by a local dealer. They were kept in containers in fresh water at 20°C under a 12-hr light/12-hr dark photoperiod. Animals were not fed and were used a week after arrival. Care of the animals was according to the Guidelines for the Care and Use of Laboratory Animals published by the Greek Government (160/1991), based on European Union regulations (86/609).

Membrane preparation

Frogs were decapitated, hearts were immediately excised, and ventricles were homogenized at 4°C in 9 v/w of buffer A (50 mM Tris, 100 mM NaCl and 2 mM EDTA, adjusted to pH 7.4 with HCl) using an Ultra-turrax homogenizer. Several hearts were used (approx 2 g) for each membrane preparation. 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 centrifugation and resuspension steps were repeated two more times. After a final centrifugation, the pellets were resuspended in buffer B at the appropriate tissue concentration (0.1 g original tissue weight per milliliter) and kept at −70°C until use. Thawed suspensions were rehomogenized using a ground glass homogenizer before use. Protein concentration was determined by the method of Bradford ('76).

Radioligand binding

Radioligand binding using [^{3}H]prazosin as the ligand was performed as previously described (Lazou et al., '94). Briefly, aliquots of the membrane suspensions (100 μl) were incubated with [^{3}H]prazosin in a total volume of 1 ml buffer B at 25°C for 60 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 (Amersham Pharmacia Biotech, Merck, Hellas, Greece) scintillation counter. Nonspecific binding

was determined in the presence of 20 μM prazosin. To determine the affinity (K_d) and the maximal binding capacity (B_{max}) of [^{3}H]prazosin to cardiac a_1-adrenoceptors, saturation curves were constructed by incubating membranes with increasing concentrations of [^{3}H]prazosin (0.05–18 nM) and the data were analyzed by the method of Scatchard. Competition binding experiments were carried out at 0.8 nM [^{3}H]prazosin with increasing concentrations of agonists.

Heart perfusions and prelabeling of phosphoinositide (PI) pools

After rapid excision, hearts were immersed in ice-cold saline and then mounted on a two-way recirculating Langendorff perfusion apparatus. The heart and the perfusion fluids were kept in temperature-controlled chambers (25°C). The standard perfusion medium was amphibian Ringer containing (in mM): NaCl 103, $CaCl_2$ 1.8, $NaHCO_3$ 23.8, Na_2HPO_4 0.6, KCl 2.5, $MgCl_2$ 1.8, and glucose, pH 7.4. The perfusion fluid was equilibrated with a gas mixture containing 95% O_2/5% CO_2, and perfusion pressure was maintained constant at 45 cm H_2O (4.41 kPa).

In all experiments, a 15-min equilibration period was allowed during which hearts were perfused with the standard perfusion medium. This was followed by a 2-hr perfusion with medium containing *myo*-[^{3}H]inositol and finally a 5-min perfusion with standard perfusion medium. At the end of this period, LiCl was added to the perfusion medium to a final concentration of 10 mM, and the perfusion was continued for another 10 min before switching to a perfusion medium containing 10 mM LiCl and adrenaline (0.3–300 μM). Perfusion with this medium was continued for 45 min.

Measurement of PI hydrolysis

At the end of the perfusion period, hearts still attached to the perfusing system were quickly frozen between aluminum tongs cooled to the temperature of liquid N_2. Frozen perfusate as well as connective tissue was removed and the frozen muscle was immediately homogenized in 5 vol wt. of 0.8 M $HClO_4$. Following centrifugation, the extract was neutralized to pH 7.0 with 0.8 M KOH after 1M Tris base was added to a final concentration of 10 mM. Precipitated $KClO_4$ was removed by centrifugation and the supernatant fraction was used for the separation of [^{3}H]inositol phosphates.

[^{3}H]inositol phosphates were separated by a method based on that of Berridge et al. ('83). The entire neutralized cell extract was chromatographed on a 0.5- × 2.5-cm column of Bio-Rad AG1x8 formate form (100–200 mesh) equilibrated with water. Each column was washed with water (2 ml), 5 mM disodium tetraborate/60 mM sodium formate (3 ml), and finally with 3 ml 0.1 M formic acid/1.0 M ammonium formate; the last wash was retained. Fifteen milliliters of scintillation fluid was added to the final wash. Radioactivity was counted on a liquid scintillation counter.

Data analyses

The results are presented as means ± SEM of n experiments. Curve fitting was performed using the 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 than a one-site model as assessed by the use of the F-test ($P < 0.05$). IC_{50} values from competition experiments were converted to K_i values using the Cheng-Prusoff equation (Cheng and Prusoff, '73) after ensuring that the Hill coefficient (n_H) was not different from unity. 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

Characterization of α_1-adrenoceptor binding in membrane fractions from heart

Binding experiments were carried out using [^{3}H]prazosin, commonly classified as a specific α_1-adrenergic receptor antagonist. Preliminary experiments indicated that [^{3}H]prazosin bound to membranes prepared from frog heart in a saturable and reversible mode. Specific binding was linearly dependent on tissue concentration (2–15 mg of original heart wt) and reached a plateau at 45 min (results not shown).

The number and affinity of α_1-adrenergic binding sites were assessed by incubating heart membranes with [^{3}H]prazosin ranging from 0.05 to 18 nM (Fig. 1). Scatchard plots for [^{3}H]prazosin

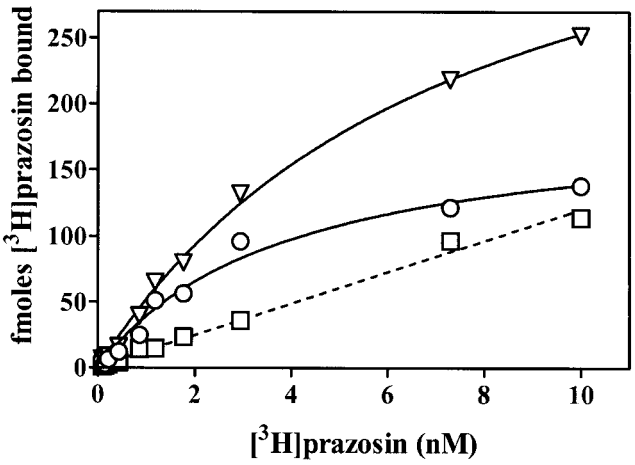

Fig. 1. Saturation curves of [^{3}H]-prazosin binding to purified membranes from frog heart. Membranes (100 μl containing 10 mg of original tissue weight) were incubated at 25°C for 60 min with increasing concentrations of [^{3}H]prazosin ranging from 0.05 to 10 nM. Total (▽) and nonspecific binding (□) were determined in the absence and presence of 20 μM prazosin (○). Specific binding was calculated by subtracting nonspecific from total binding. The best fit was determined with a one-site model. The plot is a representative of four similar experiments run in duplicate.

binding were linear, indicating a homogeneous class of binding sites. Analysis of saturation curves by nonlinear regression revealed an equilibrium binding constant (K_d) of 4.19 ± 0.56 nM and a mean receptor density (B_{max}) of 14.66 ± 1.61 fmol/mg original wet weight. To further evaluate the specificity of [^{3}H]prazosin binding, displacement experiments were performed in the presence of 0.8 nM [^{3}H]prazosin and increasing concentrations of unlabeled prazosin (Fig. 2). Nonlinear regression analysis of competition curves gave a K_d of 6.19 ± 1.9 nM and a B_{max} of 15.61 ± 5.96 fmol/mg original wet weight. These results are in close agreement with the values obtained from the saturation binding experiments.

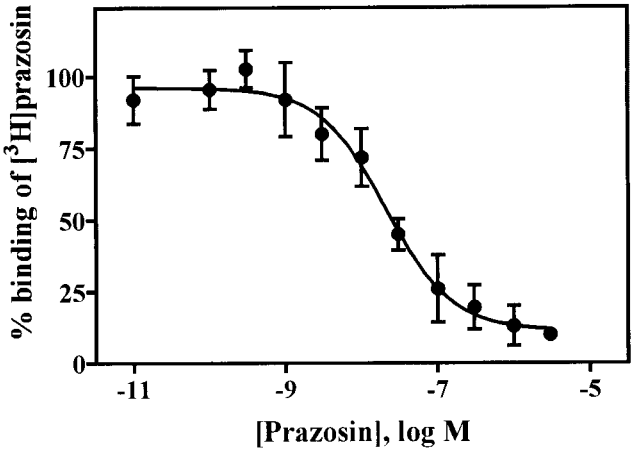

Fig. 2. Homologous competition curve for prazosin. Membrane fractions of heart were prepared and incubated with increasing concentrations of prazosin in the presence of 0.8 nM [^{3}H]prazosin. [^{3}H]prazosin binding is expressed as a percentage of total binding in the absence of prazosin. All values represent the mean ± SEM of five different experiments. Where no error bars are shown, their size is smaller than the size of the symbol.

The binding of [^{3}H]prazosin to heart membranes in the presence of various adrenergic agonists was also investigated. Adrenaline, noradrenaline, and phenylephrine, in the presence of DL-propranolol, competed with binding of [^{3}H]prazosin. Competition curves were monocomponent (Fig. 3). Values of pKi for adrenaline, noradrenaline, and phenylephrine were 4.97 ± 0.20, 5.09 ± 0.04, and 4.96 ± 0.14, respectively, and n_H values were as follows: adrenaline 1.04 ± 0.11, noradrenaline 0.94 ± 0.06, and phenylephrine 0.98 ± 0.21 (Table 1). These results show that the affinities of each individual agonist for α_1-adrenoceptor subtypes are equal.

Stimulation of PI hydrolysis by adrenaline in the heart

The effect of adrenaline on the production of [^{3}H]inositol phosphates in the frog heart prelabeled with [^{3}H]inositol was studied. The experiments were carried out both in the absence and presence of propranol to block the β-adrenergic activity of adrenaline. In the presence of maximally effective concentrations of adrenaline (100 μM), PI hydrolysis in frog heart was linear with time (results not shown). Adrenaline stimulated PI hydrolysis in a concentration-dependent manner. The data from individual experiments used to compile the composite curve in Fig. 4 were fitted to a sigmoid curve, and the derived data are shown in Table 2. In the absence of propranolol, adrenaline

TABLE 1. Binding parameters derived from the competition by adrenaline, noradrenaline, phenylephrine, and prazosin for [^{3}H]prazosin binding sites in membranes from frog heart

	pK_I	n_H
Adrenaline	4.97±0.20	1.04±0.11
Noradrenaline	5.09±0.04	0.94±0.06
Phenylephrine	4.96±0.14	0.98±0.09
Prazosin	8.26±0.22	1.05±0.07

Note: Competition curves were performed as described in Materials and Methods. Data were best fitted to either a one-site or two-site model. Values are the means±SEM from three to five separate determinations using different membrane preparations. pK_i is the negative log of K_i; n_H is the Hill coefficient.

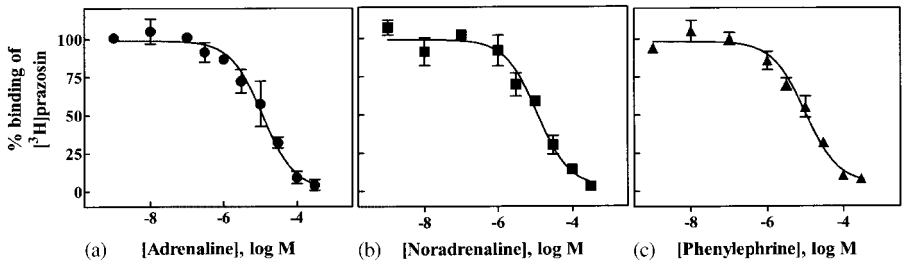

Fig. 3. Competition between [^{3}H]prazosin and (**A**) adrenaline, (**B**) noradrenaline, and (**C**) phenylephrine for binding to membrane preparations of frog heart. Membrane fractions of heart were prepared and incubated with various concentrations of agonists in the presence of 0.8 nM [^{3}H]prazosin as described in Materials and Methods. [^{3}H]prazosin binding is expressed as a percentage of binding in the absence of agonists following subtraction of blanks. A one-site curve was significantly better than a two-site curve ($P < 0.001$). Data are the mean ± SEM of three to four 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.

stimulated PI hydrolysis by 8.5-fold over the basal rate with an EC_{50} value of about 31 μM. The presence of propranolol did not affect the maximal stimulation; however, it produced a small shift of the curve to the left that was not significant, and the computed EC_{50} was 21 μM. The EC_{50} value for the stimulation of PI hydrolysis closely resembles the affinity constant for adrenaline observed in the radioligand binding, indicating that the cellular action was mediated by the same receptor identified in the binding studies.

DISCUSSION

The present study confirms the presence of α_1-adrenoceptors in the heart of amphibians and furthermore provides clear evidence for an adrenaline-induced phosphoinositide hydrolysis mediated through these receptors in the isolated perfused heart. To our knowledge, this is the first direct evidence supporting the presence of functional α_1-adrenergic receptors in the heart of a lower vertebrate.

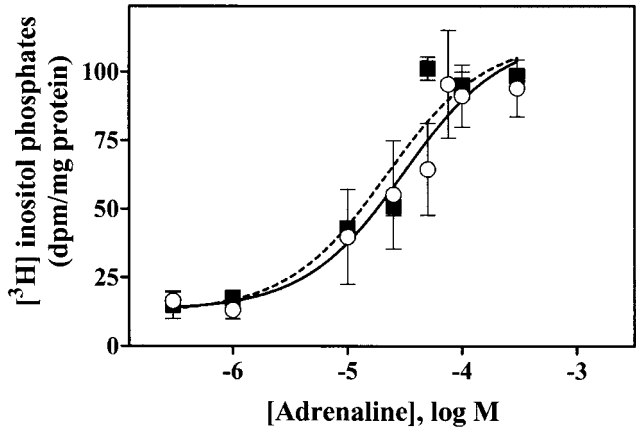

Fig. 4. Stimulation of phosphoinositide hydrolysis by adrenaline. Hearts were prelabeled with [^{3}H]inositol as described in Materials and Methods and then perfused with various concentrations of adrenaline in the absence (○) or presence (■) of 20-fold molar excess of DL-propranolol for 45 min. [^{3}H]inositol phosphates were isolated and data fitted to sigmoid curves. Each data point is the mean ± SEM of five to eight different experiments.

Cardiac cells from amphibian species have been widely used as model systems for the study of the electrophysiological properties of the heart, and the role of β- adrenergic receptors has been clearly described. However, few studies have been carried out on the presence and the functional role of α-adrenergic receptors. The participation of these receptors in the positive inotropic effect of catecholamines in the amphibian heart has been questioned (Morad et al., '78; Soustre and Rakotonirina, '81). In the frog heart, Chiu and Chu ('89) reported that at low temperatures a catecholamine effect was exerted by α-adrenoceptors with the β-adrenoceptor population being scarce or even absent at this temperature. In contrast, Herman et al. ('96) showed that α_1-, α_2-, and β-adrenergic receptors coexist in the heart of the American bullfrog *R. catesbeiana,* and they reported no change in β-adrenergic receptors in

TABLE 2. Stimulation of phosphoinositide hydrolysis by adrenaline in the perfused frog heart

Addition	pEC_{50},-log M	Extrapolated maximal response % basal
Adrenaline	4.51 ± 0.36	857
Adrenaline+ propranolol	4.66 ± 0.16	900

Note: Experiments were performed and data were fitted to sigmoid curves as described in Material and Methods. From sigmoid curves, extrapolated maximal rate of phospho[^{3}H]inositide (PI) hydrolysis was expressed relative to derived basal rate of hydrolysis data to give extrapolated maximal response. pEC_{50}, −log of half maximum effective concentration. When present, DL-propranolol was in 20-fold molar excess relative to adrenaline concentrations.

warm-acclimated frogs and in α-adrenergic receptors in cold-acclimated ones. However, there is no detailed characterization of α_1-adrenoceptors and their functional role in the heart of lower vertebrate species.

To reexamine the possibility that α_1-adrenergic receptors are actually present in the frog heart, binding studies were carried out using membranes prepared from heart. Saturation experiments using [^{3}H]prazosin and competition experiments using α_1-adrenoceptor agonists and selective antagonists were undertaken. Analysis of the data obtained in the present study suggested a single class of binding sites for [^{3}H]prazosin displaying a K_d of about 4.19 nM (Fig. 1). This value is in close agreement to that obtained in competition experiments where the potency of prazosin in inhibiting 50% of tracer binding was calculated to be about 6.19 nM (Fig. 2, Table 1). Since this is the first detailed study for the characterization of α_1-adrenergic receptors in the heart of a lower vertebrate species, these values can only be compared to those reported for mammalian heart. It can be noted that the affinity of α_1-adrenoceptors for prazosin is two orders of magnitude lower in the frog heart than in the rat heart, where K_d was reported to range from 25 to 170 pM (Hanft and Gross, '89; Lazou et al., '94; Seraskeris et al., 2001). In a study in the bullfrog heart ventricle and atrium, the authors were unable to determine the K_d because of the very low number of binding sites present (Herman et al., '96). As to the maximal binding density, the value obtained in this study, 14.66 fmol/mg original wet weight., markedly differs from that reported by Herman et al. ('96) in the American bullfrog heart. Apart from the fact that different experimental protocols have been followed to determine nonspecific binding in the two studies, these differences may reflect interspecies variability in cell surface α_1-adrenoceptor density.

A potential problem in the characterization of receptors in the frog is that the wide range of specific drugs routinely used for these purposes have been tested in mammalian tissues, whereas the stated specificity may be different when applied to lower vertebrate systems. In this regard, it is important to show that agonists alter some biological effect. Binding of agonists to α_1-adrenergic receptors has been related to stimulation of phosphoinositide turnover and subsequent increase in intracellular inositol phosphates and calcium levels (Zhong and Minneman, '99; Varma and Deng, 2000). Therefore, we studied the production of inositol phosphates stimulated by adrenaline in the perfused frog heart. Increasing concentrations of adrenaline resulted in a classic dose-response increase in phosphoinositide hydrolysis (Fig. 4). Maximal stimulation was about 8.5-fold over control, which is comparable to that observed in the rat heart (Table 2, Lazou et al., '94). However, the EC_{50} of 31 μM (Table 2) is much lower (about two orders of magnitude) than that reported for rat heart. The EC_{50} value for adrenaline was slightly decreased (21 μM) in the presence of propranolol. This is presumably partly attributable to inhibition of binding of adrenaline to β-adrenoceptors. The pEC_{50} value for the stimulation of PI hydrolysis by adrenaline correlates well with the corresponding pK_i value for this agonist in [^{3}H]prazosin binding competition assays (Tables 1 and 2). This supports close coupling between α_1-adrenoceptor occupation and phosphoinositide hydrolysis and suggests that the cellular action was mediated by the same receptor identified in the binding studies.

The fact that α_1-adrenoceptors are coupled to phosphoinositide hydrolysis in frog heart suggests that these receptors may participate in signaling events that determine cardiac physiology. It remains to be established whether these receptors contribute to signal transduction leading to cell growth and gene expression, as in other cell systems (Spector et al., '97; Lazou et al., '98; Williams et al., '98). If this also happens in the frog heart, apart from the evolutionary importance, this system may provide a useful tool for studying signaling events.

In summary, our data clearly indicate that α_1-adrenoceptors are present in frog cardiac cells and are coupled to phosphoinositide hydrolysis and the production of inositol phosphates, which is stimulated by adrenaline in the perfused heart.

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