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European Journal of Pharmacology 417 (2001) 27–35

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Angiotensin receptor in the heart of *Bothrops jararaca* snake

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Received 19 October 2000; received in revised form 26 February 2001; accepted 2 March 2001

Abstract

Angiotensin II interacts with specific cell surface angiotensin AT_1 and AT_2 receptors and, in some vertebrates, with an atypical angiotensin AT receptor. This study was designed to characterize the angiotensin receptor in the heart of *Bothrops jararaca* snake. A specific and saturable angiotensin II binding site was detected in cardiac membranes and yielded $K_d = 7.34 \pm 1.41$ nM and $B_{max} = 72.49 \pm 18$ fmol/mg protein. Competition-binding studies showed an angiotensin receptor with low affinity to both angiotensin receptor antagonists, losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) and PD123319 ((*s*)-1-(4-[dimethylamino]-3-methylphenyl)methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylate). Studies on the intracellular signaling pathways showed that phospholipase C/inositol phosphate breakdown and adenylylcyclase/cyclic AMP generation were not coupled with this angiotensin receptor. An adenylylcyclase enzyme sensitive to forskolin was detected. The results indicate the presence of an angiotensin receptor in the heart of *B. jararaca* snake pharmacologically distinct from angiotensin AT_1 and AT_2 receptors. It seems to belong to a new class of angiotensin receptors, like some other atypical angiotensin AT receptors that have already been described.

Keywords: Angiotensin receptor; Angiotensin AT_1 receptor-selective antagonist; Angiotensin AT_2 receptor-selective antagonist; Inositol phosphate; Adenylylcyclase forskolin-sensitive; Heart

1. Introduction

Angiotensin II, one of the main active peptides of the renin–angiotensin system, exerts its multiple functions on cardiovascular, endocrine and neuronal targets by interacting with specific cell surface and G protein-coupled receptors (Timmermans et al., 1993). Based on pharmacological (Chiu et al., 1989; Whitebread et al., 1989) and molecular studies (Sasaki et al., 1991; Murphy et al., 1991; Kambayashi et al., 1993; Mukoyama et al., 1993), at least two functionally distinct angiotensin receptors have been identified. They are distinguished by their differential sensitivity to two classes of compounds, typified by losartan (2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[(2′-(1*H*-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole) and PD123319 ((*s*)-1-(4-[dimethylamino]-3-methylphenyl) methyl-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1 *H*-imidazo[4,5-*c*]pyridine-6-carboxylate) or PD123177 (1-(4-amino-3-methylphenyl)-methyl-3-(diphenylacetyl)-4,5,6,7-tetrahydro-1 *H*-imidazo[4,5-*c*]pyridine-6-carboxylate), which are angiotensin AT_1 and AT_2 receptor-selective antagonists, respectively (Timmermans et al., 1993).

The majority of angiotensin II responses are attributed to activation of the angiotensin AT_1 receptor, which is widely distributed in the tissues. The angiotensin AT_1 receptor is coupled to several intracellular signaling pathways by activating phospholipase C, D and A_2, as well as voltage-dependent Ca^{2+} channels, and by causing adenylylcyclase inhibition (Catt et al., 1993; Griendling et al., 1997). Futhermore, it was also demonstrated that the angiotensin AT_1 receptor can also activate Tyr and Ser/Thr protein kinases (Duff et al., 1995; Berk and Corson, 1997; Sayeski et al., 1998).

Angiotensin AT_2 receptors, on the other hand, have a more restricted distribution, being found in the adrenal medulla, brain and reproductive tissues (Griendling et al., 1996). It is highly expressed in various fetal tissues and is up-regulated after vascular injury, myocardiac infarction and wound healing (Viswanathan and Saavedra, 1992; Nio et al., 1995). The intracellular signal transduction mecha-

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)00894-9

nism coupled to the angiotensin AT_2 receptor is not yet completely elucidated. It involves Tyr and Ser/Thr protein phosphatase activation (Kang et al., 1995; Yamada et al., 1996) and, as a consequence, mitogen-activated protein kinase (MAP kinase) inactivation, opening of delayed-rectifier K^+ channels (Kang et al., 1993), and closing of T-type Ca^{2+} channels (Buisson et al., 1995). Phospholipase A_2 signaling pathway activation has been also reported (Jaiswal et al., 1992).

The discovery of the selective non-peptide angiotensin antagonists was an important step not only to characterize and localize the angiotensin AT_1 and AT_2 receptors, but also to identify other angiotensin II binding sites that, pharmacologically, are not similar to angiotensin AT_1 or AT_2 receptors (Bergsma, 1994). These sites, present in different cells and tissues of several vertebrate species, have in common a low affinity to both losartan and PD123139/PD123177 (Sandberg et al., 1991; Aiyar et al., 1994; Chaki and Inagami, 1992a; Murphy et al., 1993; Smith et al., 1994; De Oliveira et al., 1995). They are coupled to phospholipase C/inositol phosphate breakdown (Murphy et al., 1993), and guanylate cyclase/cyclic GMP activation (Chaki and Inagami, 1992b).

The *Bothrops jararaca* snake is known to have all the components of the renin–angiotensin system (Lavras et al.,1978; Gervitz et al., 1987). The system plays a role in the cardiovascular and steroidogenic functions of this reptile (Breno and Picarelli, 1992; Lázari et al., 1994). However, differences in the potency of some angiotensin analogs were detected in the vasopressor effect and uterine muscle contraction (Breno and Picarelli, 1992; Lázari et al., 1994). Although the enzymes and substrates of the renin–angiotensin system have been characterized in *B. jararaca*, the specific angiotensin receptor type involved in the vasopressor response has not yet been reported upon. The present study was, therefore, undertaken in order to characterize the angiotensin receptor in the heart of *B. jararaca* snake, by using radioligand and functional studies with angiotensin receptor-nonselective and -selective antagonists.

2. Materials and methods

2.1. Animals

Adult male or female *B. jararaca* snakes weighing 130–300 g, were captured, classified by the Laboratório de Herpetologia, Instituto Butantan, and maintained for at least 15 days under controlled environmental conditions: 12-h light, 12-h dark lighting schedule, at 21–27°C and 65% relative humidity (Breno et al., 1990).

2.2. Membrane preparation

Snakes were decapitated, the heart was quickly removed, minced and homogenized in 25 mM Tris–HCl (pH 7.4) containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.25 M sucrose with an Ultra-Turrax homogenizer (T-25, IKA Labortechnik, Saufeni, Germany). The homogenate was centrifuged at $1000 \times g$ for 10 min. The supernatant was then centrifuged at $58{,}000 \times g$ for 30 min. The final pellet was resuspended in the binding buffer (25 mM Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), using a Dounce homogenizer and stored at −70°C. All procedures were carried out at 4°C, and all solutions contained freshly added 1 mM phenylmethylsulfonyl fluoride to inhibit proteolysis. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as a standard.

2.3. Membrane binding assay

Preliminary studies served to determine the appropriate incubation time, temperature and protein concentration of *B. jararaca* cardiac membrane. Membrane preparations from heart, with protein concentration ranging from 50 to 400 μg/0.5 ml of binding buffer 25 mM Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA and freshly added 1 mM phenylmethylsulfonyl fluoride and 100 μg/ml bacitracin, were used to determine appropriate conditions for [^{3}H]angiotensin II binding studies. [^{3}H]Angiotensin II specific binding in cardiac membrane was similar in the absence or presence of bacitracin. The time course study showed that specific binding reached a plateau by 1 h at 4°C as well as at 30°C. At 4°C, nonspecific binding was lower than at 30°C and it was about 30%, near the K_d value. According to these results, a membrane protein concentration of 200 μg/0.5 ml of binding buffer 25 mM Tris–HCl, pH 7.4, containing 5 mM $MgCl_2$, 1 mM EDTA and freshly added 1 mM phenylmethylsulfonyl fluoride was chosen and the binding studies carried out for 1 h at 4°C. We can assume that in these conditions of the assay, the ligand metabolism was inhibited.

2.3.1. Saturation-binding experiments

Cardiac membrane (200 μg/0.5 ml) was incubated with [^{3}H]angiotensin II ((5- L-isoleucine), [tyrosyl-3,5-^{3}H(N)] angiotensin II) (1–16 nM) (specific activity 30–60 Ci/mmol) in the absence (total binding) or presence of [Asp^1, Ile^5]angiotensin II (10^{-4} M) (nonspecific binding), for 1 h at 4°C. Specific binding was calculated as the difference between the total and the nonspecific binding. All experiments were performed in duplicate. After incubation, the reaction was stopped by rapid filtration through a glass fiber filter (GF/B) under vacuum, followed by three washes. The filters were partially dried under vacuum, placed in scintillation vials containing Aquasol II. The amount of radioactivity was determined in a scintillation β counter (LS 6000IC Beckman, Palo Alto, CA, USA).

2.3.2. Competition binding experiments

Cardiac membrane (200 μg/0.5 ml) was incubated with [^{3}H]angiotensin II (7 nM) in the absence and presence of increasing concentrations of unlabelled agonists ([Asp^1, Ile^5]angiotensin II; [Asp^1, Val^5]angiotensin II; [Asn^1, Val^5]angiotensin II) and antagonists ([Sar^1, Ile^8]angiotensin II; [Sar^1, Ala^8]angiotensin II; losartan; PD123319) for 1 h at 4°C. The binding reaction was stopped as described above.

2.3.3. Data analysis

Saturation and displacement binding data were analysed using a nonlinear least-square interative curve-fitting program GraphPad Prism (GraphPad PrismSoftware, San Diego, CA, USA). A mathematical model for one or two sites was applied. The equilibrium dissociation constant (K_d) and the maximum number of binding sites (B_{max}) were determined from a nonlinear regression plot (Bylund and Toews, 1993). The inhibition constant (K_i) was determined from displacement curves, using the equation of Cheng and Prusoff (1973). Angiotensin ll antagonist receptor affinity values were expressed as the negative logarithms of their respective K_i values (pK_i).

2.4. Measurement of total [^{3}H]inositol phosphate

The heart was removed, opened and incubated, under constant agitation, with nutrient solution of the following composition (mM): NaCl 147.17; KCl 4.95; $CaCl_2 \cdot 2H_2O$ 2.75; $MgSO_4 \cdot 7H_2O$ 1.21; $NaH_2PO_4 \cdot H_2O$ 1.2; $NaHCO_3$ 29.6; glucose 5.5 (pH 7.3–7.7) (Yamanouye et al., 1992), containing 5 μCi of *myo*-[1,2- ^{3}H]inositol for 80 min at 30°C, followed by addition of LiCl (10 mM). After 30 min, the tissues were incubated in the absence (basal level) or presence of angiotensin II or NaF plus $AlCl_3$, at the concentration shown for each specific experiment, and incubation was continued for 1 min. Tissues were washed three times with nutrient solution, transferred into 2 ml of methanol:chloroform (2:1 v/v) at 4°C and homogenized (Ultra-Turrrax T25 homogenizer). Chloroform (0.62 ml) and H_2O (0.93 ml) were added to the homogenate, followed by centrifugation (2000 × *g*, 10 min, 4°C) to separate the aqueous and organic phases (Fox et al., 1985).

Total [^{3}H]inositol phosphate was separated as previously described by Ascoli et al. (1989) with the following modification. Briefly, the aqueous layer was mixed with 1-ml anion-exchange resin (Dowex AG-X8, formate form, 200–400 mesh), allowed to equilibrate for 30 min at room temperature, and centrifuged (1000 × *g*, 5 min, 4°C). The resin was then washed, sequentially, with 4 ml of *myo*-inositol and 2 ml of 5 mM sodium tetraborate/60 mM sodium formate. Thereafter, 2 ml of 0.1 M formic acid/1 M ammonium formate was mixed with the resin and incubated for 30 min at room temperature. The total [^{3}H]inositol phosphate was eluted and placed in scintillation vials containing Insta-gel XF. The amount of radioactivity was determined in a scintillation β counter. Total [^{3}H]inositol phosphate was expressed as dpm/100 mg of tissue.

2.5. Measurement of intracellular cyclic AMP content

The heart was removed, opened and allowed to equilibrate for 10 min in nutrient solution at 30°C. A 3-isobutyl-1-methylxanthine (IBMX) (10^{-4} M) was then added for 10 min at 30°C, thereafter the incubation was continued for 10 min in the absence (basal level) or presence of [Asp^1, Ile^5]angiotensin II (10^{-6} and 10^{-4} M) or forskolin (10^{-5} M). In another series of experiments, the heart was also incubated with forskolin (10^{-5} M) plus [Asp^1, Ile^5]angiotensin II (10^{-4} M), under conditions similar to those described above. The reaction was stopped by freezing the tissues in liquid nitrogen, followed by homogenization (Ultra-Turrax T-25 homogenizer) in 2 ml of 3% perchloric acid. The homogenates were neutralized (pH 7) with 30% potassium bicarbonate and centrifuged at 10,000 × *g*, 20 min at 4°C. The intracellular cyclic AMP level was measured in the perchloric acid-soluble supernatant, by using a cyclic AMP [^{3}H] assay system kit, according to the manufacturer's instructions. The intracellular cyclic AMP levels were expressed as pmol/mg of protein.

2.6. Statistical analysis

The data are expressed as means ± S.E.M. Statistical analysis was carried out using an analysis of variance (ANOVA) followed by the Bonferroni test for multiple comparisons, or by the two-tailed Student's *t*-test to compare two values (Snedecor and Cochran, 1980). *P* values < 0.05 were accepted as significant.

2.7. Drugs and radiochemicals

[Asp^1, Ile^5]Angiotensin II, [Asp^1, Val^5]angiotensin II, [Asn^1, Val^5]angiotensin II, [Sar^1, Ala^8]angiotensin II, [Sar^1, Ile^8]angiotensin II, were purchased from Sigma (St. Louis, MO). Losartan and PD 123319 were a gift from DuPont Merck Pharmaceutical (Wilmington, DE) and Parke Davis Pharmaceutical Research Division (Ann Arbor, MI), respectively. (5-L-isoleucine), [tyrosyl-3,5- ^{3}H(*N*)] Angiotensin II (30–60 Ci/mmol) and *myo*-[1,2- ^{3}H]inositol (47.0 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Cyclic AMP [^{3}H] assay system kits were purchased from Amersham International (Little Chalfont, Buckinghamshire, UK). AG® 1-X8 (200–400 mesh) resin and Bio Rad protein assay were purchased from Bio Rad Laboratories (Richmond, CA). Insta-Gel XF was purchased from Packard (Meriden, CT). All chemicals not specified here were from Sigma or Merck (Darmstadt, Germany).

3. Results

3.1. [^{3}H]Angiotensin II binding in cardiac membranes

The binding of [^{3}H]angiotensin II for 1 h at 4°C to cardiac membranes was specific and saturable. A nonlinear regression analysis of specific binding fitted best a one-site model, suggesting the presence of a single class of high-affinity sites (Fig. 1). Analysis of three experiments performed in duplicate yielded a dissociation constant (K_d) of 7.34 ± 1.41 nM and binding capacity (B_{max}) of 72.49 ± 18 fmol/mg protein.

3.2. Displacement of [^{3}H]angiotensin II bound to cardiac membranes

The curves for displacement of [^{3}H]angiotensin II bound to cardiac membrane induced by angiotensin analogs, angiotensin receptor-nonselective and -selective antagonists are shown in Fig. 2. The angiotensin analogs completely displaced the [^{3}H]angiotensin II bound to cardiac membranes. Futhermore, [Asn^1, Val^5]angiotensin II was less potent than [Asp^1, Ile^5]angiotensin II and [Asp^1, Val^5]angiotensin II (Fig. 2A). Non-selective angiotensin receptor antagonists, [Sar^1, Ile^8]angiotensin II and [Sar^1, Ala^8]angiotensin II, also displaced the [^{3}H]angiotensin II bound to cardiac membranes (Fig. 2B). The displacement curves best fitted a one-site model. The angiotensin AT_1 and AT_2 receptor-selective antagonists, losartan and PD123319, failed to displace the [^{3}H]angiotensin II bound to cardiac membrane, even up to micromolar concentrations (Fig. 2C). The pK_i values for angiotensin analogs, angiotensin receptor-nonselective and -selective antagonists are summarized in Table 1. The order of potency was: [Asp^1, Ile^5]angiotensin II = [Asp^1, Val^5]angiotensin II = [Sar^1, Ile^8]angiotensin II > [Asn^1, Val^5]angiotensin II ≫ [Sar^1, Ala^8]angiotensin II ≫≫ losartan = PD123319.

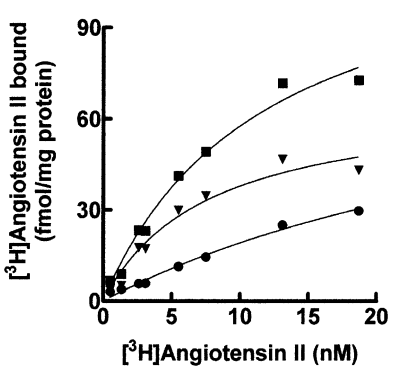

Fig. 1. Saturation curves of [^{3}H]angiotensin II binding to cardiac membrane of *B. jararaca* snake. [^{3}H]Angiotensin II (1–16 nM) was incubated with membranes (200 μg protein/0.5 ml) in the absence (total binding, ■), or presence of unlabeled [Asp^1, Ile^5]angiotensin II (10^{-4} M) (non-specific binding, ●) for 1 h at 4°C. Specific binding (▼) was calculated as the difference between the total and non-specific binding. Results are representative of three separate experiments performed in duplicate.

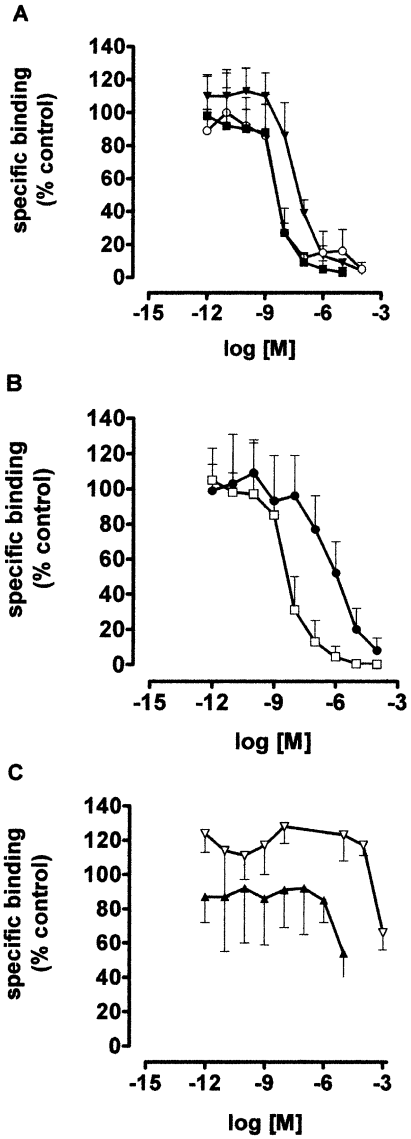

Fig. 2. Displacement curves [^{3}H]angiotensin II binding to cardiac membrane of *B. jararaca* snake, using angiotensin receptor analogs and antagonists. Membranes (200 μg protein/0.5 ml) were incubated with [^{3}H]angiotensin II (7 nM) in the absence or presence of increasing concentrations of unlabeled [Asp^1, Ile^5]angiotensin II (■), [Asp^1, Val^5]angiotensin II (○), [Asn^1, Val^5]angiotensin II (▼) (A), [Sar^1, Ala^8]angiotensin II (●), [Sar^1, Ile^8]angiotensin II (□) (B), losartan (▲) and PD123319 (▽) (C), for 1 h, at 4°C. The data are plotted as percentages of the binding in the absence of angiotensin receptor analogs and antagonists. Points and vertical lines represent the means ± S.E.M. of three to six experiments performed in duplicate.

Based on these data, the cardiac *B. jararaca* angiotensin receptor seems not to be similar to the angiotensin AT_1 and AT_2 receptors already described.

3.3. Effect of angiotensin on total [^{3}H]inositol phosphate accumulation

[Asp^1, Ile^5]Angiotensin II, at concentration of 10^{-8} M, was not able to modify the basal levels of total inositol

Table 1
pK_i values for angiotensin receptor analogs and antagonists in heart isolated from the *B. jararaca* snake
Data are means ± S.E.M. of number of experiments in parenthesis.

Agonists and antagonists	pK_i
[Asp[1], Ile[5]]Angiotensin II	8.66 ± 0.19 (5)
[Asp[1], Val[5]]Angiotensin II	8.66 ± 0.46 (4)
[Asn[1], Val[5]]Angiotensin II	7.65 ± 0.20 (4)
[Sar[1], Ile[8]]Angiotensin II	8.54 ± 0.22 (4)
[Sar[1], Ala[8]]Angiotensin II	6.28 ± 0.32 (6)
Losartan	> 4 (5)
PD123319	> 4 (3)

phosphate in the heart of *B. jararaca* (Fig. 3). In a further series of experiments that we carried out using a wide range of angiotensin concentrations (10^{-10}–10^{-4} M) and diferent incubation times (1–10 min), we could not reveal any increase in total inositol phosphate (data not shown), suggesting that the snake cardiac angiotensin receptor is not coupled to phospholipase C-mediated phosphoinositide hydrolysis.

To investigate the presence of a G protein associated to a phospholipase C/inositol phosphate breakdown pathway in cardiac tissue of the snake, we used NaF in the presence of $AlCl_3$. The fluoroaluminate compound (AlF^{-4}) produced has been described as an activator of the G protein. In fact, it mimics GTP action on the G protein and the Al^- is a cofactor, important for fluoroaluminate efficiency (Bigay et al., 1985; Zeng et al., 1989; Higashijima et al., 1991; Sondek et al., 1994). Fig. 3 shows that NaF in the presence of $AlCl_3$, at two concentrations, for 1 min of incubation, failed to produce any alteration of the basal levels of total inositol phosphate. Futhermore, the fluoroaluminate produced after 30 min of incubation with NaF

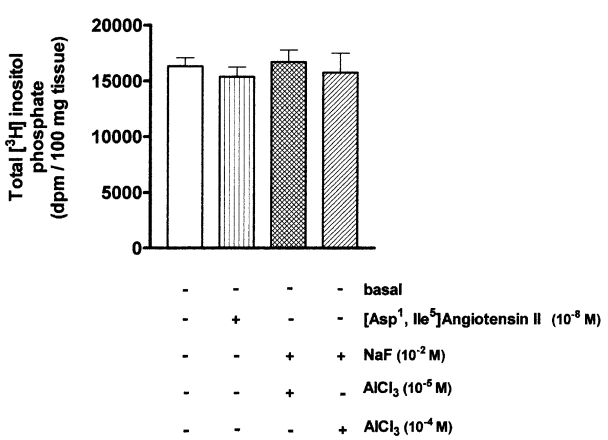

Fig. 3. Effect of [Asp[1], Ile[5]]angiotensin II and NaF plus $AlCl_3$ on total [^{3}H]inositol phosphate accumulation in heart of *B. jararaca* snake. Cardiac tissue was previously incubated with *myo*-[1–2-^{3}H]inositol (5 μCi) for 80 min at 30°C, followed by addition of LiCl (10^{-2} M). After 30 min, the tissue was incubated in the absence (basal level—open bar) or presence of [Asp[1], Ile[5]]angiotensin II (10^{-8} M) or NaF (10^{-2} M) plus $AlCl_3$ (10^{-5} and 10^{-4} M) for 1 min. Bars and vertical lines represent the means ± S.E.M. of 3 to 14 experiments.

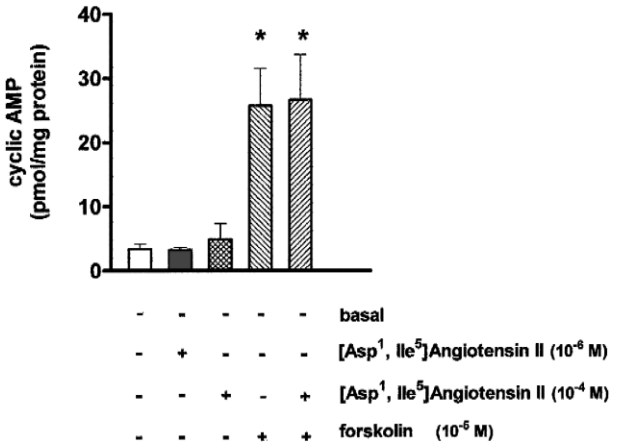

Fig. 4. Effect of [Asp[1], Ile[5]]angiotensin II on basal and forskolin-induced intracellular cyclic AMP accumulation in heart of *B. jararaca* snake. Cardiac tissue was previously incubated with IBMX (10^{-4} M) for 10 min at 30°C, thereafter the incubation was continued for 10 min in the absence (basal level—open bar) or presence of angiotensin II (10^{-6} and 10^{-4} M), forskolin (10^{-5} M) or forskolin (10^{-5} M) plus angiotensin II (10^{-4} M). Bars and vertical lines represent the means ± S.E.M. of two to four experiments. * Significantly different from basal level, $P < 0.05$.

plus $AlCl_3$ was also ineffective on total inositol phosphate accumulation (data not shown).

3.4. Effect of angiotensin on intracellular cyclic AMP accumulation

[Asp[1], Ile[5]]Angiotensin II, 10^{-6} and 10^{-4} M, did not interfere with basal levels of cyclic AMP in the heart of *B. jararaca* (Fig. 4). Forskolin produced an increase (sixfold) in intracellular cyclic AMP accumulation in cardiac tissue of *B. jararaca*. On the other hand, [Asp[1], Ile[5]]angiotensin II, 10^{-4} M, was not able to reduce the intracellular cyclic AMP accumulation induced by forskolin (Fig. 4). These results indicated that, in *B. jararaca* heart, the angiotensin II receptor is not coupled to the adenylylcyclase/cyclic AMP system.

4. Discussion

Angiotensin AT_1 and AT_2 receptors are present in the heart of several vertebrate species (Rogg et al., 1990; Baker et al., 1992; Sechi et al., 1992; Scott et al., 1992), the angiotensin AT_1 receptor being responsible for the angiotensin II-positive chronotropic and inotropic effects. Apart from mechanical effects on the heart, the angiotensin AT_1 receptor also exerts a role in ventricular hypertrophy, a cardiac growth process associated with hypertension (Kim and Iwao, 2000), whereas an opposed role is attributed to the angiotensin AT_2 receptor, because of its antiproliferative actions (Inagami et al., 1999).

Angiotensin II causes a dose-dependent vasopressor effect in the *B. jararaca* cardiovascular system, due in part

to catecholamine release (Breno and Picarelli, 1992). Although direct and indirect angiotensin cardiovascular actions have already been described in this snake, the type of angiotensin receptor responsible for these effects has not been reported.

In the present study, the angiotensin receptor was characterized in the heart of the snake *B. jararaca*. Radioligand studies showed that [^{3}H]angiotensin II binds to one class of specific binding sites in heart membranes. This finding is in agreement with results of previous studies with vascular and cardiac systems of mammals, showing one population of binding sites with dissociation constant in the nanomolar range (Baker et al., 1984; Delisée et al., 1993; Fujimoto et al., 1992; Grover et al., 1994). Aiyar et al. (1994), using the membrane from *Xenopus laevis* heart, detected one binding site for angiotensin with a K_d of 0.104 ± 0.002 nM. Conversely, studies with amphibian *X. laevis* heart, reported two populations of angiotensin binding sities, giving a high-affinity site with a K_d of 1.6 ± 0.18 nM and a low-affinity site with a K_d of 22 ± 2.5 nM (Sandberg et al., 1991).

In order to characterize the type of angiotensin receptor present in the heart of *B. jararaca* snake, several angiotensin receptor agonists and antagonists were examined for their ability to compete with [^{3}H]angiotensin II for its binding sites. The angiotensin analogs and the angiotensin receptor-nonselective antagonists, produced monophasic displacement curves consistent with the labelling of an apparently homogenous population of angiotensin binding sites.

The angiotensin analog, [Asn^1, Val^5]angiotensin II, showed less affinity than [Asp^1, Ile^5]angiotensin II and [Asp^1, Val^5]angiotensin II at angiotensin binding sites. This is in agreement with previously data for blood pressure and uterine muscle contraction of *B. jararaca* snake (Breno and Picarelli, 1992; Lázari et al., 1994), rabbit aorta (Helmer, 1964) and fowl blood pressure (Nishimura et al., 1992). Futhermore, the endogenous angiotensin peptides present in the *B. jararaca* snake are [Asp^1, Ile^5]angiotensin II and [Asp^1, Val^5, Tyr^9]angiotensin I (Borgheresi et al., 1996). Thus, [Asn^1, Val^5]angiotensin II may have a spatial arrangement less efficient for causing *B. jararaca* angiotensin receptor activation.

The affinities of the angiotensin receptor-nonselective antagonists, [Sar^1, Ile^8]angiotensin II and [Sar^1, Ala^8]angiotensin II, are similar to those of mammalian angiotensin binding sites (Dudley et al., 1990; Whitebread et al., 1989; Murphy et al., 1992). However, [Sar^1, Ile^8]angiotensin II was more potent than [Sar^1, Ala^8]angiotensin II at the angiotensin binding sites of *B. jararaca* heart. Similar properties for their differential affinities were also reported for the turkey adrenal and amphibian myocardial-cloned angiotensin II receptor (Murphy et al., 1993; Sandberg et al., 1991).

In the cardiac membrane of *B. jararaca*, the angiotensin receptors have very low affinity for both losartan (angiotensin AT_1 receptor-selective antagonist) and PD123319 (angiotensin AT_2 receptor-selective antagonist). The affinity was, however, similar to that reported for *X. laevis* cardiac angiotensin receptor (Sandberg et al., 1991). This contrasts with the comparatively higher affinity for losartan found at the cloned ferret-angiotensin-AT_1 receptor (Gosselin et al., 2000), as well as losartan and CGP42112A (nicotinic acid-Tyr-(*N*-benzoylcarbonyl-Arg)-Lys-His-Pro-Ile-OH) affinities found at the cloned angiotensin receptor of turkey adrenal (Murphy et al., 1993). Therefore, the angiotensin II receptor present in the *B. jararaca* heart is not similar to those described for mammalian angiotensin AT_1 or AT_2 receptors.

Phospholipase C/phospholipid breakdown is one of the main intracellular signaling pathways used by angiotensin AT_1 receptors. Angiotensin, at different concentrations and incubation times was not able to induce a significant change in the total inositol phosphate level of *B. jararaca* heart. These data suggest that the angiotensin receptor in this organ is not coupled to phospholipase C-mediated phosphoinositide hydrolysis. However, in chick cardiomyocytes (Baker et al., 1989) and in fish hepatocytes (Olivares-Reyes et al., 1997), where a losartan-insensitive angiotensin receptor is also present, the angiotensin receptors are coupled to the phospholipase C signal pathway.

Sodium fluoride, in the presence of $AlCl_3$, produces the fluoroaluminate compound that activates members of the heterotrimeric G protein ($G_{\alpha\beta\gamma}$) family by binding to the inactive $G_\alpha \cdot GDP$, near the site occupied by the γ-phosphate in $G_\alpha \cdot GTP$ (Higashijima et al., 1991; Sondek et al., 1994). The fluoroaluminate produced inositol 1,4,5 triphosphate accumulation and calcium mobilization in ovine endometrium (Graf et al., 1998), *X. laevis* oocyte (Moon et al., 1997), rabbit femoral artery (Ratz and Blackmore, 1990), rat caudal artery (Zeng et al., 1989) and guinea pig myometrium (Marc et al., 1988). Therefore, we examined the ability of fluoroaluminate to enhance the accumulation of total inositol phosphate in *B. jararaca* heart. Fluoroaluminate did not induce a stimulatory effect on total inositol phosphate accumulation in the *B. jararaca* heart after either 1 or 30 min of incubation. The increase of $AlCl_3$ concentration (100 μM), as reported by Marc et al. (1988) was also ineffective on total inositol phosphate accumulation. Recent results from our laboratory have shown that carbachol induces a concentration-dependent rise in the accumulation of total [^{3}H]inositol phosphate in myometrium from estrogen-treated rats (Abdalla et al., 2000). In the heart of several species, muscarinic acetylcholine receptors stimulation by high concentrations of acetylcholine or carbachol (usually $> 10^{-5}$ M) causes a positive inotropic effect. It has been speculated that this effect is linked to the phospholipase C/inositol–triphosphate/diacylglycerol pathway (for review see Caulfield, 1993; Mery et al., 1997; Giessler et al., 1999). We also used carbachol to increase the intracellular total inositol phosphate content in the heart of *B. jararaca*. Carbachol

(10^{-4} M) did not change the basal level of total [^{3}H]inositol phosphate in this organ (data not shown). Thus, all these data provide indirect evidence that this G protein present in *B. jararaca* heart is not structurally similar to the well-characterized G proteins.

Another transducer mechanism coupled to the angiotensin AT_1 receptor, although little involved, is the adenylylcyclase/cyclic AMP pathway. [Asp^1, Ile^5]Angiotensin II did not induce either a stimulatory or inhibitory response in the snake heart, suggesting the presence of an angiotensin receptor not associated to the adenylylcyclase/cyclic AMP pathway. However, our data showed an adenylylcyclase in *B. jararaca* heart similar to that present in mammals, since forskolin induced an increase in the cyclic AMP generation.

In conclusion, the present results provide evidence that the angiotensin receptor in the cardiac tissue of *B. jararaca* snake is pharmacologically distinct from the angiotensin AT_1 and AT_2 receptors. It seems to belong to a new class of angiotensin receptors, like some other atypical angiotensin receptors already described. The understanding of pharmacological differences in the action of non-peptide antagonists in different animal species gives us an opportunity to identify determinants of the binding and to develop new therapeutic tools.

Acknowledgements

The authors are grateful to Espedita M. Jesus, Maria Damiana da Silva (Universidade Federal de São Paulo) and to Lúcia Manoel Chagas, Lindonéia dos Santos, Maria Zelma da Silva, Wanda R. Carrela da Silva (Instituto Butantan) for the technical assistance. We also want to thank DuPont Merck Pharmaceutical and Parke Davis Pharmaceutical Research Division for the gift of the angiotensin AT_1 receptor-selective antagonist and AT_2 receptor-selective antagonist, respectively. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Brazil) (Doctoral fellowship to M.C.B. and Researcher fellowship to C.S.P.). This work was presented in part at the XIIIth International Congress of Pharmacology, München, Germany, 1998.

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