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Ca^{2+} Mobilization in Adult Rat Cardiomyocytes by Angiotensin Type 1 and 2 Receptors

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ABSTRACT. The role of angiotensin II (AngII) in the regulation of heart function under normal and pathological conditions has been well documented. Although two types of AngII receptors (AT_1 and AT_2 receptors) are found in equal proportions in the rat heart, most studies have focused primarily on AT_1 receptor-coupled events. In this study, the contribution of both types of AngII receptors to cardiac function was evaluated by measuring intracellular calcium ($[Ca^{2+}]_i$) levels at ambient temperature in freshly isolated adult rat ventricular cardiomyocytes. Exposure of cardiomyocytes to AngII (0.01 to 10 μM) resulted in an immediate and sustained increase in $[Ca^{2+}]_i$ in a concentration-dependent manner. The increase in $[Ca^{2+}]_i$ in cardiomyocytes by AngII was blocked by either losartan or compound PD123319 (1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid), non-peptide antagonists of the AT_1 and AT_2 receptors, respectively. The specificity of the action of these antagonists was verified by their inability to alter the basal levels of $[Ca^{2+}]_i$ as well as KCl- or ATP-induced increases in $[Ca^{2+}]_i$. AngII was also observed to initiate spontaneous beating activity in cardiomyocytes, which was prevented by both losartan and compound PD123319 in a concentration-dependent manner (0.01 to 10 μM). These data indicate that the activation of both AT_1 and AT_2 receptors may stimulate a signalling pathway that influences $[Ca^{2+}]_i$ and spontaneous beating activity in cardiomyocytes. BIOCHEM PHARMACOL **55**;9:1413–1418, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. angiotensin II; Fura-2/AM; intracellular calcium; losartan; PD123319; cardiomyocytes

AngII†, which is primarily considered to be a hemodynamic regulator, is also known to modulate cardiac function via two distinct mechanisms. The rapid action of AngII relates to changes in cardiomyocyte contractile activity involving both the inotropic and chronotropic properties of the heart [1, 2]. In addition, AngII acts as a cardiac growth factor [3] and contributes to the adaptive cardiac hypertrophy over a prolonged period in response to some pathological disease conditions [2, 4]. Thus, AngII can be seen to influence heart function on both a short- and a long-term basis by acting directly on the myocardium. Pharmacological studies have identified at least two distinct subtypes of AngII receptors, designated as AT_1 and AT_2 receptors [5]. The development of specific non-peptide receptor antagonists has been key to the pharmacological definition of these receptor subtypes as well as the study of their relative contribution to the effects of AngII. The best characterized receptor antagonists are losartan and compound PD123319 (1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid), which are specific to the AT_1 and AT_2 receptors, respectively [5]. Previous studies have shown that AT_1 receptor antagonists block both the contractile [6, 7] and mitogenic [3, 8, 9] actions of AngII in cardiac tissue. It has been observed, however, that rat cardiomyocytes express both the AT_1 and AT_2 receptor subtypes in equal proportions [10], and that AT_2 receptor expression in cardiac tissues is increased by cardiac ischemia [11], ischemia–reperfusion [12], heart failure and myocardial infarction [13]. The abundance of the AT_2 receptors and their regulation in response to some specific pathophysiologic conditions suggest that the AT_2 receptors, like the AT_1 receptors, may influence cardiac function.

It is now known that Ca^{2+} is an important signalling cation in the pathways that mediate the pressor and mitogenic actions of AngII [14, 15]. The inotropic and chronotropic effects of AngII have been shown to be dependent on changes in Ca^{2+} movements in the myocardium [16]. Exposure of neonatal cultured cardiomyocytes to AngII has been shown to produce an inward flux of Ca^{2+}, resulting in both transient and sustained rises in the cytosolic free Ca^{2+} [16, 17]. Although the AT_1 receptors have been linked to these effects [3, 9], very few studies have carried out a parallel evaluation of the effects of the

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† *Abbreviations:* AngII, angiotensin II; AT_1 and AT_2, angiotensin II type-1 and type-2 receptors; $[Ca^{2+}]_i$, intracellular calcium; Fura-2/AM, Fura-2/acetomethyl ester; IP_3, inositol 1,4,5-trisphosphate; and K-H, Krebs–Henseleit.

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AT_2 receptor antagonist on cardiomyocytes. Based on evidence that a link between the AT_2 receptors and Ca^{2+} exists in rat aortic smooth muscle cells [18] and NG108-15 neuronal cells [19], this investigation was designed to determine whether the AT_2 receptor also influenced Ca^{2+} movement in the heart. Since Feolde *et al.* [6] have demonstrated that AT_2 receptors are rapidly down-regulated in cultured cardiomyocytes, we examined the effects of this peptide on Ca^{2+} mobilization in freshly isolated adult rat cardiomyocytes by using the Fura-2/AM technique to monitor changes in $[Ca^{2+}]_i$. This method [20, 21] allows the assessment of the effects of AngII on cardiomyocytes that are independent of both changes in hemodynamics and the impact of nonmyocyte cells. The role of both the AT_1 and AT_2 receptor subtypes in AngII-dependent stimulation of $[Ca^{2+}]_i$ mobilization was evaluated in this study by the use of the nonpeptide receptor antagonists losartan and compound PD123319 [5].

MATERIALS AND METHODS

Materials

AngII and BSA were purchased from the Sigma Chemical Co. Joklik medium was from Gibco-BRL, collagenase (CLS2) was obtained from the Worthington Biochemical Co., and Fura-2/AM was supplied by Molecular Probes, Inc. The angiotensin receptor antagonists losartan and compound PD123319 were provided by DuPont–Merck and Parke–Davis, respectively.

Isolation of Adult Rat Cardiomyocytes

Cardiomyocytes were isolated from male Sprague–Dawley rats (300–350 g) by collagenase digestion of the ventricular tissue according to a modification [21, 22] of the method described by Powell and Twist [23]. Briefly, the heart was removed from rats after an injection of heparin sodium (1000 U/100 g), xylazine (10 mg/kg), and ketamine (60 mg/kg), and immediately immersed in ice-cold (Ca^{2+}-free) buffer (pH 7.4) containing 90 mM of NaCl, 10 mM of KCl, 1.2 mM of KH_2PO_4, 5 mM of $MgSO_4 \cdot 7H_2O$, 15 mM of $NaHCO_3$, 30 mM of taurine, and 10 mM of glucose. This medium was oxygenated with 95% O_2/5% CO_2 for at least 30 min. The heart was cannulated on a Langendorff apparatus via the aorta, perfused with the Ca^{2+}-free buffer in a non-circulating manner (5–10 min), and subsequently perfused with recirculating Ca^{2+}-free buffer supplemented with 0.1% collagenase, 0.1% BSA, and 50 μM of $CaCl_2$ (30 min). Following removal of the atria, the ventricles were subjected to additional digestion in collagenase solution containing 1% BSA with agitation at 37°. The cell suspension was collected every 10 min, and the first 3–4 harvests were pooled. The total cell pellet was restored gradually to a final Ca^{2+} concentration of 1 mM by 250 μM, 500 μM, 750 μM, and 1 mM in 15-min intervals. The assessment of cell viability based on the morphology revealed that 80–90% of these cardiomyocytes were rod-shaped. The cellular viability of the cardiomyocytes was not affected upon exposure to 10^{-5} M AngII, losartan, or compound PD123319.

Measurement of Intracellular Free Calcium

Freshly isolated adult rat cardiomyocytes were incubated with 5 μM of Fura-2/AM in K-H buffer (pH 7.4) containing 120 mM of NaCl, 4.74 mM of KCl, 1.2 mM of KH_2PO_4, 1.2 mM of $MgSO_4 \cdot 7H_2O$, 25 mM of $NaHCO_3$, 1 mM of $CaCl_2$, 10 mM of glucose, and 1% BSA that had been oxygenated previously for 40 min at 37° [21, 22]. The cells were washed six times with BSA-free K-H buffer to remove the BSA, and the cell concentration was adjusted to 5×10^5 cells/mL. The removal of BSA from the cardiomyocyte preparation was necessary to demonstrate AngII-induced changes in $[Ca^{2+}]_i$ as previously noted [20]. Fluorescent signals from 1.7 mL of suspended cells were measured at room temperature with an SLM DMX-1100 dual-wavelength spectrofluorometer at 340- and 380-nm excitation and 510-nm emission wavelengths [20, 21]. $[Ca^{2+}]_i$ was calculated according to the equation of Grynkiewicz *et al.* [24]. R_{max} and R_{min} values were determined by inclusion of 20 μL of Triton X-100 (0.01% final concentration) and 40 μL of EGTA (10-mM final concentration), respectively. The resting $[Ca^{2+}]_i$ levels and the maximal increase evoked by agonists were calculated according to the formula $[Ca^{2+}]_i = 224 \times R - R_{min}/R_{max} - R \times Sf2/Sb2$, where R is the ratio of fluorescence intensities at excitation wavelengths 340 and 380 nm; and Sf2 and Sb2 are the fluorescence proportionality coefficients obtained at 380 nm (excitation wavelength) under R_{min} and R_{max} conditions, respectively. AngII (0.01 to 10 μM) was added directly to cells in the fluorometer after recording the basal values. Receptor antagonists losartan and compound PD123319 were preincubated for 5–10 min prior to exposure of cardiomyocytes to AngII.

Measurement of Myocyte Contractility

Cardiomyocytes (1×10^4 cells/10 μL) were examined visually at 10× magnification. No spontaneous beating of cells prior to AngII addition was observed. AngII was added to the cells with minimal disturbance, and after 1 min of stimulation the number of beating cells in one field was measured over 1 min. The average of at least 3–5 fields was used to assess the efficacy of each treatment. A 5-min pretreatment of cardiomyocytes with losartan or compound PD123319 prior to AngII addition was used to assess the contribution of the AT_1 and AT_2 receptors. All experiments were conducted at room temperature.

Statistical Evaluation

Student's *t*-test was used to evaluate differences in each group; $P < 0.05$ was considered statistically significant.

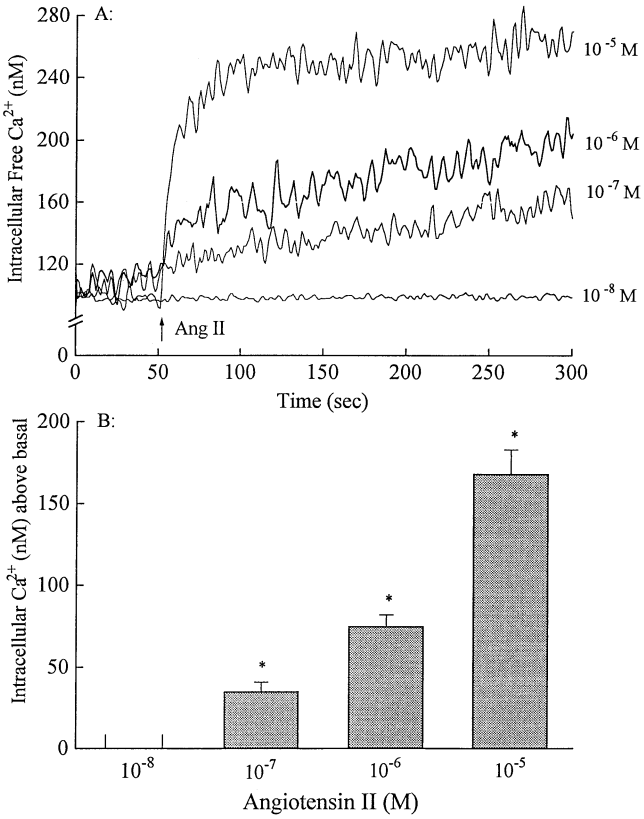

FIG. 1. Effect of AngII on intracellular free calcium in adult rat cardiomyocytes. (A) Typical tracings of changes in intracellular free calcium due to AngII are shown. Various concentrations were added directly to the cuvette once the baseline value was established. (B) Absolute changes in $[Ca^{2+}]_i$ are presented as means ± SEM of five experiments. *$P < 0.05$, compared with basal level. All measurements were conducted at room temperature.

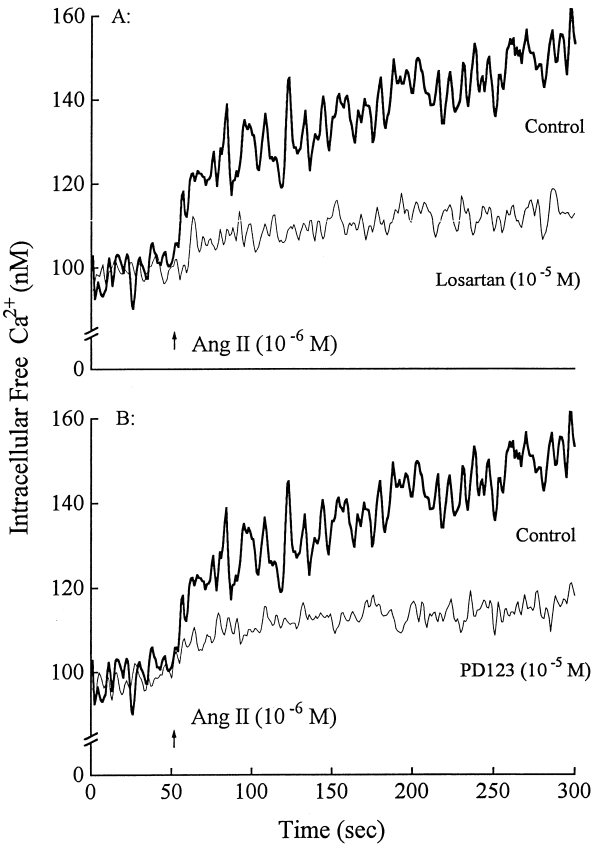

FIG. 2. Effect of angiotensin receptor antagonists on the change in intracellular free calcium induced by AngII. Losartan (10^{-5} M, panel A) and compound PD123319 (10^{-5} M, panel B) were incubated individually with cardiomyocytes for 5–10 min at room temperature prior to the addition of AngII (10^{-6} M). Similar results were observed in three independent experiments.

RESULTS AND DISCUSSION

The action of AngII (0.01 to 10 μM) on freshly dissociated adult rat cardiomyocytes was evaluated in this study, and the data are shown in Fig. 1. AngII produced an increase in $[Ca^{2+}]_i$, as measured by Fura-2/AM fluorescence spectroscopy, that remained elevated for more than 5 min after AngII addition (Fig. 1A). The peak fluorescence intensity measured in these experiments was dependent upon the concentration of AngII; 0.1 μM was the lowest effective concentration that was capable of increasing $[Ca^{2+}]_i$ (Fig. 1). It was noted, however, that the increase in free $[Ca^{2+}]_i$ was sustained for at least 10 min at all AngII concentrations examined (data not shown).

To establish the receptor subtypes involved in AngII-induced mobilization of Ca^{2+}, the ability of non-peptide receptor antagonists to block the effects of AngII was measured. An AngII concentration of 1 μM was used in these experiments. The necessity for this high concentration is related presumably to the requirement for BSA, which interferes with AngII in this system [20], during the cardiomyocyte isolation procedure. Cardiomyocytes were preincubated with various concentrations of receptor antagonist for 5–10 min prior to the addition of AngII. As shown in Fig. 2, losartan (10 μM), the AT_1 receptor antagonist, and compound PD123319 (10 μM), the AT_2 receptor antagonist, blocked the increase in $[Ca^{2+}]_i$ due to AngII (1 μM). Although both receptor antagonists reduced the fluorescence intensity produced by AngII (Fig. 2, A and B), neither affected $[Ca^{2+}]_i$ in the absence of agonist (data not shown). It was also determined that the inhibitory effects of both losartan and compound PD123319 were concentration dependent (Fig. 3). The requirement for elevated antagonist concentrations is likely a consequence of the AngII concentrations used in this experiment. To verify the specificity of the effects of these antagonists, their ability to inhibit the change in $[Ca^{2+}]_i$ produced by exposure to KCl or ATP was evaluated. Neither losartan nor compound PD123319 was effective in blocking the increase in $[Ca^{2+}]_i$ induced by 30 mM of KCl (data not shown). Similarly, the receptor-dependent increase in $[Ca^{2+}]_i$ produced by ATP was unaffected by either losartan or PD123319 (data not shown). A detailed summary of these data regarding the action of AngII receptor antagonists on AngII- or KCl-induced changes in $[Ca^{2+}]_i$ is presented in Table 1. The results obtained in this study indicate that the inhibition of $[Ca^{2+}]_i$ observed with

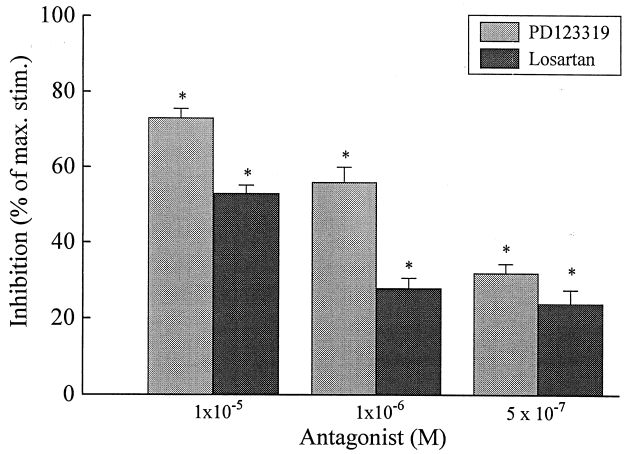

FIG. 3. Concentration-dependent effects of angiotensin receptor antagonists on intracellular free calcium in rat cardiomyocytes. Various concentrations of losartan or compound PD123319 (5×10^{-7} to 1×10^{-5} M) were incubated with cardiomyocytes for 5–10 min at room temperature. Angiotensin II (AngII) was subsequently added to the cuvette after basal values were determined. Maximum stimulation was set as the value with AngII (10^{-6} M) without antagonist present. The data are expressed as means ± SEM of five experiments. The absolute value for basal $[Ca^{2+}]_i$ was 92 nM, and the absolute value for $[Ca^{2+}]_i$ at maximal stimulation was 148 μM. *$P < 0.05$, compared with control values.

losartan and compound PD123319 was specific for AngII and that neither antagonist alone was capable of influencing $[Ca^{2+}]_i$, indicating that the action of these antagonists may be specific in nature.

To correlate the effects of AngII and the receptor antagonists on $[Ca^{2+}]_i$ with functional parameters in cardiomyocytes, we examined the actions of these agents on AngII-induced beating of cardiomyocytes. It should be noted that the cardiomyocytes used in this study do not contract spontaneously. Therefore, the effect of AngII on the total number of beating cells observed in an average microscopic field could be evaluated visually. The addition of AngII induced a rhythmic contraction of the cardiomyocytes; the increase in the number of contracting cells was

TABLE 1. Intracellular Ca^{2+} increases stimulated by KCl, ATP, and AngII in cardiac myocytes of rats

	Peak increase (% of maximum response)		
	KCl (30 mM)	ATP (50 μM)	AngII (10^{-6} M)
Control	100 ± 8	81 ± 4	58 ± 5
Losartan (10^{-5} M)	119 ± 13	79 ± 12	12 ± 0.6*
PD123319 (10^{-5} M)	96 ± 6	73 ± 8	20 ± 4*

Values are means ± SEM of five experiments. Losartan and PD123319 were added to the cuvette and measured for 10 min before KCl, ATP, or AngII was added directly to the cuvette. Basal intracellular Ca^{2+} concentrations were 104–110 ± 5–7 nM in different groups. Maximum response was defined as the increase in $[Ca^{2+}]_i$ over basal obtained with 30 mM of KCl. Maximal $[Ca^{2+}]_i$ in the presence of 30 mM of KCl was 155–163 ± 6–10 nM.

*$P < 0.05$, compared with control.

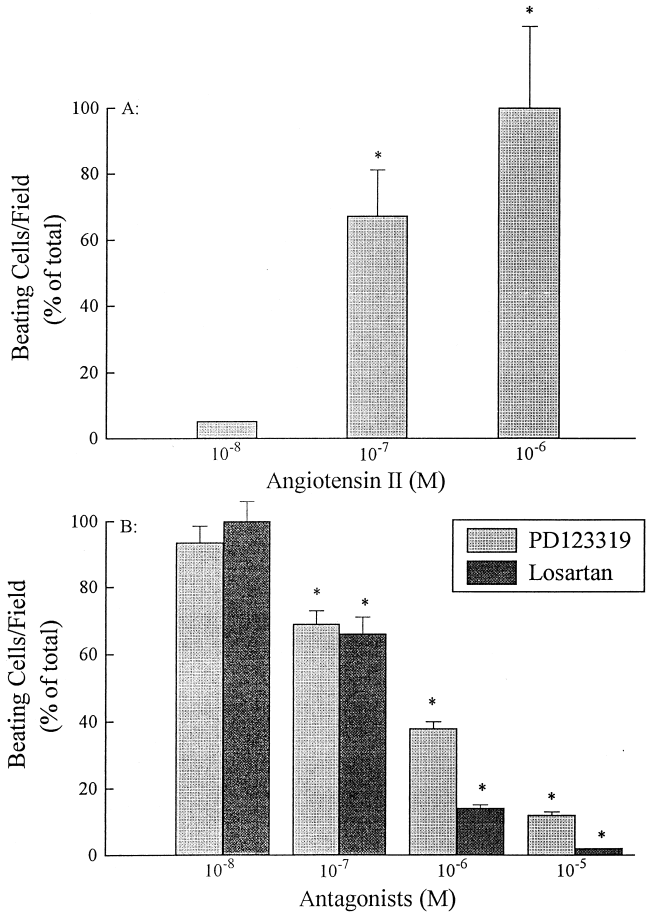

FIG. 4. Effect of AngII on adult rat cardiomyocyte function. The total number of beats/field of freshly dispersed cardiomyocytes in the presence of AngII was monitored by light microscopy at room temperature. (A) Concentration-dependent effect of AngII on the total number of beats per field. (B) Influence of the receptor antagonists losartan and compound PD123319 (10^{-8}–10^{-5} M) on the total number of beats in the presence of AngII (10^{-6} M). Approximately 350 cells were visible in each field. *$P < 0.05$, compared with maximum stimulation values by AngII. The proportion of rod-shaped cells was not altered over the course of the experiment (max 15 min).

dependent upon AngII concentration (0.01 to 1 μM) (Fig. 4A). Pretreatment of cardiomyocytes with either losartan or compound PD123319 (0.01 to 10 μM) blocked AngII-induced cell contraction in a concentration-dependent manner (Fig. 4B). These data indicate that both angiotensin receptor subtypes are required for inducing spontaneous beating of cardiomyocytes by AngII.

In this study, exposure of adult cardiomyocytes to AngII resulted in a concentration-dependent increase in $[Ca^{2+}]_i$ that was sustained for at least 10 min. Such an increase in $[Ca^{2+}]_i$ can be seen to augment the contractile force development by the heart upon exposure to AngII. Although previous studies have suggested that AngII exerts its contractile effects via modulation of basal free Ca^{2+} concentration rather than the sensitivity of the contractile apparatus to Ca^{2+} [25, 26], a recent study by Lefroy *et al.* [27] concluded that AngII does not stimulate contraction

in isolated rat cardiomyocytes. However, it should be emphasized that the inability of these investigators [27] to observe the effect of AngII on contractile force development may be due to the presence of BSA in the incubation medium because this protein has been reported recently to interfere with the action of AngII [20]. Alternatively, the loss of AngII receptors during the preparation of cardiomyocytes employed by Lefroy *et al.* [27] cannot be ruled out. Nonetheless, the results reported here indicate that the addition of AngII to these adult cardiomyocytes, which do not contract spontaneously, stimulated rhythmical beating of the cells in a concentration-dependent manner. Whether the induction of spontaneous beating of cardiomyocytes by AngII is related to the increase in $[Ca^{2+}]_i$ or vice versa cannot be determined on the basis of the information available in the literature.

Although angiotensin receptor density is higher in neonatal hearts, both the AT_1 and AT_2 receptor subtypes are present in nearly equal amounts in the hearts of fetal, neonatal, and adult Sprague–Dawley rats [10]; however, most of the actions of AngII have been attributed to AT_1 receptor activation. Since the AT_2 receptor has been linked to both apoptosis [28] and cardioprotection after ischemia–reperfusion [12], its abundance in the heart suggests it may have some role in normal cardiac development and function. The novel finding presented in this report is that the AT_2 receptors, in addition to the AT_1 receptors, are required for the AngII-dependent increase in $[Ca^{2+}]_i$. In support of this finding, a similar requirement for activation of both the AT_1 and AT_2 receptors for sustained $[Ca^{2+}]_i$ increases was reported recently in rat aortic smooth muscle cells [17] and NG108-15 neuronal cells [19]. The discrepancy with other reports, which observed that only the AT_1 receptor mediated changes in $[Ca^{2+}]_i$, may be attributed to differences in the experimental systems. Many of the earlier studies were conducted with neonatal cardiomyocytes [3, 6, 16], which are spontaneously contractile due to the leakiness of their plasma membrane to extracellular Ca^{2+}. Similarly, previous studies that utilized cultured cardiomyocytes may have been misleading since the AT_2 receptors have been reported to be rapidly lost in cardiomyocyte culture [6], and the use of freshly isolated cardiomyocytes as we have done circumvents this problem. The involvement of both AT_1 and AT_2 receptors in AngII-mediated $[Ca^{2+}]_i$ increases becomes particularly relevant since antagonists to both receptor subtypes also blocked the initiation of spontaneous beating by AngII in these cardiomyocytes. This supports speculation that the binding to both AT_1 and AT_2 receptors is necessary to activate a signal transduction system for increasing $[Ca^{2+}]_i$ as well as inducing the rhythmic contraction of cardiomyocytes. Further experimentation, however, will be necessary to determine if these receptors operate through distinct, but convergent, pathways, or influence separate processes in the same pathway. Additionally, alternative techniques will be required since the Fura-2/AM method can distinguish neither the source of the Ca^{2+} nor its subcellular location (i.e. cytosolic vs nuclear).

The signal transduction pathways connecting AT_1 receptor activation to Ca^{2+} mobilization are well-characterized and involve G-protein-linked activation of phospholipase C and the production of IP_3 [29]. A similar understanding of AT_2 receptor-associated signalling systems, however, remains elusive. While the AT_2 receptor has been identified recently as a member of the seven transmembrane domain receptor superfamily, its association with G-protein signal pathways is controversial. Recently, an uncharacterized G-protein was shown to couple the AT_2 receptor pathways in NG108-15 cells to both protein tyrosine phosphatase and Ca^{2+} current activities [19]. In addition, the production of arachidonic acid by the AT_2-dependent activation of phospholipase A_2 has been shown to result in the stimulation of both K^+ and Ca^{2+} channels in neonatal rat ventricular cardiomyocyte cultures [30], distinct from those coupled to the AT_1 receptor. Thus, these data support the premise that the AT_2 receptor promotes Ca^{2+} release in cardiomyocytes that is different from the production of IP_3 by AT_1-linked signal transduction mechanisms.

In addition to the role of Ca^{2+} as a signalling component of the contractile pathway, evidence is accumulating that demonstrates that Ca^{2+} plays an important role in regulating cell proliferation. For instance, the activation of protein kinase C by Ca^{2+} stimulates the expression of early response genes and increases protein synthesis in neonatal cardiomyocytes [3, 31]. Likewise, AngII elicited a sustained increase in smooth muscle cell $[Ca^{2+}]_i$ [16], similar to the response that provides Ca^{2+} for crucial stages of the cell cycle following growth factor stimulation [31]. Since AngII acts as a hypertrophic agent for cardiomyocytes as well as a mediator of cell contractility [2], the elevation of $[Ca^{2+}]_i$ has some potential long-term implications. This correlation between sustained Ca^{2+} release and cell growth indicates that these events may be closely coupled, further indicating that the AngII-dependent change in $[Ca^{2+}]_i$ observed in this cardiomyocyte system may have a dual function. Furthermore, the modulation of $[Ca^{2+}]_i$ levels by both receptor subtypes suggests that the AT_2 receptors, as well as the AT_1 receptors, could mediate the mitogenic actions of AngII. Up-regulation of AT_2 receptor expression or function has been noted in cardiovascular disease processes involving tissue remodelling or repair such as cardiac ischemia [11], ischemia–reperfusion [12], post-myocardial infarction [13], diabetes [32], and hypertension [33]. A direct link between the AT_2 receptors and cell growth has been reported in vascular smooth muscle cells [34, 35]. The potential involvement of these receptors in both cardiomyocyte contractile activity and growth thus warrants further investigation since blockade of the AT_1 receptors alone, which is accompanied by a compensatory increase in circulating AngII, may result in AngII exerting undefined and possibly unwanted effects through the unblocked AT_2 receptors.

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