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Effect of Angiotensin II on Ca^{2+} Efflux from Freshly Isolated Adult Rat Cardiomyocytes

Possible Involvement of Na^+/Ca^{2+} Exchanger

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ABSTRACT. In the present study, we examined the effect of angiotensin II on Ca^{2+} efflux from freshly isolated adult rat cardiomyocytes. Angiotensin II stimulated the efflux of $^{45}Ca^{2+}$ from the cells in a concentration-dependent manner, at least in pharmacological doses of 10^{-8} M to 10^{-5} M. The $^{45}Ca^{2+}$ efflux was inhibited by the type 1 angiotensin II receptor antagonist losartan, but not by the type 2 antagonist PD 123319. Angiotensin II also induced an increase in cytosolic free calcium ($[Ca^{2+}]_i$) and inositol trisphosphate formation within the cardiomyocytes. Angiotensin II-induced $^{45}Ca^{2+}$ efflux and the increase in $[Ca^{2+}]_i$ were both inhibited by thapsigargin, a specific inhibitor of the sarcoplasmic reticulum Ca^{2+} pump. The $^{45}Ca^{2+}$ efflux was not affected by removal of the extracellular Ca^{2+} but was dependent on the presence of extracellular Na^+. In addition, angiotensin II caused $^{22}Na^+$ influx into the cells. These results indicate that angiotensin II stimulates Na^+-dependent $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes, probably through its stimulatory effect on the plasma membrane type 1 angiotensin II receptors. Angiotensin II-induced increase in $[Ca^{2+}]_i$ may cause an activation of Na^+/Ca^{2+} exchange which finally results in the stimulation of $^{45}Ca^{2+}$ efflux from the cells. Since it is reported that Na^+/Ca^{2+} exchange is important in calcium homeostasis within the cells, angiotensin II may play some role in the reduction of intracellular Ca^{2+} from isolated adult rat cardiomyocytes. BIOCHEM PHARMACOL 55;4:481–487, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. angiotensin II; Ca^{2+} efflux; Na^+/Ca^{2+} exchange; losartan; PD123319; rat; cardiomyocyte

Angiotensin II (Ang II)§ is known to act as a growth factor in the heart and can cause both inotropic and chronotropic changes [1]. It has also been proposed that effects of Ang II on the heart are mediated in part by an interaction with the sympathetic nervous system [2]. The existence of type 1 (AT1) and type 2 (AT2) Ang II receptors has been demonstrated in the myocardium [3]. Activation of AT1 by Ang II causes a positive inotropic effect on the heart [4]. It is assumed that the intracellular mechanism of Ang II-induced effects is due to phosphoinositide hydrolysis, which causes the elevation of cytosolic free calcium ($[Ca^{2+}]_i$) [5]. An increase in $[Ca^{2+}]_i$ is considered to trigger a positive inotropic effect on the heart. However, this increased $[Ca^{2+}]_i$ should be restored to a resting level to respond to subsequent stimulation.

On the other hand, it is reported that the Na^+/Ca^{2+} exchanger, which removes $[Ca^{2+}]_i$ from the cardiomyocytes in exchange for extracellular Na^+, is activated in the failing heart [6]. Since Ang II can cause cardiac hypertrophy [1], Ang II may also activate the Na^+/Ca^{2+} exchanger to extrude $[Ca^{2+}]_i$ from the cells to maintain intracellular calcium homeostasis. In addition to inducing a rise in $[Ca^{2+}]_i$ in cardiomyocytes, Ang II may simultaneously stimulate the extrusion of this elevated $[Ca^{2+}]_i$ to a resting level as a counteraction for the increase in $[Ca^{2+}]_i$ of the cells.

Therefore, we examined the effect of Ang II on Ca^{2+} efflux from freshly isolated adult rat cardiomyocytes. We also investigated the mechanism of Ca^{2+} efflux from the cells. Results suggest that Ang II stimulates Na^+-dependent $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes, probably through its stimulatory effect on the plasma membrane AT1. Ang II-induced increase in $[Ca^{2+}]_i$ may cause an activation of Na^+/Ca^{2+} exchange which finally results in the stimulation of $^{45}Ca^{2+}$ efflux from the cells.

MATERIALS AND METHODS

Preparation of Adult Rat Cardiomyocytes

Treatment of animals was based on the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). Isolation of adult rat cardiomyocytes was

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§ *Abbreviations*: Ang II, angiotensin II; $[Ca^{2+}]_i$, cytosolic free calcium; K–H solution, Krebs–Henseleit bicarbonate buffer solution; IP_3, inositol 1,4,5-trisphosphate.

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performed by the method of Mitra and Morad with minor modifications [7]. Male Sprague–Dawley rats weighing 250–300 g were used. The rats were anesthetized by inhalation of saturated diethylether gas and were administered heparin (1,000 U/kg) intravenously. The heart was excised and the aorta cannulated in a Langendorff perfusion model [8]. The heart was then perfused with calcium-free buffer medium (medium A: 135 mM NaCl, 5.6 mM KCl, 1.0 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 10.0 mM HEPES, 0.2% bovine serum albumin (BSA), adjusted with NaOH to pH 7.30) for 3 min. Thereafter, the perfusion medium was changed to a cell dispersing medium, medium B (medium A supplemented with 0.2% collagenase I and 0.04% protease XIV) for 12 min. After digestion, these enzymes were washed out with medium C (medium A containing 220 μM of $CaCl_2$) for 3 min. After the completion of perfusion, the heart was removed from the column and placed in a Petri dish containing 25 mL of medium D (medium C supplemented with 2% BSA). Small pieces of tissue (3 × 3 mm) were cut from the ventricle and gently shaken in 25 mL of medium D, thus releasing the dispersed cells. The cells were resuspended once in 25 mL of a calcium-containing medium to prevent Ca^{2+} paradox [9] (0.44 mM of $CaCl_2$ with medium D, a stepwise increase in Ca^{2+} concentration) and centrifuged at 300 rpm for 30 sec. Afterwards, the cells were washed once with modified Krebs–Henseleit bicarbonate buffer solution (K–H solution) (135 mM NaCl, 5.6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 10 mM glucose, adjusted with HCl to pH 7.40) oxygenated with 95% of O_2 and 5% of CO_2 gas mixture. Finally, the isolated cardiomyocytes were resuspended in 5 mL of K–H solution. The number of counted intact, rod-shaped cells using a hemocytometer was approximately 1.0×10^6 cells/mL, and these cells were quiescent when observed by microscope.

$^{45}Ca^{2+}$ Efflux Assay

The isolated adult rat cardiomyocytes were incubated with 5 mL of K–H solution containing $^{45}CaCl_2$ (4 μCi/mL) for one hour at 37°. Then 1.5 mL volumes of the cell suspension (*ca.* 1.5×10^6 cells) were resuspended and centrifuged twice with 10 mL of K–H solution and were applied to the hand-made superfusion column consisting of 20 μm pore-size filter. This column is cone-shaped, 5 cm long and 1 cm in diameter, and the cells were stratified on the filter. K–H solution was dropped onto the cells to keep the medium volume constant (*ca.* 1 mL), and the effluent from the column was collected. The cells were then superfused with K–H solution for 15 min to remove the unincorporated $^{45}Ca^{2+}$ (the flow rate was approximately 1 mL/min). Afterwards, the effluent was collected 15 times at intervals every 30 sec (*ca.* 1 mL/min) to determine the basal efflux level. The cells were then superfused with the reaction mixture with or without test agents, and the effluent was collected 15 times at intervals every 30 sec to determine the agonist-stimulated $^{45}Ca^{2+}$ efflux levels. After agonist stimulation, the cells in the column were collected and dissolved in 1 mL of 1% Triton X-100 solution to determine the residual $^{45}Ca^{2+}$ in the cells. Extracellulary administered Ang II at 10^{-5} M did not contract the cardiomyocytes, which was confirmed by a microscopic observation. Samples were counted in 10 mL of liquid scintillation fluid for a 2-min period. The total radioactivity of $^{45}Ca^{2+}$ in the cells was determined as the sum of the radioactivity in each fraction and the residual radioactivity, and this value was used to calculate the fractional release of Ca^{2+} from the cells in each period.

Measurement of Intracellular Ca^{2+} by Fura-2

Intracellular Ca^{2+} level in suspension of cardiomyocytes was measured using the fluorescent Ca^{2+} indicator 1-[6-Amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pentaacetoxymethyl ester (fura-2). The cells (1×10^6 cells) suspended in 1 mL K–H solution containing 2 μM fura-2/acetoxymethylester and incubated at 37° for 20 min. Then, the cell suspension was washed twice with K–H solution and transferred to a measuring cuvette in a Ca^{2+} analyzer, CAF-100 (Nihon Bunko Co.). The temperature was maintained at 37°, and the medium in the cuvette was stirred with a micro-magnetic stirrer. Fluorescence was measured in cardiomyocytes in suspension with excitation and emission wave lengths of 340/380 nm and 510 nm, respectively. The intracellular Ca^{2+} level was determined using the equation described previously by Grynkiewicz *et al.* [10].

$$[Ca^{2+}]_i \text{ (nM)} = 225 \text{ nM} \times \frac{(F-F_{min})}{(F_{max}-F)},$$

where F was the measured fluorescence intensity, F_{max} was the fluorescence intensity after cell lysis and F_{min} was the fluorescence intensity in the presence of EGTA (10 mM final concentration).

Measurement of IP_3 Formation within the Cells

Ang II-induced inositol trisphosphate (IP_3) formation was measured using a [^{3}H] assay system kit [11]. Briefly, 1 mL of cell suspension (1×10^6 cells) was incubated at 37° for 1 min with or without test agents, and the reaction was stopped by the addition of an equal volume of 15% trichloroacetic acid. To sediment the precipitates, the test tube was centrifuged at 15 × *g* for 20 min and the produced IP_3 in the supernatant was assayed with the D-myo-[^{3}H] inositol 1,4,5-trisphosphate assay system (7.5 nCi/mL). Results were expressed as a percentage of the control value.

Measurement of $^{22}Na^+$ Influx into the Cells

The isolated adult rat cardiomyocytes were incubated with 1 mL of K–H solution containing $^{22}Na^+$ (3 μCi/mL) in the presence or absence of test agents for 5 min at 37°. After

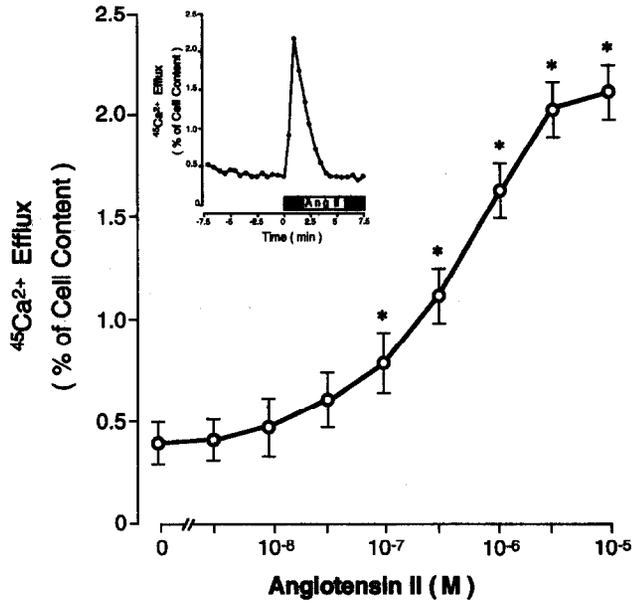

FIG. 1. Concentration-response curve for Ang II-induced $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes. Cells were preloaded with $^{45}Ca^{2+}$ as described in the text. After the stabilization of basal efflux level, Ang II was added and the cells were superfused for the next 7.5 min. Results are expressed as the peak values of the efflux (see inset which shows the time-course of 10^{-5} M Ang II-induced $^{45}Ca^{2+}$ efflux). Data are means ± SE for 3 separate experiments. The asterisk represents the statistical significance from the control value ($P < 0.01$).

incubation, the medium was discarded and the cells were washed 3 times with 1 mL of ice-cold K–H solution. Then the intracellular $^{22}Na^{+}$ was extracted with 1 mL of 1% Triton X-100 and measured in a gamma counter as described previously [12].

Statistics

The ANOVA two-way analysis of variance was used to determine the significance among groups, after which the modified *t*-test with the Bonferroni correction was used for comparison between individual groups. A value of $P < 0.01$ was considered to be statistically significant.

Chemicals

$^{45}CaCl_2$, $^{22}NaCl$ and the D-myo-[^{3}H] inositol 1,4,5-trisphosphate assay system were obtained from Amersham Corp. Angiotensin II was purchased from Peptide Institute Inc. Losartan was kindly donated by E. I. du Pont de Nemours and Company. PD123319 was a gift from Warner–Lambert Co. Thapsigargin was obtained from Sigma Chemical Co. Fura-2/acetoxymethylester was obtained from Dojin Chemical Institute. All other chemicals used were commercial products of reagent grade.

RESULTS

Effect of Ang II on $^{45}Ca^{2+}$ Efflux from Freshly Isolated Adult Rat Cardiomyocytes

The stimulatory effect of Ang II on $^{45}Ca^{2+}$ efflux was dose-dependent at concentrations of 10^{-8} to 10^{-5} M (Fig. 1). As shown in the inset of Fig. 1, the efflux of $^{45}Ca^{2+}$ increased to a peak value within approximately 1 min after Ang II addition. The peak value with 10^{-5} M Ang II was 2.2% ± 0.11% of total $^{45}Ca^{2+}$ within the cells. After the peak, the efflux level decreased rapidly within the next 5 min. The efflux then returned to almost prestimulation levels.

Effects of Ang II Receptor Antagonists on Ang II-Induced $^{45}Ca^{2+}$ Efflux from the Cells

The influence of nonpeptide Ang II receptor antagonists on Ca^{2+} efflux induced by Ang II was examined. A putative AT1 antagonist, losartan, and a putative AT2 antagonist, PD123319, were employed [13, 14]. These antagonists were applied onto the columns 5 min prior to Ang II stimulation. Figure 2 shows the effects of losartan and PD123319 on the submaximal $^{45}Ca^{2+}$ efflux from the cells stimulated by 10^{-6} M Ang II. Ang II-induced Ca^{2+} efflux was almost completely abolished by losartan at a concentration of 10^{-5} M. However, 10^{-5} M PD123319 failed to inhibit its efflux. These results suggest that Ang II-induced $^{45}Ca^{2+}$ efflux was mediated through AT1 on the plasma membrane of the cells.

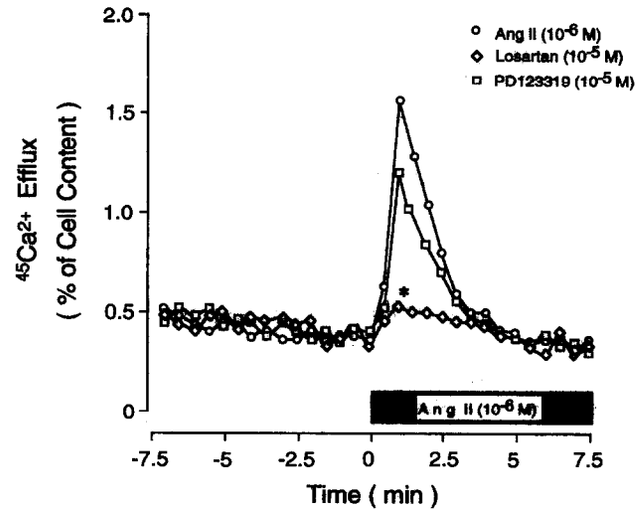

FIG. 2. Effects of AT1 and AT2 antagonists on Ang II-induced $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes. Cells were preloaded with $^{45}Ca^{2+}$ as described in the text. The AT1 antagonist, losartan (10^{-5} M), or AT2 antagonist, PD123319 (10^{-5} M), was added 5 min prior to submaximal concentration of Ang II (10^{-6} M) stimulation. Data are means ± SE for 3 separate experiments. The maximal standard error was 0.08%. The peak level with losartan was significantly less than that with Ang II only ($P < 0.01$).

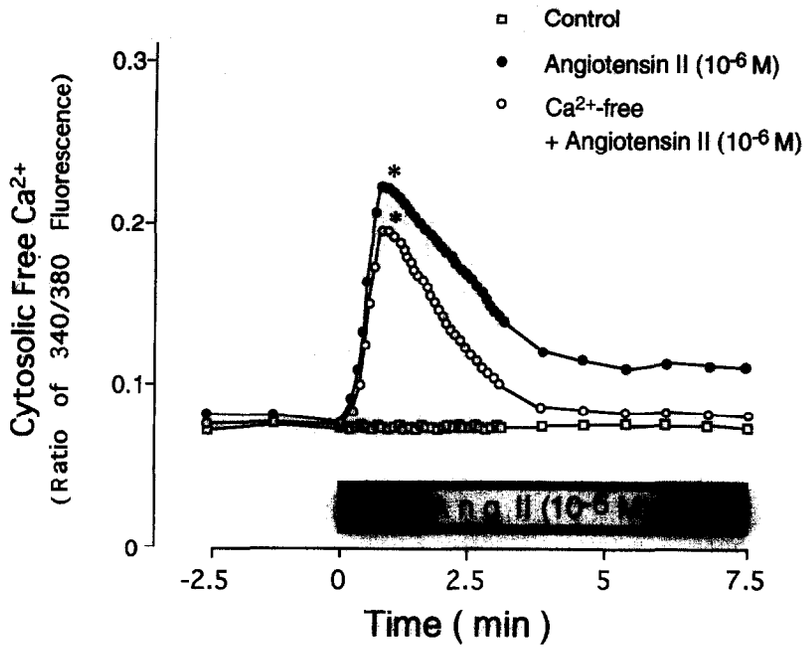

FIG. 3. Effects of Ang II on intracellular free Ca^{2+} level ($[Ca^{2+}]_i$) in the presence or absence of extracellular Ca^{2+}. Cells were preloaded with 2 μM fura-2/acetoxymethylester as described in "Materials and Methods." The Ang II (10^{-6} M)-induced change in $[Ca^{2+}]_i$ was measured under the presence or absence of extracellular Ca^{2+}. The calculated 200 nM of $[Ca^{2+}]_i$ equals the ratio of 340/380 fluorescence of 0.31 according to the equation as described in the text. Data are means ± SE for 3 separate experiments. The maximal standard error was 9.7%. The asterisk represents the statistical significance from the control value ($P < 0.01$).

Effects of Ang II on Intracellular Free Ca^{2+} Level in the Presence or Absence of Extracellular Ca^{2+}

As shown in Fig. 3, Ang II (10^{-6} M) increased $[Ca^{2+}]_i$ to a peak of approximately 150 nM, followed by a gradual decrease to a steady level of significantly more than the control level. On incubation of the cells in Ca^{2+}-free medium, Ang II also induced a peak of $[Ca^{2+}]_i$, but the level then returned to that of the control.

Effect of Ang II on Inositol Trisphosphate Formation in Cardiomyocytes in the Presence or Absence of Extracellular Ca^{2+}

As shown in Fig. 4, application of 10^{-5} M Ang II caused a ca. 80% increase in IP_3 formation during 1 min incubation. The Ang II-induced IP_3 level was slightly decreased by removal of Ca^{2+} from the medium, but was approximately 50% higher than the control level. Since Ang II caused an increase in $[Ca^{2+}]_i$ even though it was in Ca^{2+}-free medium, it is conceivable that the rise in $[Ca^{2+}]_i$ may be due to its release from sarcoplasmic reticulum induced by phosphoinositide breakdown rather than the influx of Ca^{2+} from outside the plasma membrane.

Effect of Thapsigargin, a Specific Inhibitor of Sarcoplasmic Reticulum Ca^{2+} Pump, on Ang II-Induced $^{45}Ca^{2+}$ Efflux and Increase in $[Ca^{2+}]_i$

Thapsigargin at 10^{-5} M, a specific inhibitor of sarcoplasmic reticulum Ca^{2+} pump, inhibited $^{45}Ca^{2+}$ efflux induced by Ang II (10^{-5} M) as well as the Ang II-induced increase in $[Ca^{2+}]_i$ (Fig. 5). From these results, it is assumed that Ca^{2+} release from sarcoplasmic reticulum may be essential to Ang II-induced Ca^{2+} efflux from cardiomyocytes.

Influence of Removal of Extracellular Ca^{2+} or Na^+ on Ang II-Stimulated $^{45}Ca^{2+}$ Efflux

In Ca^{2+}-removed medium, the ingress mode of Ca^{2+} transport via exchanger is blocked. The removal of Na^+ from the incubation medium inhibits Na^+/Ca^{2+} exchange, which extrudes intracellular Ca^{2+} and allows Na^+ influx in turn. These Ca^{2+}- and Na^+-deficient mediums were applied onto the columns 2.5 min prior to Ang II administration. As shown in Table 1, Ang II-induced $^{45}Ca^{2+}$ efflux was not influenced by the absence of extracellular Ca^{2+}.

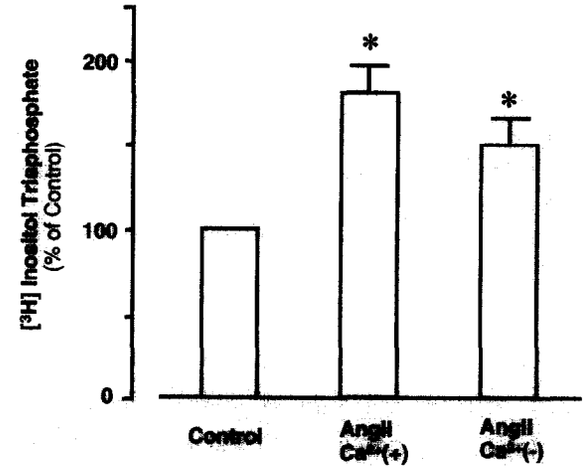

FIG. 4. Effects of Ang II on inositol trisphosphate formation in cardiomyocytes in the presence or absence of extracellular Ca^{2+}. Cells were incubated at 37° for 1 min with or without 10^{-5} M of Ang II in the presence or absence of extracellular Ca^{2+}. Accumulation of [^{3}H] inositol trisphosphate was assayed as described in "Materials and Methods." Data are means ± SE for 3 separate experiments. The asterisk represents the statistical significance from the control value ($P < 0.01$).

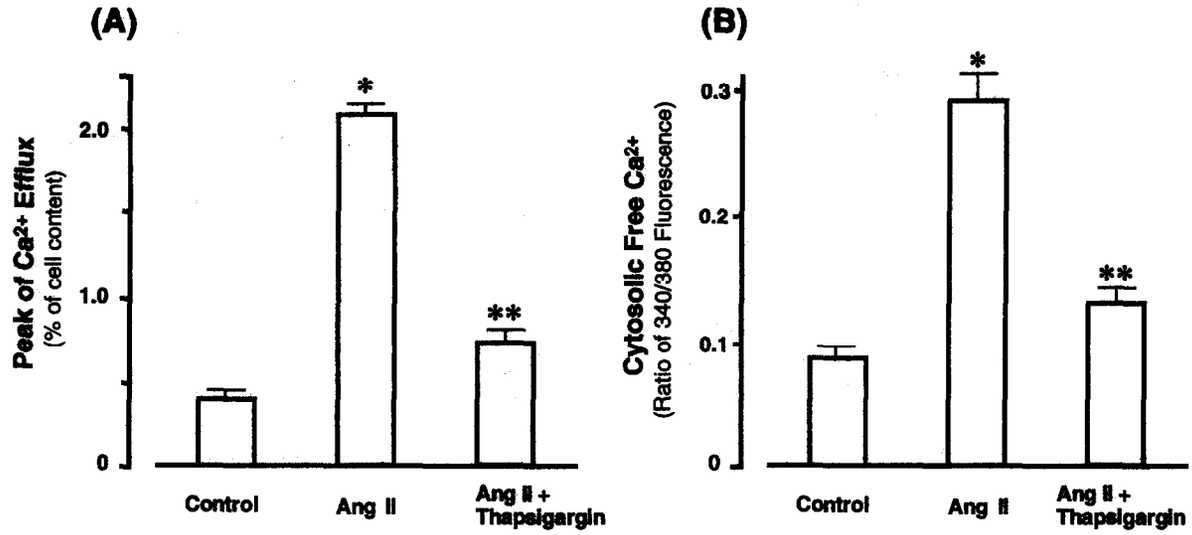

FIG. 5. Effect of thapsigargin (10^{-5} M) on Ang II-induced $^{45}Ca^{2+}$ efflux (A) and increase in cytosolic free Ca^{2+} level (B). Thapsigargin was added 5 min prior to Ang II (10^{-5} M) stimulation in both experiments. In the experiment of $^{45}Ca^{2+}$ efflux, Ang II-induced Ca^{2+} efflux was measured after the stabilization of basal efflux level. Ang II (10^{-5} M)-induced change in $[Ca^{2+}]_i$ was measured under the presence and absence of thapsigargin in a Ca^{2+}-containing medium. Data are means ± SE for 3 separate experiments. The peak levels with Ang II alone were significantly greater than the control value (*P < 0.01). The peak levels with thapsigargin were significantly less than the levels with Ang II alone (P < 0.01).**

However, the complete replacement of Na^+-free medium by sucrose significantly inhibited Ang II-induced $^{45}Ca^{2+}$ efflux from the cells. From these results, it is assumed that the effect of Ang II in stimulating Ca^{2+} efflux across the plasma membrane may be mediated by an extracellular Na^+-dependent mechanism in isolated adult rat cardiomyocytes, presumably through the Na^+/Ca^{2+} exchange mechanism.

Effect of Ang II on $^{22}Na^+$ Influx into the Cells

To evaluate the possibility that Ang II-induced $^{45}Ca^{2+}$ efflux from the cells is mediated by the Na^+/Ca^{2+} exchange mechanism, we examined the effect of Ang II on $^{22}Na^+$ influx into the cardiomyocytes. Experiments were performed using normal medium and Ca^{2+}-removed medium. Table 2 shows that $^{22}Na^+$ influx into the cells was induced by Ang II, which in turn stimulated $^{45}Ca^{2+}$ efflux from the cells. Under the condition of Ca^{2+}-free medium, Ang II also increased $^{22}Na^+$ influx into the cells. Therefore, Ang II-induced $^{45}Ca^{2+}$ efflux from the cells, which is mediated by the Na^+/Ca^{2+} exchange mechanism, was suggested.

TABLE 1. Influences of Ca^{2+}- and Na^+-deficient medium on Ang II-induced $^{45}Ca^{2+}$ efflux from freshly isolated adult rat cardiomyocytes

	Peak of Ca^{2+} Efflux (% of cell content)
Control	0.41 ± 0.05
Ang II	2.2 ± 0.11*
Ca^{2+} (−) medium	2.0 ± 0.08*
Na^+ (−) medium	0.72 ± 0.05

Cells were preloaded with $^{45}Ca^{2+}$ as described in the text. After the stabilization of basal efflux level, the medium was changed to Ca^{2+}- or Na^+-deficient medium 2.5 min prior to Ang II administration. Then, Ang II (10^{-5} M) was added, and the cells were superfused for 7.5 min. Na^+-deficient medium was prepared with sucrose instead of Na^+. Data are means ± SE for 3 separate experiments. The asterisk represents the statistical significance from the control value ($P < 0.01$).

DISCUSSION

It is known that Ang II causes inotropic and chronotropic effects on the heart [1]. The mechanism of the Ang II-induced inotropic effect is attributed to an increase in $[Ca^{2+}]_i$ in cardiomyocytes [4]. Increased $[Ca^{2+}]_i$ should be restored to a physiological level because cumulative elevation in $[Ca^{2+}]_i$ leads to irreversible injury in cardiomyocytes. Results shown in Fig. 1 revealed that Ang II causes a significant Ca^{2+} efflux from cardiomyocytes in a concentration-dependent manner, at least in pharmacological doses of 10^{-8} M to 10^{-5} M. However, the local renin-angiotensin system [15] may produce a higher local concentration of Ang II at the receptor site in the heart than that in the plasma. The concentrations of Ang II used in

TABLE 2. Effect of Ang II on $^{22}Na^+$ influx into freshly isolated adult rat cardiomyocytes

	$^{22}Na^+$ Influx (nmol/10^6 cells)	% of Control
Control	20.2 ± 1.7	
Ang II	36.5 ± 2.8*	181
Ca^{2+} (−) medium	32.6 ± 2.1*	161

Cells were incubated with $^{22}Na^+$ in the presence or absence of Ang II (10^{-5} M) and with or without Ca^{2+} in the medium for 5 min as described in the text. Data are means ± SE for 3 separate experiments. The asterisk represents the statistical significance from the control value ($P < 0.01$).

the present study were considered to be higher than those in the plasma.

As the existence of Ang II receptors in cardiomyocytes has been reported, Ang II may affect the receptors in the cells. These Ang II receptors could be classified into two major subgroups, i.e. AT1 and AT2 [16]. Next, we examined the effects of the specific AT1 antagonist, losartan, and the AT2 antagonist, PD123319, on the efflux of Ca^{2+} from the cardiomyocytes [13, 14]. As shown in Fig. 2, Ang II-induced Ca^{2+} efflux was almost completely inhibited by losartan, but not by PD123319 at a concentration for both of 10^{-5} M. From these results, it may be assumed that the stimulatory effect of Ang II on Ca^{2+} efflux from cardiomyocytes is mediated by AT1. Since we confirmed Ang II-induced increase in $[Ca^{2+}]_i$ (Fig. 3), Ang II may also simultaneously stimulate the extrusion of this elevated $[Ca^{2+}]_i$ as a counteraction for increase in $[Ca^{2+}]_i$ to maintain the calcium homeostasis within the cells. As shown in Fig. 5, thapsigargin, a specific inhibitor of sarcoplasmic reticulum Ca^{2+} pump which antagonizes the release of Ca^{2+} from sarcoplasmic reticulum indirectly, inhibited the Ca^{2+} efflux to the same extent as the inhibition of $[Ca^{2+}]_i$ increase. These results suggest that Ca^{2+} release from sarcoplasmic reticulum may trigger the Ca^{2+} efflux from the plasma membrane of the cardiomyocytes.

To investigate the intracellular mechanism of Ang II-induced Ca^{2+} efflux from cardiomyocytes, we examined the effects of Ca^{2+} or Na^+ deprivation from the medium on this efflux. It has been reported that the decline in $[Ca^{2+}]_i$ may be due mainly to the mechanism of Na^+/Ca^{2+} exchange at the plasma membrane and Ca^{2+} uptake into the sarcoplasmic reticulum [17]. As shown in Table 1, Ang II-induced Ca^{2+} efflux from cardiomyocytes was dependent on the presence of extracellular Na^+, but not on Ca^{2+}. The removal of Na^+ from the incubation medium represents the inhibition of Na^+/Ca^{2+} exchange to extrude intracellular Ca^{2+} outside the cells and in turn allow Na^+ influx. These results suggest that Ang II stimulated Ca^{2+} efflux from cardiomyocytes through the mechanism of Na^+/Ca^{2+} exchange. The finding that Ang II caused a significant $^{22}Na^+$ influx into the cells also supports this idea (Table 2). Thus, it is assumed that the acceleration of Na^+/Ca^{2+} exchange through the plasma membrane plays a key role in Ang II-induced Ca^{2+} efflux from cardiomyocytes.

Activation of Ang II receptors in cardiomyocytes is coupled with various signal transduction processes, which include stimulation of the slow Ca^{2+} channel [18] and acceleration of the hydrolysis of phosphoinositide [5], or activation of protein kinase C [19]. In the present study, we confirmed that Ang II activates the formation of IP_3 within the cells (Fig. 4). Considering the finding that phosphoinositide hydrolysis causes the activation of Na^+/Ca^{2+} exchanger in cardiac cells [20], it may be reasonable to hypothesize that Ang II stimulates the exchanger to extrude Ca^{2+} outside the cells through the activation of phosphoinositide hydrolysis. These results are consistent with the report of Ballard and Schaffer that Ang II activates the Na^+/Ca^{2+} exchanger in rat heart when evaluated by G protein activity [21]. Moreover, it has also been reported that Ang II induces Ca^{2+} efflux from bovine adrenal chromaffin cells through the Na^+/Ca^{2+} exchange mechanism [22]. From these findings, it may be reasonable to speculate that Ang II-induced Ca^{2+} efflux from adult rat cardiomyocytes may be mediated by the mechanism of Na^+/Ca^{2+} exchange, which in turn may accelerate Ca^{2+} efflux from the cardiomyocytes by an increase in $[Ca^{2+}]_i$ resulting from inositol trisphosphate turnover via AT1 receptor stimulation.

It is reported that the Na^+/Ca^{2+} exchanger is activated in the failing heart [23]. Ang II may activate the Na^+/Ca^{2+} exchanger concomitantly with the augmentation of cell proliferation. However, the exact mechanism which causes Ca^{2+} efflux from the cardiomyocytes is still unclear. We are further investigating the intracellular mechanism involved in Ang II-induced Ca^{2+} efflux from freshly isolated adult rat cardiomyocytes.

In conclusion, Ang II stimulates Ca^{2+} efflux from adult rat cardiomyocytes probably through the AT1 receptor, at least in pharmacological doses. The underlying mechanism which causes Ca^{2+} efflux from the cells is still not entirely understood; however, it can be explained at least in part by Na^+/Ca^{2+} exchange through the plasma membrane.

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