Angiotensin II Increases Spontaneous Contractile Frequency and Stimulates Calcium Current in Cultured Neonatal Rat Heart Myocytes: Insights into the Underlying Biochemical Mechanisms

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The effect of angiotensin II on cultured neonatal rat heart myocytes was studied by measuring changes in cell length, the magnitude and kinetics of the calcium current, and changes in cyclic adenosine 3',5'-monophosphate (cAMP) and phosphoinositide metabolism. Spontaneous beating frequency of multicellular networks was increased by angiotensin II with a maximal increase of 100% above control values at concentrations of 5 nM or greater. The half-maximal response occurred at 0.6 nM angiotensin II. Shortening amplitude, shortening velocity, and relaxation velocity decreased concomitantly with the increasing contractile rate. In voltage-clamped single myocytes, both steady-state and transient components of the calcium current were increased by the addition of angiotensin II. Angiotensin II had no effect on either control or isoproterenol-stimulated adenylate cyclase activity in myocyte membranes. Neither the basal levels nor the isoproterenol-stimulated cAMP accumulation in intact cells was affected by addition of hormone. In myocytes labeled with [3H]inositol, angiotensin II stimulated the formation of [3H]inositol phosphates. One minute after addition of 5 nM angiotensin II, inositol monophosphate and inositol bisphosphate levels were increased to 73% and 99%, respectively, above control values and remained elevated at 10 minutes. Inositol trisphosphate levels were not significantly different from control values at either time point. Nifedipine (10 μM) had no effect on angiotensin II-induced increases in [3H]inositol phosphates. We conclude that the increases in both spontaneous beating rate and calcium current in angiotensin II-stimulated cultured neonatal heart cells are not dependent on cAMP or inositol trisphosphate levels but may involve sustained phosphoinositide hydrolysis. (Circulation Research 1988; 62:524–534)

Angiotensin II (All) is an important vasoactive peptide that regulates cellular responses in a number of tissues.1,2 While the heart has not traditionally been considered a target tissue for All, this hormone has been reported to increase the strength of myocardial contraction and modify electrical behavior in several species.3–5 Although the physiological mechanisms by which All brings about its effects remain uncertain, several important findings have been reported. 1) There are specific, high-affinity receptors for All on both cultured neonatal rat heart myocytes and sarclemal membranes from bovine and rabbit ventricle.6–9 2) All increases the slow inward current (I_s) in rabbit papillary muscle, perfused embryonic chick heart, and bovine Purkinje fibers.5,6 3) There is an increase in twitch tension associated with the application of All to rabbit papillary muscle and bovine Purkinje fibers.5,6

There is little information on the molecular mechanisms that couple All receptor binding to changes in I_s and contractile behavior in cardiac cells. In contrast, the calcium mobilizing effects of All in noncardiac tissues are well documented.7 All has been shown both to inhibit the adenylate cyclase system in the liver,8 anterior pituitary,9 aorta,10 and adrenal cortex,11 and to stimulate phosphoinositide hydrolysis in the same tissues.14–17

In the present report, the cultured neonatal rat heart myocyte system was utilized to study the actions of All. We report here that All stimulates both contractile frequency in spontaneously beating cultured myocytes and sarclemmal calcium channel current (I_Ca) in voltage clamped myocytes. Further, the All-mediated changes in I_Ca and contractile behavior occur through a cyclic adenosine 3',5'-monophosphate (cAMP)-independent mechanism. Finally, we document that All stimulates phosphoinositide hydrolysis in cardiac myocytes, providing new insights into the mechanism of action of All in heart cells.
Preparation of Cultured Rat Heart Myocytes

Cardiac myocytes were isolated from hearts of 1–2-day-old Sprague-Dawley rats by a trypsin dispersion procedure and cultured as previously described. For most of the experiments described here, cells were grown in either 24-well or 4-well (1.7 cm) tissue culture dishes. The electrophysiological experiments were performed on myocytes grown on plastic coverslips in 35-mm culture dishes.

Preparation of Membranes From Myocyte Cultures

A crude microsomal preparation was prepared from the cultured myocytes for use in the adenylate cyclase assays. Confluent monolayers of cells in 10-cm culture dishes were harvested on days 4–6. The dishes were placed on ice, and the growth medium was removed. Ten milliliters of ice-cold buffer A (10 mM Tris and 250 mM sucrose, pH 7.4) was added to the dish, and the cells were detached by scraping with a rubber policeman. The cell suspension was filtered through an eight-pole, low-band pass, active filter with a cutoff frequency of 25 Hz, and then centrifuged at 300g for 10 minutes at 0° C. The cell pellet was resuspended in 10 ml of buffer A, homogenized in a Teflon glass homogenizer at 0° C, and centrifuged at 120,000g for 30 minutes. This total particulate fraction was washed with 10 ml of buffer B [50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Tris (HEPES-Tris), pH 7.4], rehomogenized, and centrifuged at 120,000g for 30 minutes at 0° C. The pellet was resuspended in buffer B at an approximate concentration of 1–2 mg protein/ml. The membranes were rapidly frozen on dry ice and stored at −70° C. Adenylate cyclase activity was stable for at least 2 weeks. Protein was determined by the method of Bradford, using bovine serum albumin (BSA) as a standard.

Measurement of Contractile Responses

The contractile responses of a spontaneously beating multicellular network were measured using an optical video dimension analysis system as previously described. Shortening and relaxation velocities were obtained electronically by differentiating the length signal. The output from the video dimension analyzer was digitized with a digital audio processor (PCM-501ES, Sony, Park Ridge, New Jersey) modified after Bezanilla by Unitrade, Philadelphia, Pennsylvania. Relatively high doses of All were applied to the cells, 100 nM, since BSA had to be eliminated from the medium because it interfered with the formation of electrode-cell seal. Previous studies have shown that it is essential to have BSA in the medium to prevent proteolysis of the hormone in the culture dish. The series resistance of the pipette attached to a cell with a ruptured patch was generally twice that of the pipette alone. After compensation, there usually remained an additional 2–3 MΩ of uncompensated series resistance. Experiments were performed in the “whole-cell” voltage clamp configuration at 22° C. With sodium and potassium channels blocked by external TTX and internal cesium, respectively, voltage clamp pulses (feedback resistor 1 GΩ; 8900, Dagan, Minneapolis, Minnesota) were applied to investigate I_{Ca}. Cadmium (0.1 mM) was added before the end of each experiment, and I_{Ca} was measured as the cadmium-sensitive current. Data were stored on videotape (VCR7200, Sanyo, Tokyo, Japan) after digitization with a digital audio processor (PCM-501ES, Sony, Park Ridge, New Jersey) modified after Bezanilla by Unitrade, Philadelphia, Pennsylvania. Relative high doses of All were applied to the cells, 100 nM, since BSA had to be eliminated from the medium because it interfered with the formation of electrode-cell seal. Previous studies have shown that it is essential to have BSA in the medium to prevent proteolysis of the hormone in these cultures; therefore, it was not possible to accurately control the effective concentrations of All in these experiments, but it is certainly lower than 100 nM.

Curve Fitting Procedure

The current records were analyzed off-line using VACUUM MKII (M.B.C. Systems, Mount Washington Station, Baltimore, Maryland) software and Data Translation hardware (DT 2805, Marlborough, Massachusetts). Current records were fit by a nonlinear least squares method to a single exponential function. The amplitude of I_{Ca} was estimated at t = 0. The data for the steady-state activation (d_{i}) and
inactivation ($f_0$) parameters were fit to the following Boltzmann equations:

$$d_x = \frac{1}{1 + e^{-(V_x - V_{h})/k}}$$

$$f_x = \frac{1}{1 + e^{-(V_x - V_{k})/k}}$$

where $V$ is the membrane potential and $V_h$ and $k$ are constants.

**Adenylate Cyclase Assay**

Adenylate cyclase activity in membranes from cultured myocytes was determined according to the method of Salomon et al. Briefly, reaction mixtures (final volume 0.1 ml) contained [alpha-32P]ATP (40-70 cpm/pmoll, ICN Radiochemicals, Irvine, California), 1 mM ATP, 1 mM CAMP, 0.25% BSA, 1 mM isobutyl-methylxanthine, 20 mM creatine phosphate, creatine phosphokinase (100 units/mI), adenosine deaminase (3.75 units/ml), myocyte membranes (40-60 µg), 0.1 mM GTP, 80 mM HEPES-Tris (pH 7.4) and MgCl$_2$, 0.1 mM isoproterenol, and 100 nM AII as indicated. The reaction was initiated by the addition of membranes and allowed to proceed at 35°C for 10 minutes. The reaction was terminated by adding 100 µl of stopping solution (2% sodium dodecyl sulfate, 40 mM ATP, 1.4 mM cAMP, pH 7.4). After addition of [3H]cAMP (about 5,000 cpm/tube to monitor cAMP recovery; ICN Radiochemicals), [32P]cAMP was purified by sequential chromatography on Dowex (Bio-Rad Laboratories, Rockville Center, New York) and alumina columns. Free Mg$^{2+}$ concentrations were calculated by solving for multiple equilibria.

**cAMP Determination**

Myocytes, in 1 ml DMEM containing 25 mM HEPES and 0.25% BSA, pH 7.4, were exposed to AII (100 nM) for 5 minutes at 37°C in 95% O$_2$-5% CO$_2$. Isoproterenol (1 µM) was then added, and the cells were incubated for an additional 1 minute. Time course studies revealed that isoproterenol-stimulated cAMP levels were maximal by this time. Reactions were terminated by rapidly aspirating the incubation medium and adding 0.5 ml of ice-cold perchloric acid (6%). The acid extracts were neutralized with 40 µl of 5 M K$_2$CO$_3$. cAMP in the neutral extracts was measured using a standard radioimmunoassay procedure (cAMP Radioimmunoassay Kit, New England Nuclear, Boston, Massachusetts).

Pilot experiments using [3H]cAMP revealed that 90 ± 5% of the cAMP was recovered in the neutralized extracts. For the results reported here, the data were analyzed assuming 90% recovery. Protein in experimental wells was taken as the mean of the protein determinations in three nonassayed wells on the same culture dish, with a variation of less than 10%. Protein was extracted in 0.0125% sodium dodecyl sulfate and measured according to the method of Bradford, using BSA as a standard.

**Measurement of Inositol Phosphates**

Myocytes were grown for at least 4 days and then incubated an additional 48 hours in DMEM containing 10% fetal calf serum, 1% penicillin, 1% streptomycin, [H]inositol (20 µCi/ml; New England Nuclear), pH 7.4. The wells were washed several times with 1 ml of assay medium (DME medium containing 25 mM HEPES, 0.25% BSA, pH 7.4, gassed with 95% O$_2$-5% CO$_2$). Myocytes, in 1 ml of assay medium, were exposed to 5 nM AII for the indicated times. Control wells were treated in an identical fashion except that hormone was not added. In experiments where nifedipine (10 µM) was added, cells were incubated with the calcium channel blocker for 1 minute prior to addition of 5 nM AII. Reactions were terminated by addition of perchloric acid and extracts were neutralized as described above. [H]Inositol phosphates in the neutral extracts were separated, collected, and counted according to the method of Downes and Wusteman. Statistical significance was determined by analysis of variance (ANOVA).

**Results**

**Effect of Angiotensin II on Myocyte Contractile Behavior**

In the initial phase of this study, the effect of AII on the contractile behavior of cultured neonatal rat heart myocytes was characterized. As shown in Figure 1, AII increases the spontaneous beating rate and decreases shortening amplitude. AII (5 nM) increases beating frequency in a time-dependent fashion and maximal stimulation is achieved approximately 3 minutes after addition of hormone (Figure 1A). There is also a decrease in shortening amplitude that develops over time after cells are exposed to 5 nM AII (Figure 1A). The average maximal increase in rate from a number of experiments is 99 ± 4% above control values (n = 3). The average maximal decrease in amplitude is 49 ± 3% below control values (n = 3). As another measure of contractile force, the effect of AII on shortening and relaxation velocities was determined. In response to 5 nM AII, both shortening velocity and relaxation velocity decrease over time, reaching a plateau by approximately 3 minutes after addition of hormone (Figure 1B). The average AII-stimulated maximal decrease in shortening velocity is 46 ± 9% below control values (n = 3). The average maximal decrease in relaxation velocity is 49 ± 2% below control values (n = 3).

The potency of AII in stimulating contractile frequency is shown in Figure 2. AII maximally stimulates beating frequency to 100% above control values at concentrations of 5 nM or greater. The half-maximal chronotropic response occurs at a concentration of 0.6 nM AII (EC$_{50}$). This response is specific because the AI antagonist Sar$_1$Ala$_8$All (40 nM) completely inhibits the increase in beating frequency evoked by 5 nM AII. Further, the AII response is decreased in the presence of 1 nM Sar$_1$Ala$_8$-All at all concentrations examined.

It is possible that the AII-mediated decrease in both shortening velocity and shortening amplitude are functions of the increase in contractile frequency evoked by AII. This issue was addressed by examining the force-frequency relation using myocytes that were electrically stimulated. Within the frequency range of...
To further examine force-frequency relations in these cultured cardiac cells, isoproterenol was used to increase beating frequency. Isoproterenol (10 nM) increased beating frequency by 30% in 3 minutes. However, in contrast to the responses of the cells to All, isoproterenol resulted in increases in twitch amplitude and contraction velocity, by 41% and 78%, respectively (data not shown). These expected positive inotropic responses of the cells to a β-agonist further underscore the novel negative inotropic effects of All in this system.

**Effect of Angiotensin II on Calcium Channel Current**

One explanation for the increase in beating rate observed with All is that $I_{Ca}$ is elevated by the hormone. Accordingly, $I_{Ca}$ was recorded under control conditions and in the presence of 100 nM All with Ca$^{2+}$ (1 mM) as the principal charge carrier. To examine only the role of "L-type" calcium channels in whole-cell current records, the holding potential was set to −50 mV.26 If any "T-type" channels were present in this preparation, they would be inactivated by this holding potential. Depolarizing pulses of 200-msec duration to potentials between −45 and +60 mV were applied at 0.5 Hz. Five features of $I_{Ca}$ were noted (Figure 4): 1) A steady-state current is observed following activation and inactivation, 2) Both the transient and steady-state components of $I_{Ca}$ show a bell-shaped dependence on voltage. $I_{Ca}$ increases from zero at −40 mV to a maximum at around 0 mV and then decreases as the membrane potential approaches +55 mV, 3) The time-dependent component of $I_{Ca}$ reverses direction at +55 mV. 4) Over the voltage range 0 to +55 mV, the rate of inactivation decreases with increasing depolar-
FIGURE 3. Effect of myocyte beating rate on control and angiotensin II (All)-stimulated shortening amplitude and shortening velocity. A: Time courses of shortening amplitude and shortening velocity for electrically driven cells. In this representative experiment of three, cells were electrically stimulated to indicated frequencies with pulses of 1-msec duration at 50 volts. Contractions were recorded before (■) and after (○) switching superfusion from control media to media containing 1 nM All. Each point represents average value over a 20-second interval. B: Sample records of shortening amplitude and shortening velocity in cells electrically driven to a constant beating rate. Myocytes were stimulated with pulses of 1-msec duration at 50 volts to a constant rate of 160 beats/min.

Steady-State Calcium Channel Current (d_ and f_)

To investigate the steady-state component of I_c observed in Figure 4D, we measured d_ and f_ variables (Figure 5). To examine d_, the membrane potential was held at -50 mV. The tail current (I_m) was measured upon returning to the holding potential following a 10-msec pulse to V_m (Figure 5A). To control for "rundown" all trials to V_m were alternated with trials to V_{max} (the test potential that gives the largest tail current) and the ratio of the resulting tail currents (I_m/I_{max}) was determined (Figure 5B). The smooth curve shows the best-fit line described by a Boltzmann equation.

To investigate f_, the membrane potential was held at -50 mV. Following a 200-msec voltage clamp step to V_{max} the membrane potential was returned to the holding potential for 10 msec and then depolarized to the evaluation potential (+5 mV) for 200 msec. To control for "rundown," all trials to V_m were alternated with trials to V_{max} (the test potential that gives the largest current transient at the evaluation potential), and the ratio of the resulting currents (I_m/I_{max}) was determined (Figure 5C). The smooth curve shows the best-fit line described by a Boltzmann equation.

In Figure 5D, both d_ and f_ have been plotted. These results show a large overlap of the two curves (hatched area). The voltage-dependence of the overlap of d_ and f_ constitutes a voltage range where significant steady-state I_c should be seen.

Effect of Angiotensin II on d_ and f_

To measure the effect of All on d_ and f_ we used the same protocols described above. All produces a small shift of d_ to more negative voltages (Figure 5B). All decreases V_i without materially affecting k (see Figure 5 legend). The mean shift of V_i is -2.8 ± 1.1 mV (n = 5; p < 0.05, two-tailed t test). All produces a small shift of f_ to more positive voltages (Figure 5C). All increases V_i without materially affecting k (see Figure 5). The mean shift of V_i is +2.6 ± 1.3 mV (n = 5; p < 0.05, two-tailed t test). The effect of All on both d_ and f_ is shown in Figure 5D. All evokes a slight increase in the magnitude of the overlap of d_ and f_.

To calculate the shape of the current-voltage relation of the transient component of I_c from d_ and f_, we have assumed that the kinetics of activation are always much faster than the kinetics of inactivation. In addition, we have adjusted a voltage-independent "relative conductance" term, g_c, to give us the best fit line by eye to the data points. The expression used is

\[ I_{\text{max}}(V) = g \times (V_m - E_{\text{channel}}) \times [(1 - f_r(V_m)) \times d_r(V_m)] 
\]

where V_m is the membrane potential and E_{channel} is the reversal potential for the current. The smooth curves generated by Equation 1, which make use of the
Effect of Angiotensin II on cAMP Metabolism

It is evident from the above data that All increases $I_{ca}$ in the cultured myocytes. Since it is well known that elevated cAMP levels increase $I_{ca}$ in heart cells, the effect of All on adenylate cyclase activity in myocyte membranes was examined. As shown in Figure 6, All (100 nM) has no effect on Mg$^{2+}$-dependent adenylate cyclase activity. In contrast, isoproterenol (0.1 mM), which was included as a positive control, increases adenylate cyclase activity maximally about 13-fold (at 3.97 mM free Mg$^{2+}$). Further, All (100 nM) does not significantly affect isoproterenol-stimulated adenylate cyclase activity.

Although All does not stimulate adenylate cyclase activity, the peptide could increase cAMP levels in intact cells through mechanisms that are independent of direct receptor-adenylate cyclase interactions (e.g., inhibition of cAMP degradation). Therefore, the effect of All on cAMP levels in intact cells was determined. All, in doses as high as 100 nM, has no effect on control levels of cAMP (Figure 7). Under the same conditions,
FIGURE 5. Effect of angiotensin II (All) on steady-state activation ($d_\text{m}$) and inactivation ($f_\text{i}$) variables. A: Effect of All on tail currents used to measure $d_\text{m}$. Upper panel, original records of potential and current during a 10-msec depolarizing pulse to 0 mV and repolarization to the holding potential (-50 mV) under control conditions, in the presence of 100 nM All, and in the presence of 0.1 mM CdCl$_2$; lower panel, $I_{\text{cd}}$ (Cd sensitive difference current) from upper panel. Deactivating tail currents were fit as described in Cohen and Lederer and in “Materials and Methods” to an exponential. For the records shown, the exponential fit is displayed (solid line) with the following initial value (A) and time constants (r): control, $A=0.98$ nA, $r=1.7$ msec; All, $A=1.6$ nA, $r=1.56$ msec.

B: Effect of All on steady-state activation ($d_\text{m}$). Control, $V_\text{r}= -2.5$ mV, $k=6.5$ mV; 100 nM All, $V_\text{r}= -4.8$ mV, $k=6.4$ mV.

C: Effect of All on steady-state inactivation ($f_\text{i}$). Control, $V_\text{r}= -6.2$ mV, $k=9.5$ mV; 100 nM All, $V_\text{r}= -3.6$ mV, $k=9.6$ mV.

Effect of Angiotensin II on Phosphoinositide Hydrolysis

The above results indicate that All increases beating frequency and $I_\text{ca}$ through mechanisms that are independent of cAMP. Therefore, it is unlikely that cAMP-dependent protein kinase is responsible for the All-induced increase in $I_\text{ca}$. However, other intracellular messengers may be involved in mediating the physiological actions of All. In this regard, several studies using noncardiac tissues have correlated All-mediated functional responses with increased degradation of phosphoinositides.$^{15-17}$ Further, $\alpha_1$-adrenergic agonists, which exert positive inotropic effects on heart tissue, have been shown to increase phosphoinositide breakdown in cardiac cells.$^{33}$ Accordingly, the effect of All on phosphoinositide hydrolysis in the cultured rat heart cells was examined. Myocytes were prelabeled with [H]inositol, and the hydrolysis of phosphoinositides was monitored by measuring the formation of [H]inositol phosphates in intact cells. $^{33}$ When cultured myocytes are exposed to 5 nM All, there is a significant increase in the levels of inositol monophosphate (IP) and inositol bisphosphate (IP$_2$) at 1 minute (Figure 8). Further, these levels remain elevated 10 minutes after addition of the peptide. The increases above control
values for IP and IP₂ are 73% and 99%, respectively, at 1 minute and 59% and 232%, respectively, at 10 minutes. Inositol trisphosphate (IP₃) levels are not significantly elevated at either time point. Preliminary studies, in which 10 mM LiCl was used to block IP degradation, show that IP and IP₂ levels are increased as early as 30 and 10 seconds, respectively, after addition of 100 nM All (data not shown). Under these conditions All does not significantly elevate IP₁ levels above control values at either 5, 10, or 30 seconds (data not shown).

The above observations indicate that All evokes sustained elevation of both IP and IP₂. However, since many of the enzymes involved in phosphoinositide metabolism may be calcium-dependent, increases in inositol phosphates could be an indirect consequence of increases in IP₃ rather than a direct result of receptor-mediated activation of phosphoinositide hydrolysis. To resolve this issue, the effect of the calcium channel blocker nifedipine (10 μM) on All-stimulated inositol phosphate formation was examined. This concentration of nifedipine has been shown to completely block calcium current in voltage-clamped neonatal rat heart myocytes. At both 1 minute and 10 minutes, the All-stimulated formation of IP while All-stimulated IP₂ levels are increased slightly in the presence of the calcium channel blocker (Figure 8). The All-mediated increases over control values for IP and IP₂, in the presence of nifedipine, are 83% and 160%, respectively, at 1 minute; the increases at 10 minutes are 55% and 291%, respectively. IP levels are unchanged, compared with control values, in the presence of All plus nifedipine. Nifedipine alone does not alter control levels of IP₁, IP₂, or IP₃ (data not shown). For all three inositol phosphates, addition of nifedipine does not have a statistically significant effect on All-stimulated inositol phosphate formation at either 1 minute or 10 minutes (p>0.05).

Discussion

The cellular mechanisms associated with the physiological effects of several cardioactive agents, such as α-adrenergic and β-adrenergic agonists, have been extensively studied. In contrast, the molecular events that mediate the actions of other stimulatory agents such as the octapeptide All are less clearly understood. To better understand these mechanisms, we have examined the effects of All on both physiological and biochemical responses in cultured rat heart cells. Four important conclusions based on our findings are 1) All stimulates the spontaneous beating rate in neonatal cardiac myocytes, 2) All increases L_{\text{max}} in these same cells, 3) All-mediated stimulation of beating frequency and L_{\text{max}} is independent of cAMP, and 4) All stimulates phosphoinositide hydrolysis in cardiac cells, and this process may be involved in the All-mediated physiological effects.

Our studies show that All increases the spontaneous beating frequency of neonatal cardiac myocytes. This response is receptor-mediated as evidenced by the following: 1) The EC₅₀ is almost identical to the previously reported K_{d} for high-affinity All binding in these cells; 2) The presence of 40 nM of the antagonist Sar¹,Ala⁸-All completely blocks the All-induced increase in beating rate; and 3) 1 nM of the antagonist partially blocks the effect of All. These data are consistent with, and expand upon, previously reported work.

Negative Inotropic Effects of Angiotensin II

Concomitant with increases in beating rate, All elicits negative inotropic effects characterized by decreases in shortening amplitude, shortening velocity, and relaxation velocity. Several observations indicate that these responses are not artifacts of the increase in beating frequency. In electrically stimulated cells, the force-frequency curve remains relatively flat within the frequency range of the All response seen in spontaneously beating cells. Further, addition of All causes...

Figure 7. Effect of angiotensin II (All) on cAMP levels in intact myocytes. Myocytes were incubated with 100 nM All (stippled bar) or 1 μM isoproterenol (ISO) in the absence (solid bar) or presence (hatched bar) of 100 nM All. Control levels of cAMP (open bar) were determined in the absence of hormonal stimulation. Each histogram represents the mean ± SEM of 3–4 experiments performed in duplicate.

Figure 6. Effect of angiotensin II (All) on adenylate cyclase activity in myocyte membranes. Membranes (40–60 μg) were incubated with increasing concentrations of MgCl₂ in the presence (○) or absence (●) of 100 nM All. Membranes were also incubated in the presence of 0.1 mM isoproterenol with (○) or without (●) 100 nM All. Each point represents the mean of 2–4 experiments performed in duplicate (mean ± SEM, n=4–8).
which of these three possibilities is the most likely patch-clamp techniques should allow us to determine contractile frequency could be explained by increases in cardiac muscle. The effect of the hormone on underlying mechanisms responsible for the actions of All in cardiac tissue. The possibility that the onset of the All-mediated contractile response might involve an early, transient increase in IP_3 is unlikely since IP_3 levels were not significantly elevated above control values at either 10 minutes after addition of hormone, a time at which contractile frequency has started to increase in these preparations. This interpretation is confirmed by our finding that All increases both transient and steady-state I_{Ca} in cultured neonatal rat heart cells. These results can be attributed to increases in current due to shifts in d_0 and f_0 as well as changes in relative conductance (g). Such increases in g could occur through recruitment of previously inactive channels, increases in single channel conductance, and/or alterations in channel kinetics. Future studies using patch-clamp techniques should allow us to determine which of these three possibilities is the most likely explanation.

Cyclic Adenosine 3',5'-Monophosphate

A major goal of this study was to identify the underlying mechanisms responsible for the actions of All in cardiac tissue. The effect of the hormone on contractile frequency could be explained by increases in I_{Ca}, resulting in increased pacemaker potential. However, stimulation of I_{Ca} alone is inconsistent with negative inotropic responses evoked by All. Therefore, it is likely that other additional mechanisms are involved in the All response. It is well established that the total cardiostimulating effect of β-adrenergic agonists is mediated by multiple mechanisms in response to a single intracellular messenger, cAMP. However, our data indicate that cAMP is not important in mediating the actions of All since, under conditions where physiological responses are elicited, the hormone does not alter intracellular cAMP levels.

Phosphoinositide Hydrolysis

In contrast, under conditions where All increases spontaneous beating rate, the hormone stimulates phosphoinositide hydrolysis as well. Phosphoinositide hydrolysis is not an indirect effect of the All-mediated increase in I_{Ca} since increased inositol phosphate formation is not affected by addition of a calcium channel blocker. Two important products of the phosphoinositide pathway are inositol 1,4,5-trisphosphate, which mobilizes calcium from intracellular stores, and diacylglycerol, an activator of protein kinase C. Inositol phosphates (IP_1, IP_2, IP_3) were measured in parallel experiments, [3H]inositol-labeled myocytes were exposed to 5 nM All for 1 minute (open bar) or 10 minutes (stippled bar) prior to terminating the reaction. In parallel experiments, [3H]inositol-labeled myocytes were exposed to 10 μM nifedipine (NIF) for 1 minute prior to incubation with 5 nM All for 1 minute (solid bar) or 10 minutes (hatched bar). Each histogram represents the average percent of control from three experiments performed in duplicate (mean ± SEM). Control values (cpm) were IP_1, 170–370; IP_2, 50–100; IP_3, 400–800.
A Possible Role for Protein Kinase C

The activation of protein kinase C could explain both the negative inotropic effects and the increase in I_{Ca} evoked by All. Recently, investigators working with electrically stimulated adult rat myocardial cells and rat papillary muscle reported negative inotropic responses evoked by phorbol esters, which mimic diacylglycerol in their ability to activate protein kinase C. A 15% decrease in resting intracellular calcium concentration after phorbol ester treatment has also been reported. Other studies using noncardiac tissues have demonstrated that protein kinase C is involved in the activation of calcium current and the recruitment of new calcium channels, the phosphorylation of contractile and cytoskeletal proteins, and the regulation of smooth muscle contraction. It is also clear that protein kinase C modulates calcium channels differently in various noncardiac tissues. For example, activation of the enzyme in PC12 pheochromocytoma cells and snail neurons results in apparent decreases in calcium current. Taken together, the above observations, coupled with those reported in the present study, provide a rationale for further study of protein kinase C as a mediator of All action on heart cells.

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