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REGULATION BY ANGIOTENSIN II OF PHOSPHATE TRANSPORT IN CARDIAC MYOCYTES

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Abstract—The purpose of this study was to test the hypothesis that angiotensin II (ang II) affects the transport of inorganic phosphate in adult ventricular myocytes. Ventricular myocytes were isolated from Dahl rats and allowed to take up ^{32}P inorganic phosphate (^{32}P-P_i). The intracellular ^{32}P concentration increased rapidly and reached a peak at 5 min. Ang II, 10^{-10} to 10^{-5} M, produced a significant ($P < 0.05$) and concentration-dependent reduction in ^{32}P-P_i uptake that plateaued at 0.1 to 1.0 μM. Ang II at 1 μM produced a 30% reduction in V_{max} and a slightly greater reduction in the *Km* of ^{32}P-P_i uptake, compared to myocytes that were not exposed to ang II. The ang II receptor antagonist saralasin (Sar[1]-Val[5]-Ala[8]-angiotensin II), significantly ($P < 0.05$) antagonized the action of ang II on ^{32}P-P_i uptake. TPA (12-*O*-tetradecanoylphorbol-13-acetate) also produced a significant ($P < 0.05$) reduction of ^{32}P-P_i uptake, suggesting that protein kinase C is involved in the transduction of ang II effects on intracellular P_i. ^{32}P Efflux from myocytes, pulsed with ^{32}P-P_i and chased with P_i-free medium, was accentuated markedly by ang II; this effect was blunted by saralasin. These data suggest that ang II is capable of regulating total intracellular P_i in the heart via two actions: (i) by inhibiting uptake of P_i into the myocyte, and (ii) by increasing the efflux of phosphates out of the cell.

The octapeptide angiotensin II (ang II) has direct effects on the heart in a number of species. An increase in the force of contraction is produced by ang II in the intact heart, isolated atria and isolated papillary muscle preparations [1–8]. In the adult rat heart, ang II increases the force of contraction of the right ventricle [9], and in isolated adult rat ventricular myocytes, ang II increases contractility and delays relaxation velocity [10]. In cultured neonatal rat cardiac myocytes, ang II stimulates spontaneous contractile frequency concomitant with increases in stimulation of calcium current and polyphosphoinositide turnover [11]. The mechanism of action of ang II in the cardiomyocyte is not well understood. One potential site of action of ang II on the heart is through the regulation of intracellular phosphate.

Intracellular inorganic phosphate (P_i) may regulate metabolic processes such that increased intracellular P_i increases anerobic glycolysis [12], oxidative phosphorylation [13], and metabolic rate [14] and decreases insulin release [15]. P_i is one of the factors regulating cardiac contractility as increased intracellular P_i is associated with reduced myocardial contractility [16–19]. In cardiac cells in which creatine phosphate and, to a lesser extent, ATP are subject to rapid turnover, the size of the P_i pool fluctuates according to cellular energy demands [18–20] and is determined both by the rate of hydrolysis of high energy molecules to generate P_i [18, 21] and by the rate of influx and efflux across the sarcolemma [22]. Recently, P_i movement into bovine cardiac sarcolemmal vesicles has been found to involve a sodium-dependent co-transport process [23]. Despite its importance, P_i transport in the heart has been relatively neglected as a subject of investigation. Since the regulation of the intracellular P_i pool, by alterations of P_i influx and efflux, may influence cellular energetics, we endeavored to study the influence of ang II on both the influx and efflux of P_i. The results presented here are of 2-fold importance. First, they provide evidence of a novel action of ang II in adult rat ventricular myocytes, and second, they suggest a mechanism by which the control of intracellular P_i may be accomplished in the heart.

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EXPERIMENTAL PROCEDURES

Isolated adult rat cardiac myocytes. Viable adult cardiac myocytes were isolated based on the method of Kryski *et al.* [24]. Briefly, Dahl rats, 200–300 g, were anesthetized with halothane and killed by cervical dislocation. Hearts were excised, placed in oxygenated, 35°, Joklik Modified Minimal Essential Medium (Gibco/BRL Life Technologies, Burlington, Ontario), which does not contain calcium and to which has been added 1.2 mM $MgSO_4$, 23.8 mM $NaHCO_3$ and 0.5 mM L-carnitine (initial solution). The hearts were attached to a perfusion apparatus and perfused through the aortas with the same solution oxygenated and at 35° for 5 min, after which collagenase, type II (Worthington Biochemical Corp., Freehold, NJ, U.S.A.), fatty acid free bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 25 μM $CaCl_2$ were added to the perfusate for 20 min. Heart were removed from the perfusion apparatus, trimmed of connective tissue and atria, and placed in a solution similar to the initial one plus 1% bovine serum albumin and

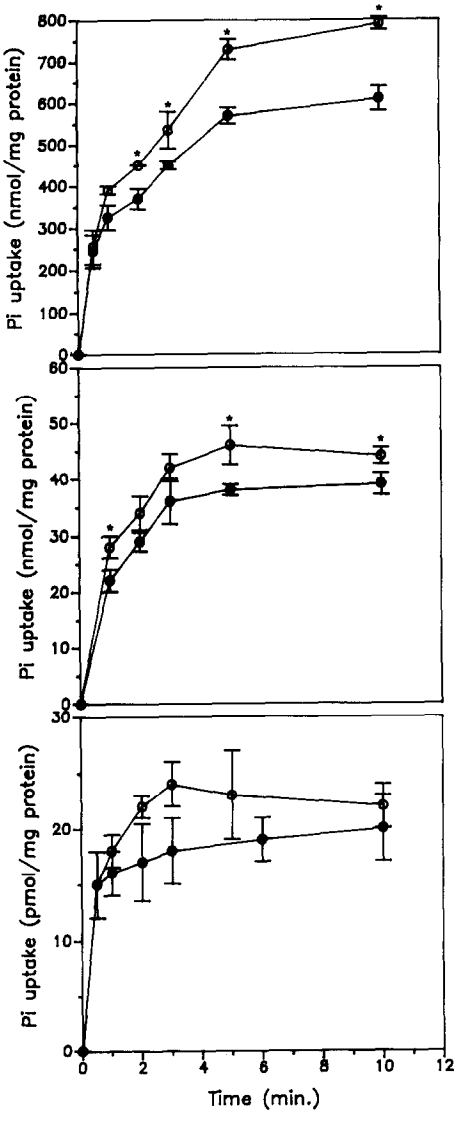

Fig. 1. Time course of P_i uptake in ventricular myocytes. Isolated ventricular myocardiocytes from 200–300 g Dahl rats were rinsed for 30 min in P_i-free DMEM before being added to ^{32}P-P_i pulse medium. Final mixtures contained either 5 mM P_i as KH_2PO_4 (upper panel), 0.5 mM P_i (middle panel) or 1 nM P_i (lower panel) plus carrier-free ^{32}P-P_i. Key: (○) untreated samples, and (●) samples containing 100 nM ang II. The uptake was stopped by rapidly pelleting the cells, washing them in cold phosphate-buffered saline, and lysing them. Experiments shown in each panel were repeated three times in triplicate. Each point is the mean ± SEM. An asterisk (*) indicates a significant difference from the treated samples at the 5% level ($P < 0.05$).

1.5 mM $CaCl_2$. The ventricles were minced and then shaken on a rotary shaker at 135 rpm at 35° after which cells were harvested. Myocytes were suspended in Dulbecco's Modified Eagle's Medium (DMEM) (Flow Laboratories, Product No. 12-332, Mississauga, Ontario) and used for experiments within 2 hr.

The isolated cells were counted in a hemocytometer chamber and assayed for viability by trypan blue exclusion. Viability was between 70 and 85%. The yield of viable cells was 20–25 million per heart.

Incorporation of ^{32}P *orthophosphate.* Isolated cells were rinsed in P_i-free DMEM and then placed for 30 min in P_i-free DMEM to lower intracellular P_i so that uptake of label was maximized. Each sample contained between 2.5 and 5.0 × 10^5 cells. Medium for uptake studies was made by adding KH_2PO_4 to an appropriate concentration plus carrier-free ^{32}P-P_i (Amersham Canada, Oakville, Ontario). Angiotensin II (Peninsula Laboratories, Belmont, CA, U.S.A.), Sar^1-Val^{5}Ala^8-angiotensin II (saralasin) (Peninsula Laboratories), isoproterenol (Sigma Chemical Co.) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Peninsula Laboratories) were dissolved in P_i-free DMEM. Uptake commenced with the addition of cells to uptake medium containing ^{32}P-P_i and ang II, saralasin or isoproterenol. Only the TPA experiments were carried out after preincubating the cells with TPA for 2 hr prior to the commencement of uptake.

To stop the uptake, cells were either diluted in 14 vol. of cold DMEM containing the same concentration of phosphate followed by centrifugation for 1 min at 50 *g* on an IEC benchtop centrifuge, or the cells were collected by rapid centrifugation for 5 sec on a Fisher micro-centrifuge (up to a maximum of 13,500 *g*). Pellets were then rinsed in ice-cold phosphate-buffered saline (PBS), centrifuged once more and lysed in 0.1% sodium dodecyl sulfate (SDS), 1% deoxycholate, 1% Triton X-100. ^{32}P Content was determined by scintillation counting in ACS (Amersham) scintillation fluid. Protein content of samples was determined by the method of Bradford [25].

Efflux of ^{32}P. Myocytes, 10^6 myocytes/sample, were incubated in P_i-free DMEM for 30 min. Samples were then pulsed for 1 hr with ^{32}P-P_i (5 μCi/mL). The medium was removed and replaced with fresh P_i-free DMEM containing ang II or saralasin. To determine the time course of release of ^{32}P, at selected times of exposure to ang II or saralasin or control, myocytes were centrifuged at 50 *g* for 45 sec and samples of supernatant were taken for scintillation counting. Although this method does not eliminate the possibility that lack of mixing of medium could leave unstirred layers, this approach was preferable to the cell damage caused by repeated centrifugation. Drug-treated and control samples should be subject to the same error in this respect. These experiments were carried out repeatedly in triplicate with SEM of less than 10%.

Data analysis. The data are presented as means ± SEM. Between group comparisons used analysis of variance, with pairwise comparisons. The kinetic parameters of uptake were calculated using Woolfe–Augustinsson–Hofstee transformation of non-linear least squares "best fit" for saturation isothermic assay data (London Software, Cleveland, OH, U.S.A.). Significance was set at the 5% level ($P < 0.05$).

RESULTS

Intracellular P_i uptake was measured over various

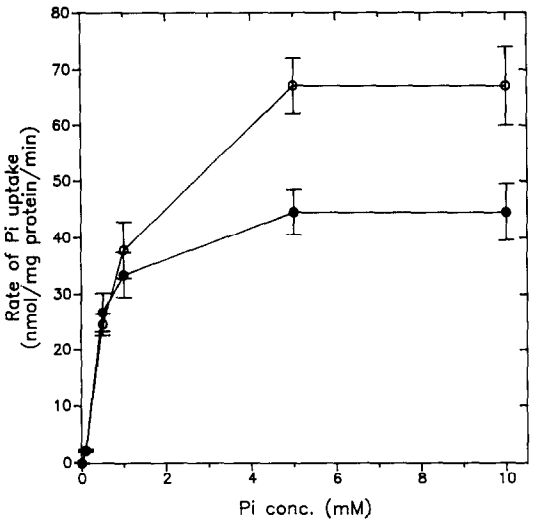

Fig. 2. Uptake of P_i in the presence of various P_i concentrations. ^{32}P-P_i uptake rates for the saturable component of total uptake were determined at 1 min with different concentrations of P_i (as KH_2PO_4) in the medium. Cardiac myocytes exposed to 100 nM ang II (●) are compared to control cells (○) not exposed to 100 nM ang II. The experiment was conducted in triplicate and the data are shown as the mean ± 1 SEM. In the control cells, V_{max} was 67 nmol/mg protein/min and K_m was 1 mM, while in ang II treated cells the V_{max} was 43 nmol/mg protein/min and K_m was 0.5 mM.

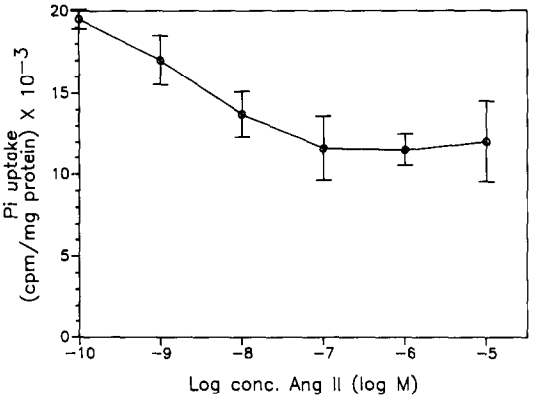

Fig. 3. Inhibition of P_i uptake in response to various ang II concentrations. The rate of uptake of ^{32}P-P_i in the presence of various concentrations of ang II in the medium was determined at 1 min of uptake when the concentration of P_i in the medium, as KH_2PO_4, was 0.5 mM. Experiments were repeated two times in triplicate. Each point is the mean ± SEM.

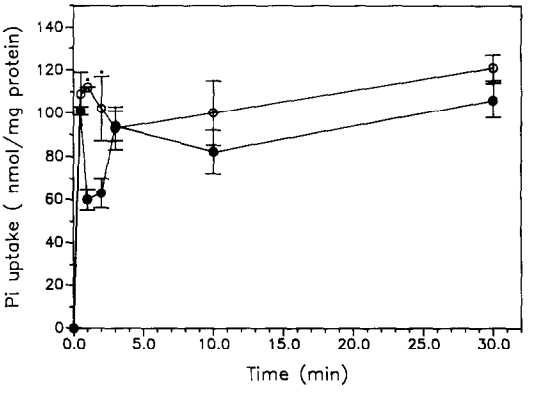

Fig. 4. Time course of ^{32}P-P_i uptake in the presence of isoproterenol. ^{32}P-P_i uptake was determined as described in Fig. 1 in the presence (●) or the absence (○) of 1 μM isoproterenol. Experiments were repeated two times in triplicate. Each point is the mean ± 1 SEM.

time periods up to 60 min. During this period the viability of isolated cardiac myocytes was unchanged as assessed by trypan blue exclusion and the absence of spontaneously contracting myocytes. Intracellular P_i concentration increased rapidly and reached a peak by 5 min, after which it remained relatively constant (Fig. 1). Ang II, 100 nM, reduced the initial uptake rate, at both millimolar and nanomolar concentrations of extracellular P_i. Based on these results, subsequent studies of uptake were conducted 1 min after incubation with ^{32}P-P_i in order to determine initial rates. The uptake curve was resolved graphically into saturable and non-saturable uptake components. The saturable component displayed Michaelis–Menten kinetics with a rapid P_i uptake rate in the presence of P_i concentrations from slightly above 0 mM P_i to 5 mM after which a plateau was apparent (Fig. 2). The kinetic parameters from six experiments, calculated using a Woolfe–Augustinsson–Hofstee transformation, indicated that ang II at 100 nM decreased the V_{max} to 72 ± 28 nmol/mg protein/min (± 1 SEM) compared to 116 ± 29 nmol/mg protein/min in control cells not exposed to ang II. The K_m was also reduced from 2.5 ± 0.1 mM P_i in control cells to 0.75 ± 0.25 mM P_i in cells exposed to ang II.

To define the relationship between inhibition of P_i uptake and the concentration of ang II, initial rates of uptake were measured at a range of ang II concentrations between 10^{-10} and 10^{-5} M (Fig. 3). Inhibition of P_i uptake was proportional to the log of ang II concentration reaching a plateau at about 0.1 μM, suggesting saturation. To determine whether the action of ang II on P_i uptake was common to that of a positive inotropic agent, untreated myocytes were compared to myocytes treated with 1 μM isoproterenol (Fig. 4). Rates of uptake until 1 min were not altered by exposure to isoproterenol. However, there was a marked transient decrease in intracellular ^{32}P concentration between 1 and 3 min in isoproterenol-treated samples, followed by a rapid recovery to levels similar to the controls.

Ang II treated cells took up significantly ($P < 0.05$) less P_i compared to controls (Fig. 5). Saralasin antagonized the inhibitory action of ang II on cellular P_i uptake. P_i uptake was 81 ± 6% of control in myocytes exposed to ang II compared to 87 ± 5% in myocytes simultaneously treated with equimolar (0.1 μM) saralasin and ang II while myocytes exposed

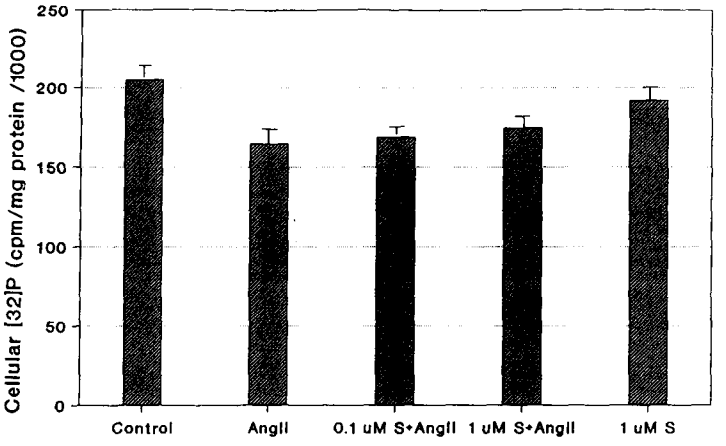

Fig. 5. Effect of the ang II antagonist saralasin on ang II-induced inhibition of P_i uptake. ^{32}P-P_i uptake was determined in the presence of saralasin (S) and angiotensin II (AngII). Uptake was determined at 1 min by assaying uptake in the presence of 0.5 mM P_i, as KH_2PO_4, in the medium. Cardiac myocytes were exposed to either 0.1 μM ang II, or 0.1 μM ang II plus 0.1 μM saralasin or 0.1 μM ang II plus 1 μM saralasin, or 1 μM saralasin alone or neither drug (control). Experiments were repeated two times in triplicate. The results are expressed as the mean ± 1 SEM, and the scale for the y-axis is divided by 1000 so that the largest number shown is 250,000 cpm/mg protein.

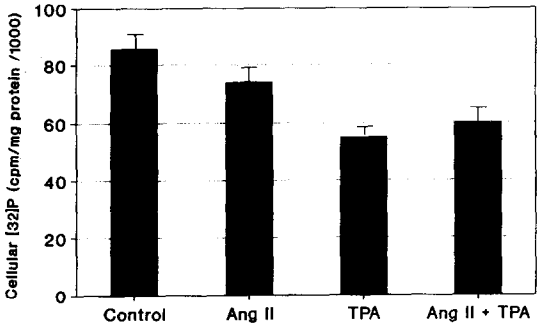

Fig. 6. Effect of TPA on P_i uptake. ^{32}P-P_i uptake was determined in the presence of 0.5 mM P_i, as KH_2PO_4. Cardiac myocytes were exposed to either 1 μM ang II or 160 nM TPA or both or neither agent (control). TPA was dissolved in 0.01% dimethyl sulfoxide. Experiments were repeated three times in triplicate. Each point is the mean ± 1 SEM, and the scale for the y-axis is divided by 1000 so that the largest number shown is 100,000 cpm/mg protein.

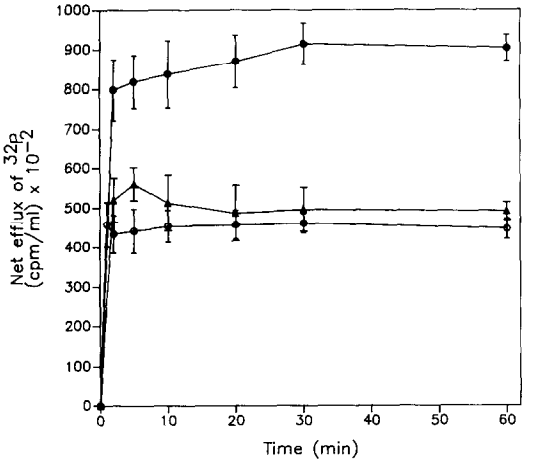

Fig. 7. Ang II stimulation of ^{32}P efflux. Cardiac myocytes (10^6 myocytes/mL) in 1 mL were incubated in 5 μCi/mL $^{32}P_i$-containing DMEM for 1 hr. The pulse was replaced by P_i-free DMEM plus 100 nM ang II (●) or 100 nM ang II with 1 μM saralasin (△) or neither drug, control (○). Cells were spun at 50 g to form a pellet. Samples (10 μL) of supernatant were removed over the time course and counted by liquid scintillation. Experiments were repeated three times in triplicate. Each point is the mean ± SEM, and the scale for the y-axis is multiplied by 10^{-2} so that the largest number shown is 100,000 cpm/mL.

to 1 μM saralasin plus ang II (0.1 μM) took up 91 ± 4% of the amount of P_i taken up by control cells. Saralasin alone produced a minimal inhibition of P_i uptake.

Exposure of cells for 1 hr to 160 nM TPA significantly ($P < 0.05$) reduced P_i uptake (Fig. 6). TPA inhibited P_i uptake by 45 ± 5%. Ang II did not produce further inhibition of P_i uptake in the presence of TPA. Viability of cells was not affected by exposure to TPA, ang II or the combination of TPA and ang II.

^{32}P Efflux from myocytes pulsed with ^{32}P-P_i and chased with P_i-free medium was measured in the medium during a time course of 60 min (Fig. 7). A rapid accumulation of extracellular ^{32}P occurred up to 10 min in the presence of 100 nM ang II but not in control cells. Cardiac myocytes exposed to 100 nM ang II, plus 100 nM saralasin expelled consistently less ^{32}P into the extracellular medium compared to those exposed to ang II alone. No increase in ^{32}P

efflux was observed in myocytes not exposed to ang II.

DISCUSSION

The present study found that P_i uptake by the cardiac myocyte is inhibited by ang II. A reduction in both V_{max} and K_m was observed. Although in principle a compound that decreases both of these parameters of uptake should increase uptake at some low substrate concentration, this was not observed in the present study perhaps because sufficiently low concentrations of P_i could not be attained in the medium. The inhibition of P_i uptake was demonstrated to be a specific ang II mediated action as the ang II competitive inhibitor, saralasin, blocked the effect of ang II on P_i uptake. Saralasin has a small partial agonistic action at the ang II receptor and itself produced a small reduction in P_i uptake.

Recent studies have shown manifold effects of ang II on adult rat heart. Isolated adult rat ventricle and ventricular myocytes respond to ang II with changes in both contractility and relaxation [9, 10]. Other effects in adult rat heart produced by ang II stimulation include induction of Egr-1 gene [26], phosphorylation of proteins in hypertrophic cells [27], and inhibition of adenylyl cyclase activity in the sarcolemma [28].

There is accumulating evidence that a complete renin-angiotensin system exists within the heart. Renin substrate, renin, angiotensin-converting enzyme activity as well as ang II have been detected in the ventricular myocyte of the adult heart [29–31]. Thus, the required components are in place for a functional angiotensin autocrine or intracrine system within the ventricular myocyte. This may implicate a role for endogenous angiotension to "regulate" P_i flux in the heart.

While ang II-induced alteration of P_i uptake may be secondary to other effects of ang II within the cell, it is not likely to be secondary to an increase in contractile frequency or force of contraction [1–11]. Isoproterenol, which produces a much greater positive inotropic action on the heart than ang II, displayed a distinctly different pattern of influence on intracellular ^{32}P compared to ang II. Initial uptake of ^{32}P-P_i was not affected significantly by isoproterenol. However, a subsequent transient decrease in intracellular ^{32}P occurs thereafter perhaps secondary to increased P_i efflux with isoproterenol [32].

Ang II-induced alteration of P_i transport may be mediated by protein kinase C. The phorbol ester TPA strongly inhibited P_i uptake and this effect was not additive to the effect of ang II. Since phorbol esters are known to activate protein kinase C [33], and in other cells such as vascular smooth muscle cells, protein kinase C has been strongly implicated as a component of the signal transduction pathway of ang II [34], it is likely that the effect of ang II on P_i uptake is transduced by protein kinase C. Support for the transduction of ang II signalling through protein kinase C also comes from studies on neonatal rat cardiac cells in which phosphoinositide turnover and calcium channel activity are stimulated by both ang II and phorbol esters [2, 35]. Furthermore, TPA induces phosphorylation of myocardial proteins which are phosphorylated by ang II stimulation [27].

Ang II also enhanced ^{32}P efflux from myocytes. This represents, at least in part, increased P_i efflux although the identity of the labeled expelled species was not ascertained and could be composed of phosphorus-containing metabolites as well. In this experiment intracellular ^{32}P was not determined simultaneously with the measured accumulation in the medium; however, it is reasonable to assume that intracellular ^{32}P would decrease since it is the sole source of ^{32}P. The action of ang II may be on an efflux mechanism or reverse exchanger transporting P_i out of the cell. It is unlikely to be due to ang II inhibition of P_i uptake which could shift the equilibrium between the intracellular P_i pool and the extracellular pool. Similar observations have been reported by Medina and Illingworth [36] from studies in which they examined the effluent ^{32}P-P_i from perfused rat hearts using a continuous flow apparatus which eliminates re-uptake of pooled counts. The present experiments extend the observation of increased P_i efflux observed by Medina and Illingworth by demonstrating that it is due to activity in the cardiomyocyte fraction of the heart. An important additional result from the present study is the partial inhibition of the ang II effect on expelled ^{32}P by the competitive ang II inhibitor saralasin, indicating that the action of ang II on efflux is mediated through ang II receptors.

In summary, ang II was found to both inhibit P_i uptake and enhance ^{32}P efflux. Thus, the net effect of ang II on intracellular P_i is to decrease P_i. The study did not measure free P_i in the cell. With this caveat, the evidence of novel ang II actions on the ventricular myocyte suggests a role for ang II as an inhibitor of intracellular P_i in these cells. Considering the relationship between P_i content and mechanical performance of the heart [16–19] and the postulated direct effect of P_i on the acto-myosin complex [17], an ang II-stimulated reduction of intracellular P_i in cardiac cells could play a role in increasing myocardial contractile force.

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