P2X7 receptor agonists pre- and postcondition the heart against ischemia-reperfusion injury by opening pannexin-1/P2X7 channels

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Submitted 28 March 2011; accepted in final form 15 June 2011

Vessey DA, Li L, Kelley M. P2X7 receptor agonists pre- and postcondition the heart against ischemia-reperfusion injury by opening pannexin-1/P2X7 channels. Am J Physiol Heart Circ Physiol 301: H881–H887, 2011. First published June 17, 2011; doi:10.1152/ajpheart.00305.2011.—Protection of the heart from ischemia-reperfusion injury can be achieved by ischemic preconditioning and ischemic postconditioning. Previous studies revealed that a complex of pannexin-1 with the P2X7 receptor forms a channel during ischemic preconditioning and ischemic postconditioning that results in the release of endogenous cardioprotectants. ATP binds to P2X7 receptors, inducing the formation of a channel in association with pannexin-1. We hypothesized that this channel would provide a pathway for the release of these same cardioprotectants. Preconditioning-isolated perfused rat hearts with 0.4 μM ATP preceding 40 min of ischemia minimized infarct size upon subsequent reperfusion (5% of risk area) and resulted in >80% recovery of left ventricular developed pressure. Postconditioning with ATP after ischemia during reperfusion was also protective (6% infarct and 72% recovery of left ventricular developed pressure). Antagonists of both pannexin-1 (carbenoxolone and melfloquine) and P2X7 receptors (brilliant blue G and A438079) blocked ATP pre- and postconditioning, indicating that ATP protection was elicited via the opening of a pannexin-1/P2X7 channel. An antagonist of binding of the endogenous cardioprotectant sphingosine 1-phosphate to its G protein-coupled receptor diminished protection by ATP, which is also consistent with an ATP-dependent release of cardioprotectants. Suramin, an antagonist of binding of ATP (and ADP) to P2Y receptors, was without effect on ATP protection. Benzoyl benzoyl-ATP, a more specific P2X7 agonist, was also a potent pre- and postconditioning agent and sensitive to blockade by pannexin-1/P2X7 channel antagonists. The data point out for the first time the potential of P2X7 agonists as cardioprotectants.

benzoyl benzoyl adenosine 5'-triphosphate; cardioprotection; G protein-coupled receptors; P2X7 receptor; hemichannels; phospho-Akt; sphingosine 1-phosphate

LOSS OF CORONARY BLOOD FLOW to the heart for extended periods of time leads to cell injury and impaired function (2, 13). Within a certain time frame, this damage is the result of events largely occurring upon subsequent reperfusion (2, 7, 9, 13, 18, 21, 38), and thus it is referred to as ischemia-reperfusion injury. However, the potentially damaging events associated with ischemia-reperfusion injury are largely preventable or reversible with appropriate treatments initiated either before (preconditioning) or immediately after (postconditioning) the index ischemia (20, 21, 24, 35, 38, 39). In fact, the use of multiple cardioprotective regimens has demonstrated that the ex vivo rat heart can recover fairly well from up to 90 min of global ischemia (33).

The earliest studies of ischemic pre- and postconditioning demonstrated that short nondamaging cycles of ischemia-reperfusion either before or immediately following the index ischemia result in cardioprotection (20, 21, 23, 35, 38, 39). This protection results from the release from myocytes of endogenous cardioprotectants that bind to cell surface G protein-coupled receptors (GPCRs) and trigger cell signaling pathways that lead to protection (3, 10, 20, 24, 25, 28, 33–35). We found that the release of these endogenous cardioprotectants from myocytes in response to ischemic preconditioning or ischemic postconditioning occurs via the opening of a channel formed by the interaction of a P2X7 purinergic receptor with a pannexin-1 hemichannel (36, 37). With ischemic preconditioning and ischemic postconditioning, the formation of this channel is triggered by a brief ischemia followed by reperfusion. It would be potentially very advantageous if this channel could be opened pharmacologically. Because of the potential of this channel as a site for intervention in preventing damage from myocardial infarction, we explored the possibility of opening this channel with P2X7 receptor agonists.

ATP is the primary endogenous agonist for the P2X7 purinergic receptor (30). The activation of the P2X7 receptor by ATP leads to the recruitment of pannexin-1 and can mediate the opening of a channel (27). Thus ATP-induced formation of pannexin-1/P2X7 channels should support cardioprotectant release from myocytes (36, 37). It could potentially be as effective as ischemia-reperfusion-induced opening of these channels. However, very little attention has been paid to the cardioprotective effects of ATP. This is partly because extra-cellular ATP can be hydrolyzed by a number of enzymes (31) and cardioprotection is thought to be due to the degradation product adenosine, which is a known cardioprotectant (3, 25). In this article, we explore the ability of ATP and another P2X7 agonist, benzoyl benzoyl-ATP (BzATP), to directly promote cardioprotection against ischemia-reperfusion injury by opening the pannexin-1/P2X7 channel with the release of endogenous cardioprotectants.

MATERIALS AND METHODS

Materials. ATP, 2(3')-O-(4-benzoyl benzoyl)-ATP (BzATP), brilliant blue G (BBG), carbenoxolone (CBX), melfloquine, suramin, triphenyltetrazolium chloride (TTC), and wortmannin were obtained from Sigma. N-Ethylhalothane-1-phosphate (SIP) was obtained from Biomol Research Laboratories. VPC23019 (VPC) was obtained from Avanti Polar Lipids. A438079 hydrochloride 3-[[5-(2,3-dichlorophenyl)-1H-tetrazol-1-yl]ethyl]pyridine hydrochloride was obtained from Tocris Bioscience.

Langendorff ex vivo-perfused heart. The use of animals in this study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, Washington DC, 1996) and was approved by the Institutional Animal Care and
Use Committee. Hearts from ca. 250-g male Sprague-Dawley rats were removed under pentobarbital sodium anesthesia and mounted on a Langendorff apparatus, and buffer was perfused as previously described (32). During the index ischemia, the heart was lowered into a thermostated chamber that maintains an ambient temperature of 37°C. Left ventricular developed pressure (LVDP) was measured using a Millar micromanometer-tipped catheter inserted into the left ventricle and is reported as a percentage of the preindex ischemia pressure. Infarct size was measured at the end of reperfusion by TTC staining, followed by computer analysis as previously described (32).

The standard protocol for producing ischemia-reperfusion injury consisted of an initial equilibration of the heart for 30 min, followed by 40 min of sustained global ischemia (index ischemia), and then 40 min of reperfusion. Pharmacological preconditioning by ATP or BzATP consisted of a 1-min perfusion with 0.4 μM ATP or 50 nM BzATP, beginning at the 15-min point of the equilibration period. When antagonists were tested for their effect on ATP or BzATP preconditioning, they were present for the entire duration of the equilibration period, including the ATP or BzATP infusion. Pharmacological postconditioning with ATP or BzATP was achieved by including 0.4 μM ATP or 50 nM BzATP in the reperfusion buffer.

When antagonists were tested for their effect on ATP or BzATP postconditioning, they were present for the entire 40 min of reperfusion. To prepare the ATP or BzATP solution, a stock solution was added to perfusion buffer. BBG (2 μM), CBX (5 μM), melفاquine (0.4 μM), VPC (1 μM), and suramin (1 μM) were administered by dissolving them directly in the perfusion buffer. These antagonists by themselves are without effect on ex vivo heart hemodynamics (left ventricular end-systolic and end-diastolic pressures, LVDP, heart rate, and maximum rate of left ventricular pressure change over 2 h of perfusion).

Western blot analysis. Hearts were homogenized in 0.13 M KCl, 20 mM HEPES (pH 7.4), 1 mM EGTA, 1 μg/ml leupeptin, and 0.25 μg/l each of aprotinin and pepstatin A. An aliquot of the homogenate was removed for testing, and the remainder was centrifuged at 100,000 g to generate a particulate and a cytosolic fraction. The particulate fraction was washed once with the isolation buffer. Western blot analysis was conducted on the homogenate as previously described (36). For Western blot analysis, the following antibodies (Cell Signaling Technology) used were as follows: Akt, phospho-Akt (Ser473, No. 3080), and p110 (No. 4060) and phosphatidylinositol 3-kinase (PI3K; p85, No. 4257; Signal Technology) used were as follows: Akt, phospho-Akt (Ser473, No. 3080), and p110 (No. 4060). Protein concentration was determined using the detergent compatible DC Protein Assay kit from Bio-Rad and used to equalize the protein concentration in all samples. Each lane was loaded with precisely 5 μg of protein.

Statistical analysis. Data are expressed as means ± SE. Statistical comparison of means was performed using ANOVA followed by Student-Newman-Keuls post hoc testing. A P value of <0.05 was considered statistically significant.

RESULTS

Ischemia-reperfusion injury occurs when ex vivo hearts, preequilibrated for 30 min, are exposed to 40 min of global ischemia (the "index ischemia"), followed by 40 min of reperfusion (control, Fig. 1). As previously shown (32–35), the recovery of hemodynamic function, assessed as LVDP, is poor, and at the end of 40 min of reperfusion, these hearts have large areas of infarction by TTC staining. Treating ex vivo hearts at the midpoint of the equilibration period (before ischemia) with a 1-min perfusion of 0.4 μM ATP greatly improved the recovery of LVDP during reperfusion (79 ± 5% of its preischemic value compared with 6 ± 2% for 1 min buffer treatment). Furthermore, ATP treatment nearly eliminated areas of infarction (infarct size was only 5 ± 1% compared with 34 ± 1% for buffer control). Continuous perfusion with 0.4 μM ATP for the entire equilibration period did not alter the response but was considered less desirable for having a washout period before index ischemia. Continuous perfusion with 0.4 μM ATP for 150 min was without effect on hemodynamic function. It can be concluded that ATP is capable of pharmacologically preconditioning the ex vivo heart.

Pharmacological postconditioning with ATP (Fig. 2) by including 0.4 μM ATP in the reperfusion medium from the start of reperfusion also resulted in an excellent recovery of LVDP during reperfusion (72 ± 2% of its preischemic value) and dramatically reduced the infarct size (6 ± 0.3%). Initiating reperfusion with a 1-min perfusion with 0.4 μM ATP was slightly less effective than the continuous 40-min perfusion, and so the latter was employed for postconditioning studies. It is clear from these data that ATP is an effective pharmacological pre- and postconditioning agent.

The effect of exogenous ATP could be due to ATP binding to either ligand-gated receptors of the P2X receptor family and/or to GPCRs of the P2Y receptor family. We previously...
produced cardioprotection. Thus, after index ischemia and reperfusion, it resulted in only a 7 ± 1 and 8 ± 2% recovery of LVDP, respectively, and significant areas of infarction (35 ± 0.4 and 32 ± 2%, respectively), both of which are statistically different (P < 0.05) relative to the ATP alone (70% recovery of LVDP and 5% infarct). Likewise, preconditioning by ATP (Fig. 1) was also abolished in the presence of the pannexin-1 channel blockers 2 μM CBX or 0.4 μM mefloquine (4 ± 0 and 9 ± 4% recovery of LVDP and 39 ± 2 and 31 ± 7% infarct size, respectively). At much higher concentrations, CBX and mefloquine can also inhibit connexins. To rule out connexin effects, we checked to see whether a selective inhibitor of connexin-43, GAP-27 (5), altered ATP preconditioning. In the presence of 40 μM GAP-27, 0.4 μM ATP still resulted in 91 ± 2% recovery of LVDP with only a 4.5 ± 0.4% infarct size. Thus any effect that CBX or mefloquine would have on connexin-43 would be inconsequential to ATP preconditioning. Thus it appears that it is a pannexin-1/P2X7 channel blockade that inhibited the primary component of preconditioning by ATP. We also evaluated the role of this channel in postconditioning by ATP. Equilibrated hearts were exposed to 40 min of index ischemia and then reperfusion with 0.4 μM ATP for the first minute of reperfusion. While protective in terms of infarct size, this procedure was not as effective at restoring hemodynamic function (50 ± 3% recovery of LVDP) as adding 0.4 μM ATP to the reperfusion buffer for the entire 40 min of reperfusion. Continuous reperfusion with 0.4 μM ATP resulted in 72 ± 2% recovery of LVDP and 6 ± 0.3% infarct size. Reperfusion with buffer containing 0.4 μM ATP plus the P2X7 receptor antagonist CBX (2 μM) or A438079 (1 μM) resulted in an extensive diminution of the cardioprotection by ATP. As shown in Fig. 2, hearts treated at reperfusion with 0.4 μM ATP in the presence of 2 μM BBG only had an 9 ± 0% recovery of LVDP and an infarct size of 36 ± 2%, and in the presence of 1 μM A438079, the recovery of LVDP was 5 ± 2% and the infarct size of 35 ± 3%. Both values are statistically different (P < 0.05), relative to reperfusion with ATP in the absence of antagonist.

ATP postconditioning was also blocked by the pannexin-1 channel blocker CBX, present at a concentration of 5 μM during the entire reperfusion phase. The recovery of LVDP was reduced from 72 ± 2% for ATP alone to 8 ± 2% in the presence of CBX, and the infarct size was increased from 6 ± 0.3 to 30 ± 1% (P < 0.05). Likewise (Fig. 2), the addition of 0.4 μM mefloquine along with ATP at reperfusion blocked postconditioning (7 ± 1% recovery of LVDP and 30 ± 2% infarct size). Thus pannexin-1 channel blockade at reperfusion also inhibited an important component of postconditioning by ATP. These combined studies suggest that ATP is able to also open a pannexin-1/P2X7 purinergic receptor channel at reperfusion that releases sufficient cardioprotectant to postcondition the heart.

Since the primary cardioprotective effect of ATP appears to be accomplished by releasing of endogenous mediators, antagonists of the binding of these released endogenous mediators to their respective GPCRs should interfere with ATP-induced cardioprotection. A primary endogenous cardioprotectant released via the pannexin-1/P2X7 channels is S1P (35–37). The binding of S1P to its receptor can be blocked by VPC (4). For ATP postconditioning (Fig. 2), the addition of 1 μM VPC demonstrates that a complex of P2X7 purinergic receptors with pannexin-1 leads to the formation of channels that allow the release endogenous cardioprotective agents (36, 37). Since the primary agonist for P2X7 receptors is ATP (31), we wanted to determine whether cardioprotection by ATP is based on the opening of the pannexin-1/P2X7 channel. To address this question, we examined the effects of selective antagonists of the rat P2X7 purinergic receptor, BBG and A438079, and of pannexin-1 hemichannels, CBX and mefloquine (1, 12, 14, 16, 22). BBG, CBX, and mefloquine, at the concentrations employed in these experiments, have been previously shown to be without effect on hemodynamic function of ex vivo hearts over 2 h of continuous perfusion and did not lead to any areas of infarction (36). Furthermore, at the concentrations used in these experiments, BBG and CBX did not interfere with pharmacological preconditioning by 0.4 μM S1P or adenosine (36). We tested A438079 and found that after >2 h of continuous perfusion, it was without effect on left ventricular end-systolic and end-diastolic and developed pressures and also did not lead to any areas of infarction. We first tested the effect of these antagonists on pharmacological preconditioning induced with a 1-min infusion of 0.4 μM ATP. As shown in Fig. 1, if preconditioning with ATP was conducted in the presence of 2 μM BBG or 1 μM A438079 to block ATP binding to the P2X7 receptor, then it failed to
along with ATP led to diminished cardioprotection. The recovery of LVDP was only 8 ± 3% and the infarct size was 28 ± 1%. This is significantly different (P < 0.05) from ATP alone (72 ± 2% LVDP and 6 ± 0.5% infarct size). Likewise, if 1 µM VPC is present along with ATP during preconditioning (Fig. 1), the recovery of LVDP is reduced to 20 ± 8% and infarct size is increased to 20 ± 3%. This decrease in ATP protection upon blocking S1P binding to cell-surface GPCRs supports the interpretation that ATP has a primary effect of inducing the release of S1P, and other cardioprotectants, via pannexin-1/P2X7 channels. The complete blockade of cardioprotection is not obtained, because other cardioprotectants, e.g., adenosine, are also released and also contribute to cardioprotection (35).

Exogenous ATP also could be acting by binding to GPCRs of the P2Y receptor family either directly or after hydrolysis to ADP. To examine the P2Y family of receptors, we studied the effect of the nonselective P2Y antagonist suramin (19, 31) on ATP pre- and postconditioning. Suramin has been shown to block P2Y receptors in isolated perfused hearts at a concentration of 1 µM (19). When hearts were pharmacologically preconditioned with ATP in the presence of 1 µM suramin (Fig. 1), the recovery of hemodynamic function and infarct size were not significantly changed (75 ± 7% LVDP and 7 ± 1% infarct size) relative to ATP alone (79 ± 5% LVDP and 5 ± 1% infarct size). Postconditioning by ATP was also relatively insensitive to 1 µM suramin (69 ± 3% LVDP and 6 ± 1% infarct size with suramin relative to 72 ± 2% LVDP and 6 ± 0.3% infarct size with ATP alone). These data add further support to the hypothesis that ATP cardioprotection is elicited primarily via the opening of the pannexin-1/P2X7 channel.

The most potent P2X7 agonist known to date is BzATP (6). In our system (Fig. 3), a 1-min infusion of 50 nM BzATP during equilibration was able to effectively precondition ex vivo hearts (76 ± 6% recovery of LVDP and an infarct size of 5 ± 0%). If preconditioning with 50 nM BzATP was conducted in the presence of 2 µM BBG, then the subsequent index ischemia and reperfusion resulted in only a 5 ± 1% recovery of LVDP and there were significant areas of infarction (38 ± 3%). Both values were statistically different (P < 0.05) relative to BzATP alone. Likewise, preconditioning with BzATP is blocked (Fig. 3) by cotreatment with 5 µM CBX (4 ± 1% LVDP and 44 ± 2% infarct). BzATP preconditioning was unaffected by the presence of 1 µM suramin (74 ± 6% LVDP and 5 ± 0.2% infarct size), suggesting specificity for P2X receptors.

Postconditioning with 50 nM BzATP present during reperfusion (Fig. 4) was also able to effectively protect ex vivo hearts (83 ± 6% recovery of LVDP and an infarct size of 5 ± 0.5%). If postconditioning with 50 nM BzATP was conducted in the presence of 2 µM BBG, then the subsequent index ischemia and reperfusion resulted in only a 7 ± 0.3% recovery of LVDP and there was a 42 ± 2% infarct size, both values being statistically different (P < 0.05) relative to BzATP alone. BzATP cardioprotection was similarly eliminated by 5 µM CBX (7 ± 3% LVDP, 38 ± 2% infarct). In contrast, 1 µM suramin had little effect on BzATP postconditioning (64 ± 4% LVDP, 5 ± 0.2% infarct), again suggesting specificity for P2X receptors.

The most prominent feature of cardioprotection in rat hearts is the GPCR-dependent activation of the PI3K signaling pathway leading to the phosphorylation of Akt (11, 17, 23, 24, 34, 37). Western blot analysis of the PI3K regulatory subunit p85 in homogenates (Fig. 5) revealed that, for untreated hearts, ischemia results in dramatically decreased levels of p85 (P < 0.5). The 110β/p85 form of PI3K is known to be responsive to GPCR agonists (23). Total Akt was revealed by Western blot analysis of homogenates (Fig. 5) to also be depleted during ischemia (P < 0.05 compared with untreated heart). ATP preconditioning results in a substantial preservation of the level of p85 and total Akt during ischemia (P < 0.05 relative to untreated heart). As a result, at the beginning of reperfusion, ATP-treated hearts are poised to rapidly generate phospho-Akt and were found to do so, presumably in response to GPCR stimulation, by released cardioprotectants during the first 10 min of reperfusion (Fig. 5). As expected, blocking the ATP opening of the pannexin-1/P2X7 channel during preconditioning with 5 µM CBX interferes with the preservation of the level of p85, total Akt, and phospho-Akt (P < 0.05 relative to absence of agonist) during ischemia and prevents the recovery of phospho-Akt levels upon reperfusion. ATP directed postconditioning (Fig. 5) was also associated with rapid recovery of phospho-Akt levels during the first 10 min of reperfusion, and this recovery is blocked by cotreatment with 5 µM CBX.
In this article we provide clear evidence that treating the ex vivo heart with low concentrations of ATP, either before or after the index ischemia, can lead to cardioprotection, i.e., ATP can effectively pharmacologically pre- and postcondition the heart. ATP is the primary endogenous agonist for the P2X7 purinergic receptor (30), and activation of the P2X7 receptor by ATP also leads to the recruitment of pannexin-1 and the opening of a nonselective channel (27). We previously demonstrated that the heart contains both pannexin-1 and P2X7 (36) and that brief ischemia can induce their interaction and lead to a channel that provides for the release from myocytes of endogenous cardioprotectants such as S1P and adenosine (36, 37). In this study we sought to provide evidence that ATP induces an interaction of P2X7 with pannexin-1, forming a channel for cardioprotectant release, and that this is the basis for the ability of ATP to protect against ischemia-reperfusion injury.

Potential targets for extracellular ATP include both ligand-gated receptors of the P2X receptor family, such as P2X7, and also GPCRs of the P2Y receptor family (6, 31). To assess the role of these different receptors in ATP protection, we made use of selective receptor antagonists. To examine the role of ATP-binding to P2X7 receptors and subsequent interaction with pannexin-1 hemichannels, we used antagonists of both P2X7 and pannexin-1. The first class of antagonists studied were those antagonizing bindings of ATP to the P2X7 receptor.

Both BBG and A438079 have been shown to be selective antagonists of the P2X7 receptor (14, 22). They are both without effect on heart function, and further study of BBG revealed that it does not interfere with pharmacological pre- or postconditioning by either S1P or adenosine (36). Both BBG and A438079 were found to effectively block pharmacological pre- and postconditioning by ATP. This indicates that ATP binding to the P2X7 receptor is required for cardioprotection by ATP.

The second class of antagonists studied was pannexin-1 antagonists. They were mefloquine and CBX (1, 16). Although both have been employed to block connexins, CBX has not been reported to be inhibitory to connexins at the concentration employed in this paper (5 μM), and likewise for mefloquine (0.4 μM). Both are considered specific for pannexin-1 at concentrations employed in this study (36). Furthermore, they are both without effect on heart function, and CBX was shown...
not to interfere with pharmacological pre- or postconditioning by either S1P or adenosine (36). At the relatively low concentrations employed in these studies, both of these antagonists effectively blocked pharmacological pre- and postconditioning by ATP. This indicates that ATP binding to the P2X7 receptor requires further interaction with pannexin-1 hemichannels to complete the cardioprotective effect. Taken together, these data provide evidence that ATP pre- and postconditioning require the formation of the nonselective pannexin-1/P2X7 channel.

The effectiveness of antagonists to pannexin-1/P2X7 channel in blocking cardioprotection by ATP suggests that the release of cardioprotectants via this channel accounts for the majority of the response to ATP. Thus it would appear that very little of the cardioprotective effect can be attributed to ATP activation of P2Y receptors. This is supported by the fact that the nonspecific P2Y receptor antagonist suramin (6, 31) did not inhibit ATP pre- or postconditioning. The fact that suramin also blocks certain P2X receptors, but not P2X7 (6, 31), further supports the specific role of the P2X7 receptor.

CBX and mefloquine have also been used to inhibit connexins, but as previously discussed (36), this only occurs at much higher concentrations than were employed here to inhibit pannexin-1. Nevertheless, the possibility that CBX or mefloquine were having effects related to inhibition of connexins was ruled out by showing that the inhibition of connexin-43, the predominant connexin in the heart, with the mimetic peptide GAP-27 (5) did not interfere with ATP preconditioning.

One might expect a small contribution to cardioprotection arising from adenosine generated from ATP by extracellular hydrolytic enzymes (31). However, adenosine-driven cardioprotection is insensitive to CBX or BBG (36), and since ATP cardioprotection is essentially eliminated by these agents, adenosine is not a significant source of protection. Thus it appears that the primary effect of ATP is to trigger the release of other cardioprotectants. The lack of effect of the P2Y antagonist suramin says that other potential endogenous cardioprotectants such as UTP, pyridoxal phosphate, ADP, etc., which have their effect via binding to P2Y receptors, either are not released via the this channel or that their release is not making as significant of a contribution as adenosine or S1P, or other possible GPCR agonists.

It can be concluded that the most likely explanation for the role of pannexin-1/P2X7 channels in ATP cardioprotection is that they are supporting the release of GPCR-targeted cardioprotectants. Primary among these cardioprotectants is S1P (35). If the release of S1P is required for ATP protection, then antagonism of S1P receptor binding should diminish the protection by ATP. Indeed, the antagonism of S1P binding to its GPCR with VPC diminished the cardioprotection provided by both ATP preconditioning and ATP postconditioning (Figs. 1 and 2). All of these data add further support to the conclusion that exogenous ATP can trigger the release of endogenous cardioprotectants via pannexin-1/P2X7 channels both before ischemia (preconditioning) or during the early stages of reperfusion following the ischemia (postconditioning).

The most potent P2X7 agonist known to date is BzATP (6). In our system BzATP at 50 nM was also able to both pre- and postcondition ex vivo hearts (Fig. 3). This cardioprotection was eliminated by the pannexin-1/P2X7 blockers BBG and CBX. Agonists such as BzATP, which are potentially more potent and selective agonists for the P2X7 receptor than ATP, might avoid some of the side effects of ATP such as a potential for the induction of arrhythmias (8).

PI3K and Akt are important components of the cardioprotection signaling pathway that leads to the key cell survival molecule phospho-Akt (11, 17, 23, 24, 34, 37). As reported previously (37) and shown in Fig. 5, after extended ischemia, non-ATP-treated cells are deficient in PI3K and total Akt and therefore poorly prepared to support signaling driven by released GPCR agonists at reperfusion. However, in hearts preconditioned with ATP, there is a preservation of these key signaling proteins during ischemia so that rapid recovery is supported at full reperfusion because of a more effective Akt phosphorylation (Fig. 5). With ATP postconditioning, these proteins rapidly recover during early reperfusion, which supports effective Akt phosphorylation and recovery.

As mentioned in RESULTS, preconditioning with 0.4 μM ATP was equally effective whether administered as a 1-min dose or a 30-min continuous perfusion. This indicates that excess ATP does not cause the channel to stay open, a situation that could lead to extensive leakage of cellular components. This is consistent with the findings in other systems where the opening of the pannexin-1/P2X7 channel was found to be transient since it is inhibited by released permeant ATP (15, 26). This results in a tight control over channel opening.

The demonstrated ability of ATP to induce pharmacological pre- and postconditioning raises the question of whether ATP plays a role in ischemic pre- or postconditioning. This remains for future studies to determine.

A possible difficulty associated with pharmacological pre- or postconditioning with ATP is that ATP potentially can trigger a wide variety of cell signaling pathways by activating a number of different P2Y and P2X receptors. One consequence of this is that high extracellular ATP is often associated with contractile, chronotropic, and arrhythmogenic effects (31). However, in our experiments a very low concentration of ATP (0.4 μM) was employed. While this concentration is sufficient for cardioprotection, it is well below the dose for these and other off-target effects (29, 31) including the arrhythmogenic dose for a nonischemic heart (8). BzATP was effective as a cardioprotectant at even lower concentrations (50 nM). Hopefully, agonists more specific for the P2X7 receptor will prove to be more effective pharmacological agents for cardioprotection than ATP.

In conclusion, we have found that ATP can pre- and postcondition the ex vivo heart and that the cardioprotection afforded by ATP results from the opening of pannexin-1/P2X7 channels releasing endogenous cardioprotectants. Furthermore, the specific P2X7 receptor agonist BzATP protects in a like manner. These studies reveal the pharmacological potential of P2X7 agonists.

GRANTS
This work was supported by a grant from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development (to D. A. Vessey).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AJP-Heart Circ Physiol • VOL 301 • SEPTEMBER 2011 • www.ajpheart.org

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