Complementary role of extracellular ATP and adenosine in ischemic preconditioning in the rat heart

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Received 30 August 2001; accepted in final form 16 January 2002

Ninomiya, Hideki, Hajime Otani, Kejie Lu, Takamichi Uchiyama, Masakuni Kido, and Hiroji Imamura. Complementary role of extracellular ATP and adenosine in ischemic preconditioning in the rat heart. Am J Physiol Heart Circ Physiol 282: H1810–H1820, 2002. First published January 17, 2002; 10.1152/ajpheart.00760.2001.—Although adenosine is an important mediator of ischemic preconditioning (IPC), its relative contribution to IPC remains unknown. Because adenosine is formed through the hydrolysis of ATP, the present study investigated the role of ATP and adenosine in IPC. Isolated and buffer-perfused rat hearts underwent IPC by three cycles of 5-min ischemia and 5-min reperfusion before 25 min of global ischemia. The rate-pressure product (RPP) 30 min after reperfusion was taken as an endpoint of functional protection. Interstitial fluid (ISF) adenosine nucleotides and adenosine were measured by cardiac microdialysis techniques. Inhibition of IPC-induced recovery of RPP was partial by the adenosine receptor antagonist 8-(p-tolylsulfonyl)theophylline (SPT; 100 μM) or by the structurally distinct P2Y purinoceptor antagonists suramin (300 μM) or reactive blue (RB; 10 μM) but was additive when SPT was given with suramin or RB. The P2X purinoceptor antagonist 2,4-diaminobutane-6-carboxylic acid (AMPA; 10 μM) or suramin or RB. ISF ATP and adenosine increased temporarily by 10-fold during IPC. AMP-C-P plus SPT, suramin, or RB. ISF ATP and adenosine increased temporarily by 10-fold during IPC. AMP-C-P augmented the increase in ISF ATP associated with the decrease in ISF adenosine. There was a reciprocal correlation between the ISF concentration of ATP and adenosine in preconditioned hearts. In addition, there was a significant correlation between ISF adenosine and ATP and the inhibitory potency of SPT and suramin or RB against functional protection conferred by IPC. These results suggest that extracellular ATP and adenosine play a complementary role in IPC through P2Y purinoceptors and adenosine receptors, respectively.

Cardiac microdialysis

ADENOSINE is known to play an important role in mediating ischemic preconditioning (IPC) in many species, including the rabbit, pig, dog, and human (33). However, the argument against the role of adenosine as the principal mediator for IPC in the rat heart has been provided by several investigators (26, 32). In addition, although ecto-5’-nucleotidase plays a major role in extracellular adenosine formation during IPC (16–18), the inhibitory effect of an ecto-5’-nucleotidase inhibitor, α,β-methylene adenosine diphosphate (AMP-CP), on myocardial protection conferred by IPC is controversial (31). Thus there is circumstantial evidence suggesting that adenosine is not the sole mediator of IPC.

Extracellular ATP is a local regulator of physiological functions in the cardiovascular system. ATP is released to the interstitial space from endothelial cells by mechanical and chemical stimuli such as bradykinin, acetylcholine, and serotonin (1, 42) and from sympathetic and parasympathetic perivascular nerves (3) and cardiomyocytes (9) in response to ischemia or hypoxia. ATP was indeed found in the coronary effluent of the isolated and perfused heart during hypoxia and postischemic reperfusion (2, 4, 40). Extracellular ATP binds to P2 purinoceptors, which constitute a large family of receptors that are ion channels (P2X purinoceptors) or that couple to G proteins (P2Y purinoceptors). Although P2Y purinoceptors are coupled with both pertussis toxin-sensitive G-protein and -insensitive G-proteins, which are distinct from adenosine receptors (which are coupled only with G-protein), these two receptors could share common intracellular signaling cascade that converges in activation of protein kinase C and yet-unidentified downstream effector (5, 8). ATP provokes physiological responses with a potency that is equal to or even greater than that of adenosine (37). Nevertheless, ATP has been paid little attention because of the existence of dogma predicting that extracellular ATP is rapidly hydrolyzed to adenosine by the action of the ecto-nucleotidase system. This notion has been supported by the fact that the predominant purine found in the coronary effluent after hypoxia or ischemia was adenosine (2, 4, 40). However, a cardiac microdialysis technique described by Van Wylen and associates (39) has made it possible to measure interstitial fluid (ISF) ATP levels in the in vivo heart. The studies performed by Kuzmin and...
associates (22, 23) have indeed demonstrated a substantial increase in ISF ATP concentrations after myocardial ischemia.

It is, therefore, assumed that ATP present in the ISF at sufficient concentrations plays a substantial role in mediating IPC. The relative contribution of adenosine and ATP to IPC can be determined by the amount of ATP released to the extracellular space, the rate of ATP degradation, i.e., activity of the ecto-nucleotidase system, and the rate of adenosine degradation. The present study tested this hypothesis by examining the inhibitory effect of an adenosine receptor antagonist, a P2 purinoceptor antagonist, or both on cardioprotection conferred by IPC and by employing a 5'-ecto-nucleotidase inhibitor to inhibit adenosine formation while augmenting ATP accumulation in the ISF. In addition, the correlation between ISF adenosine and ATP levels and the inhibitory potency of an adenosine receptor and a P2 purinoceptor antagonist against IPC-induced functional protection was analyzed. Furthermore, the rate of ATP and adenosine degradation was evaluated in vivo by treating the heart with known concentrations of ATP or adenosine. The present study demonstrates that extracellular ATP and adenosine play a complementary role in IPC through P2Y purinoceptors and adenosine receptors, respectively.

MATERIALS AND METHODS

Perfusion techniques. Male Sprague-Dawley rats weighing 250–300 g were used in the present study. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Publication No. 86-23, Revised 1985). The rats were anesthetized intraperitoneally with pentobarbital sodium (100 mg/kg). The hearts were excised and perfused as described previously (27) at a constant mean pressure of 70–75 mmHg with Krebs-Henseleit bicarbonate (KHB) buffer solution of the following composition (in mM): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 25 NaHCO3, 1.2 KH2PO4, 2.5 CaCl2, and 11 glucose; pH was 7.4 at 37°C when equilibrated with a mixture of 95% O2-5% CO2 gas.

Isovolumic left ventricular function was measured as described previously (27). The baseline measurement was performed 60 min after insertion of the microdialysis probe. Left ventricular function remained little changed from the baseline for at least another 105 min of perfusion, which matches the time frame of the entire experiment. The balloon was filled with saline to produce a left ventricular end-diastolic pressure (LVEDP) of 5–10 mmHg at baseline, and the balloon volume was kept constant throughout the experiment. Coronary flow (CF) was measured by timed collection of the coronary effluent. The hearts producing left ventricular de-pressed pressures (LVDP) of <80 mmHg or a heart rate (HR) of <240 beats/min at baseline were excluded from the study.

Experimental protocol. Ischemic preconditioning was introduced by three repeated cycles of 5-min ischemia and 5-min reperfusion. The adenosine receptor antagonist 8-(p-sulphophenyl)-theophylline (SPT; 100 μM), the structurally distinct P2Y purinoceptor antagonists suramin (300 μM) or reactive blue (RB; 10 μM), and the P2X antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid tetrasodium (PPADS; 50 μM) were included in the perfusion buffer 10 min before and during IPC. These purinoceptor antagonists were excluded from the buffer for 10 min before 25 min of global ischemia. AMP-CP (100 μM) was included in the buffer 10 min before and throughout IPC until the sustained ischemia. In the experiments in which the effects of the purinoceptor antagonists alone on the recovery of postischemic cardiac function were examined, they were included for 40 min before a 10-min washout period followed by 25 min of ischemia. AMP-CP and SPT were obtained from Sigma (Tokyo, Japan), suramin and PPADS were purchased from Alexis Biochemical (San Diego, CA), and RB was purchased from Research Biochemical International (Natick, MA).

Cardiac microdialysis. Cardiac microdialysis was performed according to the method of Van Wylen and associates (39) with some modifications. The microdialysis probes were constructed from dialysis fiber (PAN-1200, 5000 Dalton cutoff, Asahi Chemical) with a 310-μm external diameter and a 200-μm internal diameter joined to fine Silica tubing to form an 8-mm window for exchange of myocardial interstitial solutes with dialysate. A single microdialysis probe was implanted in the left ventricular free wall, approximately midway between the base and apex. Placement of probes within the myocardial wall was verified by examination of histological sections made from myocardium by dissection and visual inspection, and only data from hearts in which probes were located fully within the ventricular wall were analyzed. The dialysis probes were connected to a 1-ml gastight microsyringe and perfused using a microdialysis pump (CMA model 102, Carnegie Medicine; Stockholm, Sweden) at 2 μl/min with Ringer solution [containing (in mM) 147 NaCl, 4.0 KCl, and 2.3 CaCl2] degassed with argon to minimize oxygen delivery to the tissue during ischemia. Measurement of the baseline dialysate sample collection was delayed for 60 min after the implantation of the microdialysis probes to minimize the effect of tissue injury associated with insertion of the microdialysis probes. Dialysate was collected in 10-min aliquots, snap-frozen in liquid nitrogen, and stored at −80°C for later analysis for adenine nucleotides and adenosine by HPLC. To determine the recovery rate for ATP, ADP, AOPCP, AMP, and adenosine, the 8-mm probes were perfused at 2 μl/min while immersed in 1 μM solutions of each adenine nucleotide and adenosine in Ringer solution at 37°C. The relative recovery rates for the adenine nucleotides and adenosine (dialysate concentration/external concentration × 100%) were determined for each microdialysis probe.

HPLC analysis of purine metabolites. HPLC analysis and quantification of purine metabolites were performed as described by Harrison and associates (13). However, the HPLC column, the mobile phase buffer, and the absorbance for ultraviolet detection were modified to gain a better separation of adenine nucleotides and adenosine. Thawed 20-μl dialysate samples were injected onto a Shodex Asahipak GS-320 HQ column and eluted at a flow of 1 ml/min in 100 mM NaH2PO4 (pH 3.0) at 25°C for separation of adenine nucleotides and adenosine using an automated HPLC system (Tosoh Techno System; Tokyo, Japan). Eluent absorbance was continuously monitored at 260 nm, and peaks were identified and quantified by comparison of retention times and peak areas with known external standards run with the samples. ISF adenosine nucleotides and adenosine concentrations were calculated by correction for percent recovery for each microdialysis probe determined in vitro.

Statistics. All numerical data are expressed as means ± SE. Statistical analysis was performed with one-way ANOVA and Scheffe’s multiple-comparison test. Linear regression analysis was used to determine the correlation between ISF ATP and adenosine concentrations and between the inhibitory potency of purinoceptor antagonists and ISF adenosine or ATP concentrations. A value of P < 0.05 was considered to be statistically significant.
RESULTS

Abrogation of IPC-induced functional protection requires coadministration with adenosine receptor antagonist and P2 purinoceptor antagonist. The recovery of the rate-pressure product (RPP), LVEDP, and CF obtained 30 min after reperfusion was taken as an endpoint of myocardial protection, because functional protection was well correlated with the antinecrotic effect of IPC in this model (27). None of the purinoceptor antagonists at the concentrations tested in the present study significantly affected the baseline contractile function as well as recovery of function after reperfusion when administered alone (data not shown). RPP was significantly reduced during IPC in all groups of hearts, although SPT and coadministration of SPT with suramin tended to increase RPP during IPC (Fig. 1A). The recovery of RPP obtained 30 min after reperfusion was significantly improved in the preconditioned heart compared with the control heart. The inhibitory effect on IPC-mediated improvement of RPP by SPT, suramin, or RB alone was partial, but was additive when SPT was combined with suramin or RB. PPADS had no effect on the improved recovery of RPP conferred by IPC.

None of the purinoceptor antagonists significantly affected LVEDP during IPC (Fig. 1B). Ischemic contracture defined as a 5-mmHg increase in LVEDP from the baseline occurring after 10 min of ischemia in the control heart. The time to the onset of ischemic contracture was significantly shortened and the magnitude of ischemic contracture was significantly enhanced in the preconditioned heart. This accelerated ischemic contracture observed in the preconditioned heart has been reported previously (21, 27), although the exact mechanism of this phenomenon is unclear. Interestingly, the time to the onset of contracture was delayed and its magnitude was attenuated by treatment with SPT, suramin, or RB. Upon reperfusion, LVEDP rose further in the control heart, whereas it decreased in the preconditioned heart. The LVEDP-lowering effect of IPC was partially inhibited by SPT, suramin, or RB but was completely abrogated by combined administration of SPT with suramin or RB. PPADS had no effect on the improved recovery of LVEDP during IPC.

CF was measured as an index of coronary vascular function. CF was significantly increased during IPC (Fig. 1C). SPT, suramin, or RB partially attenuated the increase in CF induced by IPC. Coadministration of SPT with suramin or RB completely abrogated the coronary vasodilatation effect of IPC. PPADS had no significant effect on the increase in CF induced by IPC. The recovery of CF after reperfusion was significantly improved in the preconditioned heart compared with the control heart. SPT, suramin, or RB partially inhibited the improved recovery of CF conferred by IPC. However, coadministration of SPT with suramin or RB abolished the improved recovery of CF. PPADS had no significant effect on the improved recovery of CF.

AMP-CP does not block IPC-induced functional protection. Administration of AOPCP in combination with or without SPT, suramin, or RB had no significant effect on the IPC-induced depression of RPP before the sustained ischemia (Fig. 2). Upon reperfusion, pretreatment with AMP-CP alone had no significant effect on the improved recovery of LVEDP conferred by IPC. However, combined pretreatment with SPT, suramin, or RB significantly inhibited the LVEDP-lowering effect of IPC. When AMP-CP was coadministered with SPT and suramin or RB during IPC, the IPC-induced improvement of diastolic function was completely abolished.

Although the increase in CF induced by IPC was not significantly affected by AMP-CP alone, it was inhibited by combined administration with SPT (P < 0.1), suramin (P < 0.05), or RB (P < 0.05) and completely abolished by SPT plus suramin or RB. Upon reperfusion, pretreatment with AMP-CP alone had no significant effect on the improved recovery of CF conferred by IPC. However, the IPC-induced improvement of CF was significantly inhibited by combined pretreatment with SPT, suramin, or RB and completely abolished by SPT plus suramin or RB.

ISF ATP is increased by IPC and augmented by AMP-CP. The left ventricular ISF ATP level was stabilized after 60 min of insertion with microdialysis fiber. Treatment with AMP-CP significantly increased the basal level of ISF ATP (Fig. 3A). The first IPC challenge increased ISF ATP by >10-fold (from 0.06 ± 0.01 to 0.73 ± 0.2 μM). This increase in ISF ATP was significantly augmented in the presence of AMP-CP. The increase in ISF ATP was blunted during the second and third IPC challenges, and the ISF ATP level returned to baseline before the sustained ischemia. In contrast, ISF ATP remained elevated in these periods in the presence of AMP-CP. In the control heart, ISF ATP was significantly increased to 1.26 ± 0.18 μM during the early sustained ischemia. In contrast, this increase in ISF ATP was abolished in the preconditioned heart (0.18 ± 0.04 μM). ISF ATP decreased to 0.44 ± 0.08 μM during the late sustained ischemia in the control heart, whereas the ISF ATP level further declined in the preconditioned heart. ISF ATP remained significantly higher during the entire period of ischemia in the preconditioned heart treated with AMP-CP. The ISF ATP level declined toward the baseline level during 30 min of reperfusion in all groups of hearts.

ISF ADP was below the detectable level in the control heart before ischemia but was significantly increased in the preconditioned heart compared with the control heart (Fig. 3B). The increase in ISF ADP was blunted during the second and third IPC, as has been observed in ISF ATP. Because AMP-CP has the same retention time as ADP in HPLC analysis, actual ISF
Fig. 1. Effect of purinoceptor antagonists on function protection conferred by ischemic preconditioning (IPC). The adenosine receptor antagonist 8-\(p\)-sulfophenyltheophylline (SPT; 100 \(\mu\)M), the P2Y purinoceptor antagonists suramin (Su; 300 \(\mu\)M) or reactive blue (RB; 10 \(\mu\)M), and the P2X antagonist pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium (PPADS; 50 \(\mu\)M) were administered 10 min before and during IPC (hatched boxes). Solid boxes, ischemia. A: rate-pressure product (RPP); B: left ventricular end-diastolic pressure (LVEDP); C: coronary flow. ●, Control \((n = 10)\); ○, IPC \((n = 8)\); ●, SPT + IPC \((n = 10)\); ○, Su + IPC \((n = 10)\); ●, SPT + Su + IPC \((n = 7)\); ○, PPADS + IPC \((n = 8)\); ●, RB + IPC \((n = 8)\); ○, IPC + SPT + RB \((n = 7)\). Each symbol represents the mean ± SE. *\(P < 0.05\) and **\(P < 0.01\) compared with controls; †\(P < 0.05\) and ††\(P < 0.01\) compared with IPC.
Fig. 2. Effect of the ecto-5'-nucleotidase inhibitor α,β-methylene adenosine diphosphate (AMP-CP) on functional protection conferred by IPC. AMP-CP (100 μM) was administered 10 min before and during IPC and until 25 min of ischemia (gray boxes). SPT (100 μM), Su (300 μM), and RB (10 μM) were administered 10 min before and during IPC (hatched boxes). Solid boxes, ischemia. A: RPP; B: LVEDP; C: coronary flow. ○, IPC (n = 8); ▲, AMP-CP + IPC (n = 8); □, SPT + AMP-CP + IPC (n = 8); ■, Su + AMP-CP + IPC (n = 8); ▣, RB + AMP-CP (n = 8). Each symbol represents the mean ± SE of 8 experiments in each group. *P < 0.05 and **P < 0.01 compared with IPC.
ADP concentrations could not be measured in hearts treated with AMP-CP. However, ISF AMP-CP was nearly saturated with exogenous AMP-CP during IPC and >50 μM of AMP-CP retained in the ISF during the late sustained ischemia. In the control heart, ISF ADP was increased to a detectable level during the sustained ischemia. ISF ADP returned to an undetectable level during the late reperfusion in the control and the preconditioned hearts. ISF AMP-CP was almost completely washed out during 30 min of ischemia.

ISF AMP at the baseline was 0.02 ± 0.004 μM and was not significantly affected by treatment with AMP-CP before IPC (Fig. 3C). The first IPC challenge significantly increased ISF AMP (0.19 ± 0.04 μM). This increase in ISF AMP was markedly augmented in the presence of AMP-CP (0.99 ± 0.09 μM). The increase in the ISF AMP level observed in the preconditioned heart was blunted during the second and third IPC. In the control heart, the ISF AMP level increased significantly during the sustained ischemia. The increase in ISF AMP in the preconditioned heart returned toward the baseline level during the late reperfusion. ISF adenosine at the baseline was 0.16 ± 0.03 μM and was not significantly affected by treatment with AMP-CP before IPC (Fig. 3D). ISF adenosine was increased by >10-fold (1.79 ± 0.29 μM) after the first IPC. AMP-CP significantly inhibited the increase in ISF adenosine during the first IPC (0.68 ± 0.14 μM). Although ISF adenosine concentrations declined during the second and third IPC, the values were significantly higher in the preconditioned heart without AMP-CP than with AMP-CP during these periods. In the control heart, ISF adenosine increased to 4.82 ± 0.80 μM during the early sustained ischemia. This increase in ISF adenosine accumulation was significantly inhibited in the preconditioned heart (1.97 ± 0.27 μM) and was further attenuated by pretreatment with AMP-CP (1.25 ± 0.15 μM). The ISF concentration of adenosine was markedly increased during the late sustained ischemia in all groups of hearts. There was no significant difference in the ISF adenosine concentration between the control heart (16.01 ± 1.52 μM) and the preconditioned heart (13.57 ± 1.47 μM). Moreover, AMP-CP treatment only minimally (P < 0.1) affected the increase in ISF adenosine during this period (11.96 ± 1.26 μM). The ISF adenosine level remained elevated during the early reperfusion but returned to the baseline level during the late reperfusion in all groups of hearts.

**IPC increases ISF adenosine and ATP in a reciprocal fashion.** In addition to the above-noted temporal changes in adenine nucleotides and adenosine, we found that the ISF concentration of ATP and adenosine during IPC was markedly different among the preconditioned hearts, whereas the sum of total adenine nucleotides and adenosine concentration was relatively invariable among them (Fig. 4). We then analyzed the relationship between the ISF concentration of ATP and adenosine in the preconditioned heart (Fig. 5). When the ISF ATP concentration in the preconditioned heart was plotted as a function of the ISF adenosine concentration, there was a strong linear relationship ($r^2 = \ldots$).
Degradation rate of extracellular ATP and adenosine. Because the ISF concentration of the sum of total adenine nucleotides and adenosine was similar among the preconditioned hearts, we presumed that the amount of ATP released to the extracellular space and the rate of adenosine degradation may be similar among the hearts. This assumption was tested by treating the heart with a known concentration (10 μM) of ATP or adenosine. When the heart was equilibrated with buffer containing 10 μM ATP, a marked difference in the ISF concentration of ATP (ranging between 0.24 and 2.02 μM, 1.01 ± 0.28 μM, n = 6) and adenosine (ranging between 0.58 and 3.42 μM, 2.13 ± 0.45 μM) was observed among the hearts, whereas the difference in ISF concentration of the sum of total adenine nucleotides and adenosine was relatively smaller (ranging between 3.17 and 4.35 μM, 3.63 ± 0.19 μM) (Fig. 7A). This pattern of an increase in the ISF concentration of adenine nucleotides and adenosine is reminiscent of that observed in the preconditioned heart. In contrast to a marked difference in the ISF concentration of ATP and adenosine in the ATP-treated heart, the ISF concentration of adenosine was similar among hearts treated with adenosine (Fig. 7B), indicating that a relatively small individual difference exists with respect to ATP release to the extracellular space and the rate of adenosine degradation.

DISCUSSION

IPC is a receptor-mediated process in that cell surface receptor stimulation is transduced to intracellular signaling cascade and downstream effector systems (5). Among the receptors, adenosine receptors are thought to be the dominant player responsible for the induction of IPC. However, accumulating evidence suggests that adenosine receptors are not the sole mediator of IPC but that α₁-adrenoceptors (15), bradykinin receptors (11), and opioid receptors (30) may also contribute to IPC. The present study has provided evi-
Dence for the first time that extracellular ATP could play a substantial role in mediating IPC through P2Y purinoceptors.

To substantiate the hypothesis that ATP and its degradation product adenosine play a complementary role in IPC, we employed a nonselective adenosine receptor antagonist, SPT, and two structurally distinct P2Y purinoceptor antagonists, suramin and RB, to evaluate the relative contribution of ATP and adenosine to mediating IPC. Indeed, we found that IPC-induced improvement of systolic, diastolic, and coronary vascular functions during reperfusion were partially inhibited by SPT, suramin, or RB but completely abrogated by coadministration of SPT with suramin or RB. The P2X purinoceptor antagonist PPADS had no effect on myocardial protection conferred by IPC. The results suggest that IPC-induced functional protection is mediated by both adenosine and P2Y receptors. Additive contribution of these purinoceptors to IPC was further suggested by the fact that the coronary vasodilatation effect of IPC was partially inhibited by SPT, suramin, or RB but was completely abrogated by combination of SPT with suramin or RB. The validity of the interpretation of the functional data depends on the potency and selectivity of the purinoceptor antagonists tested. The concentration of SPT (100 μM) used in the present study is known to block adenosine A1 receptors completely when ISF adenosine is <10 μM in the rat heart (10). Because ISF adenosine concentrations during IPC were below this level, adenosine receptor activation was thought to be negligible in the presence of 100 μM SPT. We used a high dose of suramin (300 μM) to obtain a maximum inhibitory effect on P2Y purinoceptors. In our preliminary study, suramin at a dose of 100 μM was only marginally effective in inhibiting the functional protection afforded by IPC, whereas 500 μM suramin showed no further inhibitory effect. Because this concentration of suramin is known to antagonize both P2X and P2Y receptors (12), use of the P2X purinoceptor antagonist PPADS was needed to confirm an antagonistic effect of suramin against P2Y purinoceptors. In addition, we employed another class of P2Y purinoceptor antagonist, RB. The dose of RB (10 μM) used is known to inhibit P2Y purinoceptors selectively and completely, although nonspecific action of RB resulting from the purity cannot be eliminated (14, 28).

The potential role of extracellular ATP in mediating IPC was supported by a cardiac microdialysis study. The data demonstrated that a substantial amount of ATP is released to the extracellular space upon IPC. In the present study, ISF ATP concentration in the rat left ventricular muscle was ~0.06 μM at baseline and was increased temporarily by >10-fold during IPC in some hearts. AMP-CP treatment significantly augmented the increase in ISF ATP to a concentration comparable to that observed with ISF adenosine in the preconditioned heart without AMP-CP, and the decrease in ISF adenosine was associated with a significant increase in AMP. The mechanism by which AMP-CP increases ISF ATP is currently unknown.

Fig. 6. Relationship between the inhibitory potency of the purinoceptor antagonists against the functional protection afforded by IPC and ISF concentrations of adenosine or ATP in the preconditioned heart. The inhibitory potency of SPT (100 μM) is plotted as a function of ISF adenosine (A), and Su (300 μM; B) and reactive blue (RB; 10 μM; C) are plotted as a function of ATP concentrations. There was a significant linear correlation (r² = 0.72, P < 0.01) between the inhibitory potency of SPT and the ISF concentration of adenosine. In addition, there was also a significant linear correlation between the inhibitory potency of Su or RB and the ISF concentration of ATP (r² = 0.76 and 0.77, P < 0.01, respectively).
However, it has been demonstrated that AMP-CP treatment effectively inhibits ATP degradation (22). Although AMP-CP does not directly affect ecto-ATPase activity in vitro (29), combined accumulation of AMP-CP and purine monophosphate nucleotides such as AMP and IMP can inhibit ecto-ATPase in vivo (34, 35, 41). Thus the later mechanism may be responsible for accumulation of ATP during IPC and the sustained ischemia in the presence of AMP-CP. The validity of the values of ISF ATP depends on the accuracy of the microdialysis technique and in vitro rate of recovery to estimate ISF ATP. We waited for 60 min after the insertion of the dialysis fiber until baseline measurement of ISF ATP. This was necessary to minimize the effect of tissue injury associated with insertion of the microdialysis fiber (39). For determination of ISF ATP concentrations, we perfused the dialysate fiber at a rate of 2.0 μL/min, which allowed us to collect a 20-μL dialysate sample during each preconditioning challenge and to obtain adenine nucleotides and adenosine at a concentration high enough for HPLC analysis except for ADP, which was below a detectable level in some dialysate samples. However, it was not possible to evaluate exact concentrations of ISF adenine nucleotides and adenosine, because diffusional exchange across the dialysis fiber could be different in vivo in the beating heart. In the in vivo analysis of extracellular ATP degradation by treating the heart with 10 μM ATP, we found that dialysate ATP concentrations ranged from 0.24 to 2.02 μM (1.01 ± 0.28 μM), and a ~2.1 times higher concentration of adenosine (2.13 ± 0.45 μM) was formed in the ISF at the same time. This in vivo analysis is compatible with the observation that the ISF concentration of adenosine (1.79 μM) was ~2.5 times higher than that of ISF ATP (0.73 μM) in the preconditioned heart. The lightly higher ratio of adenosine to ATP observed in the preconditioned heart may be attributed to the contribution of cytosolic nucleotidase to ISF adenosine formation or activation of ecto-5'-nucleotidase by IPC, as has been proposed by Kitakaze and associates (16–19). It is therefore assumed that micromolar concentrations of ATP may be released into the extracellular space by IPC, and on average 10% of the released ATP retains in the ISF or on average 20% of the released ATP is degraded to adenosine.

There was a significant negative linear correlation between ISF ATP and adenosine levels during IPC. Because little individual difference was noted in the sum of ISF total adenine nucleotides and adenosine concentrations and the rate of adenosine degradation was relatively constant among the hearts, the amount of ATP released to extracellular space during IPC should be comparable among individual hearts. It is suggested that ecto-ATPase activity may be a crucial rate-limiting step in extracellular adenosine formation and that this nucleotidase activity could determine the rate of relative contribution of ATP and adenosine to IPC.

The reciprocal variation in the ISF concentration of ATP and adenosine among the hearts may explain the prevalent controversy with respect to the contribution of adenosine to IPC in some species. To address this issue, we analyzed the relationship between the ISF concentration of adenosine and ATP and the inhibitory potency of SPT and suramin or RB against the functional protection. There was a significant positive correlation between the inhibitory potency of SPT and the ISF concentration of adenosine, whereas the inhibitory potency of suramin or RB was positively correlated with the ISF concentration of ATP. The fact that an
inhibitory effect of combined administration of SPT with suramin or RB on the functional protection afforded by IPC was additive to that exerted by each alone indicates that ATP and adenosine play a complementary role in mediating IPC.

AMP-CP was employed in an attempt to elicit the contribution of ecto-5'-nucleotidase in ISF adenosine formation and functional protection afforded by IPC. However, we found that AMP-CP failed to block functional protection, whereas this ADP analog did inhibit ISF adenosine formation induced by IPC. This inhibition of ISF adenosine formation was associated with an increase in the ISF concentration of ATP. Thus the inability of AMP-CP to block the functional protection afforded by IPC may be attributed to the increase in ISF ATP. However, despite a significant reduction of the ISF concentration of adenosine, SPT was still capable of inhibiting the functional protection afforded by IPC in the presence of AMP-CP. This is probably because of the residual adenosine formation in the presence of AMP-CP concomitant with an increase in the ISF concentration of AMP, which is also a potent agonist of adenosine receptors (P1 purinoceptors). The effect of AMP-CP on IPC-mediated myocardial protection has been controversial (18, 31). The reason for such conflicting observations is currently unknown, but may be related to a difference in the ecto-ATPase activity of the heart not only among the species but also among individuals, as has been suggested in the present study. Hearts in which the ecto-ATPase activity is substantially higher in the presence of AMP-CP could introduce a preconditioning effect without an increase in ISF adenosine, whereas those in which the ISF concentration of ATP cannot be elevated by AMP-CP lose both of the purinoceptor agonists necessary to trigger IPC. This possibility remains to be investigated.

It should be noted that treatment with AMP-CP in combination with suramin or RB but not SPT attenuated IPC-induced acceleration of ischemic contracture. This finding is reminiscent of the inhibitory effect of SPT, sumanin, or RB on IPC-enhanced ischemic contracture, suggesting that the acceleration of ischemic contracture is mediated through P1 and P2Y purinoceptor-dependent processes. Acceleration of ischemic contracture by IPC has been recognized in several investigations, including our own (21, 27), although the exact mechanism of IPC-enhanced ischemic contracture remains unknown. Whether or not accelerated contracture and cardioprotection are mediated through common signaling cascades downstream of purinoceptors remains to be investigated.

The relative contribution of cytosolic and ecto-5'-nucleotidases in extracellular adenosine accumulation during hypoxia and ischemia has been a matter of debate because mammalian cardiac muscle contains equally active ecto-5'-nucleotidase and cytosolic nucleotidase (6). The majority of evidence suggests that cytosolic nucleotidase is primarily responsible for extracellular adenosine accumulation in normoxic and hypoxic hearts (2, 6, 36). However, Deussen and associates (7) showed that extracellular production of adenosine exceeded intracellular production in isolated and buffer-perfused guinea pig hearts, suggesting that ecto-5'-nucleotidase plays a predominant role over cytosolic nucleotidase in aerobic conditions. Lasley and associates (24) argue for the contribution of ecto-5'-nucleotidase to the release of adenosine during the early phase but not the late phase of myocardial ischemia. It is therefore reasonable to conclude that the predominant source of extracellular adenosine during a brief period of ischemia and reperfusion such as IPC may be extracellular ATP.

We found that IPC inhibited accumulation of ISF ATP and adenosine during early sustained ischemia. Inhibition of ISF adenosine accumulation during sustained ischemia in the preconditioned heart has already been reported by several investigators (13, 25, 38). The mechanism of blunted ISF adenosine accumulation during sustained ischemia in the preconditioned heart has been investigated by Harrison and associates (13) based on the hypothesis of accelerated ischemic depletion of intracellular adenine nucleotides by a preconditioning challenge. However, they found that reduced adenosine production was unrelated to ischemic intracellular ATP depletion. In contrast, our study demonstrated a time-dependent decrease in the ISF adenine nucleotide and adenosine level during IPC, indicating that IPC inhibition of ISF adenosine formation during early sustained ischemia is due to reduced mobilization of ATP from the extracellular store and not due to inhibition of ecto-5'-nucleotidase. In the control heart, however, ISF ATP and adenosine were increased during early sustained ischemia and AMP-CP effectively inhibited this ischemia-induced increase in adenosine, further suggesting that ATP was predominantly derived from the extracellular pool during early sustained ischemia. Moreover, such attenuated ISF adenosine formation during early sustained ischemia in the preconditioned heart appears to contradict the hypothesis that amplified adenosine formation during sustained ischemia contributes to myocardial protection (17, 19, 20). Our study rather suggests that ATP and adenosine are the triggers but not the mediators of myocardial protection conferred by IPC.

In contrast to significant inhibition of ISF adenosine formation in the preconditioned heart, there was a marked increase in ISF adenosine during late ischemia in all groups of hearts. IPC was no longer capable of inhibiting adenosine formation in late ischemia, and the increase in ISF adenosine was only minimally affected by AMP-CP. These results suggest that cytosolic nucleotidase is the dominant player of adenosine formation over ecto-5'-nucleotidase during late ischemia.

In conclusion, the present study suggests that extracellular ATP and adenosine play a complementary role in IPC through P2Y purinoceptors and adenosine receptors, respectively, in isolated and perfused rat hearts.

This work was supported in part by Research Grant 10671275 from the Ministry of Education, Science, and Culture of Japan. Present address of K. Lu: Department of Cardiothoracic Surgery, Capital University of Medical Science, Beijing Friendship Hospital, 95 Yongan Rd., Beijing, 100050 China.

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