Purinergic receptors have attracted growing interest as therapeutic targets. This perspective focuses on P2X4 receptors as a new cardioprotective target in heart failure.

Purinergic receptors were initially conceived to describe the likely existence of membrane receptors responding to adenosine triphosphate (ATP) (P2 receptors) or its breakdown product adenosine (P1 receptors) in causing nonadrenergic, noncholinergic relaxation of gut smooth muscle. Cloning of these receptors has yielded insights into their properties and signaling mechanisms, allowing classification into subfamilies of metabotropic (P2Y and P1) and ionotropic (P2X) receptors based on structure. These receptors are now associated with a number of important roles in normal and pathophysiology. Adenosine has been used extensively as a vasodilator and an antiarrhythmic; and efforts to target each adenosine receptor subtype as novel therapies are ongoing. In this Perspective, we will focus our attention on P2 receptors in the heart (see Figure 1 and the Table for basic characteristics and known cardiac distribution) and more specifically on novel aspects of the P2X4 receptor.

Potential Role of P2X4 Receptors in Response to Extracellular ATP

It has been long known that extracellular ATP can cause a modest increase in contractility and cytosolic calcium in cardiomyocytes. In adult ventricular myocytes from healthy wild-type mice, agonist 2-methylthioadenosine-5'-o-triphosphate (2-mesATP) caused an increase in a nonselective cation current that was insensitive to antagonism by suramin or pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS). The reversal potential of this current was similar to that of the cloned P2X4 receptor. The P2X4 receptor, structurally similar to others in the P2X family, have EC50s for ATP similar to P2X2,5,6, higher than P2X1,3, and approximately 1 magnitude lower than P2X7. Its unique properties include relative insensitivity suramin and PPADS and selective potentiation by ivermectin, permitting distinction from other P2X receptors. Moreover, 2-mesATP induced a greater increase of contractility in cardiomyocytes with cardiac-specific overexpression of the human P2X4 receptor than in wild-type myocytes. These results indicated that the P2X4 receptor has a physiological effect in native murine myocytes, and the signal can be augmented with transgenic overexpression. Interestingly, these transgenic animals exhibit a modest increase in basal cardiac contraction without cardiac hypertrophy or failure. Enhanced in vivo contractility is not associated with an increased basal contractility of isolated myocytes, consistent with dissipation of endogenous extracellular ATP in isolated myocytes. This further supports the idea that extracellular ATP activates overexpressed P2X4 receptor to achieve an enhanced in vivo contractile state.

Further characterization of 2-mesATP effect on P2X4-overexpressing cardiomyocytes showed that the enhanced myocyte contractile response was not accompanied by any change in L-type calcium channel current-voltage relationship. This observation, along with the lack of depolarization in response to 2-mesATP, indicates no calcium influx via the L-type calcium channel during P2X4 agonism. Among the P2X receptors, P2X4 is one of the most calcium-permeant, though the majority of entering ions are still Na+. Enhanced Na+ entry via overexpressed P2X4 channel may act to further increase local calcium by affecting the sodium calcium exchanger (Figure 2). Localized domains of calcium increase at the plasma membrane may activate calcium-dependent protein(s) in close vicinity of the receptor channel, after which diffusion of calcium may reach the sarcoplasmic reticulum (SR) to enhance its loading. Increased SR calcium loading was seen in the transgenic myocytes with exposure to agonist, providing a mechanism for P2X4 receptor-mediated increase in contractility in these mice. Whether modest increase in cytosolic calcium caused by activation of overexpressed P2X4 receptors can activate the calcineurin-NFAT prohypertrophic pathway is unknown, though in vitro evidence exists for ATP-induced (not adenosine diphosphate [ADP] or adenosine) inhibition of norepinephrine- and phenylephrine-induced hypertrophy via stimulation of unidentified P2(X) receptor(s). It is conceivable that P2X4 flux exerts primarily localized control over calcium mediated signaling (Figure 2) with minimal or no effect on Ca2+ influx in the dyadic space.

Endothelial nitric oxide synthase (eNOS) is a possible interacting protein activated by localized calcium and calmodulin. Studies on a global P2X4 knockout have demonstrated this receptor to be an important mecano-transducer in endothelial cells, with P2X4 knockout endothelial cells exhibiting abnormal shear responses. This was demonstrated to be due to a lack of force-induced Ca2+ influx and subsequent nitric oxide production. This role in maintaining flow-
induced vasodilatation is important in physiological vascular homeostasis, as a single nucleotide polymorphism leading to decreased ATP binding was recently associated with increased pulse pressure in humans.13 No cardiac performance observation has been mentioned about the P2X4 knockouts, presumably due to a lack of significant basal phenotype change, though it would be of interest to see if exposing these animals to cardiac insult may expose a diseased phenotype. Similarly, a knock-in mouse harboring the known human polymorphism may be a more relevant disease model to help elucidate potential roles of this receptor. Given a possible link between P2X4 receptors and eNOS and the known cardioprotective effect of nitric oxide and cGMP-dependent protein kinase (PKG),13 it is intriguing to suspect a role for eNOS and the known cardioprotective effect of nitric oxide and cGMP-dependent protein kinase (PKG).13,14,15 To activate endogenous P2X4 receptors in mice with heart failure, a hydrolysis-resistant adenine nucleotide (MRS2339) with agonist activity at cardiomyocyte P2X4 receptors was infused chronically in calcequestrin-overexpressing and postinfarct heart failure mice as well as in dogs with pacing-induced heart failure. MRS2339 infusion at low dosage was able to protect these animals with heart failure, similar to the protective effect exerted by cardiac-specific transgenic overexpression of the P2X4 receptor.16

Another potentially important feature of this receptor channel is its mechanosensitive properties. Increased stretch induced by laminar shear stress prevented desensitization of P2X4 channel to its ligand.17 Since stretch can induce ATP release with subsequent channel activation,11 a stretch-mediated P2X4 channel opening suggests a potential important role for P2X4 receptors in cellular response to mechanical force. In the failing heart with increased left ventricular dilatation, the P2X4 channel on cardiac myocytes may also become more activated due to mechanosensitive actions of the channel.

Additionally, P2X4 receptors have recently been demonstrated to exist on lysosome-related exocytotic vesicles. Fusion of these vesicles to the plasma membrane leads to localized calcium increase that further induces Ca2+-dependent vesicular fusion, promoting compound exocytosis.18 It is tempting to speculate that stimulation of P2X4 receptors in ischemia or heart failure may augment cardiac release of vesicular contents, which could contain cytokines and/or cardioprotective factors. Secretion of natriuretic peptides and vascular endothelial growth factor are known to be stimulated under a number of conditions that involve hypoxia and mechanical stretch.19,20 P2X4 receptor stimulation in macrophages contributes to inflammasome activation due to K+ efflux via the channel and subsequent cellular K+ depletion.21,22 The P2X4-mediated inflammatory responses may be needed for tissue repair but may also cause damage during ischemia and reperfusion. In any case, the P2X4 receptor may be a novel participant in regulating inflammatory response in heart failure or during ischemia/reperfusion.

The role of P2 receptor subtypes at the cardiomyocyte level deserves further consideration. As conventional

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP-dependent protein kinase</td>
</tr>
<tr>
<td>PPADS</td>
<td>pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid</td>
</tr>
<tr>
<td>P1</td>
<td>adenosine receptors</td>
</tr>
<tr>
<td>P2X</td>
<td>ionotropic purinergic receptors</td>
</tr>
<tr>
<td>P2Y</td>
<td>metabotropic purinergic receptors</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>UTP</td>
<td>uridine triphosphate</td>
</tr>
<tr>
<td>2-mesATP</td>
<td>2-methylthioadenosine-5'-o-triphosphate</td>
</tr>
</tbody>
</table>

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**Figure 1. Basic features of P2 receptors.** Seven P2X and 8 P2Y subtypes have been identified. P2X channels respond to ATP as their only endogenous ligand and are organized as homo- or hetero-trimers of 2 transmembrane domain subunits that form nonselective cation channels, though some have been demonstrated to allow passage of anions and large molecules.24 The P2Y receptors are G-protein–coupled receptors that have recently been shown to form hetero-oligomers as well.24 In addition to endogenous ATP and ADP, P2Y receptors respond to the pyrimidines UDP and UTP. P2Y1, P2Y2, P2Y3, and P2Y4 are coupled to Gq to activate PLCβ1, leading to IP3-mediated mobilization of intracellular calcium storage, whereas P2Y5, P2Y6, and P2Y13 couple to Gs and thus inhibit adenyl cyclase. P2Y11 uniquely couples to both Gi and Go to increase calcium and cAMP. For detailed pharmacological profile and synthetic ligands, please consult recent reviews.24,35 (Illustration: Ben Smith.)
knowledge would predict, having too much calcium and cationic influx can be damaging to the heart. Using Langendorff-perfused mouse hearts and isolated mouse ventricular myocytes, Gurung et al showed that high concentrations of ATP (10 μmol/L, 100 μmol/L) can induce delayed afterdepolarization and ectopic action potentials, which can be blocked with 100 μmol/L suramin or 30 μmol/L PPADS, but not with 10 μmol/L nifedipine.23 This seeming paradox could be explained by the different agonists and doses used in these studies. 2-mesATP is a partial agonist at P2X4 receptors with an EC50 of 10 to 100 μmol/L, as opposed to ATP, which has an EC50 of 1 to 10 μmol/L.24 Thus, low concentration (3 μmol/L) of 2-mesATP may be able to elicit an increase in contraction but not induce sufficient depolarization to cause ectopic beats and diastolic calcium buildup, which was only observed at >10 μmol/L of ATP. Additionally, the P2X4 receptor is not blocked by suramin or PPADS at the concentra-
tions used;24 thus it is unlikely that it plays a role in the arrhythmias elicited in the Gurung study. These data confirm the existence of multiple subtypes of P2 receptors in the native heart and emphasize the importance of understanding the actions of specific P2 subtypes.

Other P2 Receptors
Recent studies have also suggested differential roles played by various P2 receptors. Although earlier studies of our group had shown that 2-mesATP–stimulated positive inotropy in native rat and mouse heart models was PLC independent5 and largely suramin-insensitive,7 indicating a likely P2X4-mediated effect, a recent study has argued that in isolated mouse ventricular myocytes, P2Y11 receptors may be largely responsible for ATP-induced inotropic increase.25 They demonstrated an agonist rank order of AR-C67085Y > ATPgammaS > 2-MesATP, which fits the agonist profile of the P2Y11 receptor.25

Table. Widespread P2 Receptor Expression in Cardiac Tissue

<table>
<thead>
<tr>
<th>Reference</th>
<th>Species</th>
<th>Cardiac Region</th>
<th>Disease</th>
<th>Receptors Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banfi, C, et al; 2005</td>
<td>Human</td>
<td>LV</td>
<td>CHF and healthy</td>
<td>All at the mRNA level</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Protein not confirmed for P2X5 and P2Y2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P2X6 upregulated in CHF</td>
</tr>
<tr>
<td>Musa, H, et al; 2009</td>
<td>Human</td>
<td>RA and SAN</td>
<td>Healthy</td>
<td>mRNA for P2X4,7 and P2Y1,2,4,6,12 to 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P2X: P2X4,7 most abundant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P2Y: P2Y1,2 most abundant in RA</td>
</tr>
<tr>
<td>Rat</td>
<td>LV, RA, and SAN</td>
<td>Healthy vs CAL</td>
<td></td>
<td>Healthy:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P2X: P2X4 most abundant in all regions</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P2Y: P2Y1,12,14 most abundant in LV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CAL:</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P2X4 mRNA unregulated by 93% in the SAN, nonsignificant increase trend in the RA and LV</td>
</tr>
</tbody>
</table>

CAL indicates coronary artery ligation; CHF, congestive heart failure; LV, left ventricle; RA, right atrium; SAN, sinoatrial node. Note regional and species differences.

Figure 2. P2X4 receptor as a potential pathway to activate endogenous cardiac eNOS via localized calcium handling. The cardiac P2X4 could activate eNOS on stimulation by receptor agonist. This can be facilitated by a localized increase in calcium near eNOS, binding to local calmodulin and leading to increased eNOS activity. A calcium gradient is set up by diffusion, with minor amounts reaching the SR. NO production can lead to many downstream effects, both PKG-dependent and PKG-independent, many known to exert a salutary effect in ischemia and heart failure. CaM indicates calmodulin; NCX, sodium calcium exchanger; sGC, soluble guanylyl cyclase. (Illustration: Ben Smith.)
P2Y<sub>1</sub>-mediated contractile effect depends on PLC and cAMP, which implies increased mobilization of intracellular calcium stores, whereas the smaller contractile effect associated with P2X channels may be due to gradual augmentation of the SR store via diffusion of calcium localized near the P2X channel. The P2Y<sub>2</sub> receptor has been suggested to mediate uridine triphosphate (UTP)-conferred protection against myocardial damage after myocardial infarct, although the existence of this receptor at the protein level has not been confirmed in the human heart. Like ATP, UTP was found at increased levels in human plasma from patients with acute myocardial infarction, and previous animal studies directly measuring UTP from the cardiac vein suggest the ischemic heart as the source for UTP. It was demonstrated that in P2Y<sub>2</sub> knockout mice, the UTP-conferred reduction in infarct size and improvement in cardiac function were abrogated. Additional evidence supports stimulation of P2Y<sub>2</sub> receptors as a protective mechanism to decrease angiotensin type 1 receptor density in cardiac fibroblasts through inducible nitric oxide synthase–mediated S-nitrosylation of the p65 subunit of NF-κB, thus negatively regulating angiotensin II–mediated inflammatory response in the heart. The P2X<sub>2</sub> receptor has also been implicated in mediating effects of ischemic preconditioning and postconditioning, by interacting with connexin-I hemichannels to release multiple cardioprotectants in Langendorff ex vivo rat hearts. However, another study exposing HL-1 atrial cells to hypoxic stress in culture detected an early rise in ATP in culture, followed by increased release of cytoplasmic histone-associated DNA-fragments (indicating cell death) that were thought to be directly induced by P2Y<sub>2</sub> and P2X<sub>2</sub> receptor subtypes, whereas the P2Y<sub>4</sub> receptor was thought to exert a protective effect. Addition of apyrase to degrade extracellular ATP, or adding GAP26 or 18aGA to inhibit connexin hemichannels, also abolished hypoxic stress-associated apoptosis. These results seem to be in conflict and warrant further characterization and studies across different model systems.

**Future Directions**

Other than exocytotic vesicular release from nerve terminals, the source of extracellular purines and pyrimidines capable of acting on P2 receptors has largely been considered to stem from damaged or dying cells; hence there is a continued interest in P2 receptors in ischemia. However, ATP can be released by a variety of cell types (endothelial cells, blood components such as red blood cells and platelets, and immune cells) under physiological conditions in response to mechanical distortion, hypoxia, or stimulation by certain agents. It is highly possible that subtypes of the P2 receptor can mediate preconditioning or postconditioning to reduce injury during a more catastrophic episode. Early work on cardiac P2X<sub>4</sub> receptors as a therapeutic target in treating heart failure has pointed to a potentially rewarding translational research on purinergic signaling. Understanding the physiological roles of individual P2 receptors in the heart will be imperative as interest grows in targeting purinergic receptor signaling in treating heart failure and ischemia.

The many permutations of subtype combinations and the paucity of highly selective agonists and antagonists have made it difficult to ascertain the specific role of each P2 receptor in the heart. Genetic tools at the cellular level, accompanied by our increasing ability to differentiate cells into specific lineages, may prove efficient at elucidating cell type–specific molecular and cellular mechanisms. Cardiac- and vascular-specific knockout of P2X<sub>4</sub> receptors or knock-in of P2X<sub>4</sub> receptor polymorphism should aid in the elucidation of this receptor in different tissues in disease states. This should help us to understand the potential balance between arrhythmogenic or undesired effects versus the salutary anti-ischemic and heart failure protection benefits of P2X<sub>4</sub> receptors. In light of the most recent crystal structure obtained from zebrfish P2X<sub>4</sub>, now in the open state with ATP binding, new information on the conformational changes associated with P2X channel gating will enable guided design of new pharmacological agents. Crystal structure and site-specific mutagenesis of P2X<sub>4</sub> receptors have elucidated the ATP-binding pocket, which resides at each pair of subunit interfaces in the trimeric channel. Four positively charged lysine and arginine residues line the binding pocket for ATP, whose phosphate groups mediate ligand specificity (ATP→ADP, AMP). Lys70 and Thr189 (Lys67 and Thr186 in human homologue) are important in maintaining specificity over CTP, GTP, and UTP. The human Tyr315Cys mutation that disrupts the agonist binding is located within this pocket. Additionally, the open state of the receptor leaves relatively large gaps that could be filled in by lipids and allosteric modulators such as ivermectin. Detailed mechanisms of how the open state can be stabilized by such modulators, and more fascinatingly by mechanical forces, would be of interest in future investigations. Additional challenges in drug design include rapid adenine nucleotide degradation by extracellular nucleotidase and the negative charges on nucleotides preventing oral absorption. Development of nucleotidase-resistant ligands and prodrugs at P2X<sub>4</sub> receptors with masked charges that can be restored after oral absorption are additional goals for clinical application.

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**Disclosures**

None.

**References**

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