Characterization and mechanism of P2X receptor-mediated increase in cardiac myocyte contractility

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Shen J-B, Shutt R, Pappano A, Liang BT. Characterization and mechanism of P2X receptor-mediated increase in cardiac myocyte contractility. Am J Physiol Heart Circ Physiol 293: H3056-H3062, 2007.—Cardiac P2X purinergic receptors can mediate an increase in myocyte contractility and a potentially important role in the heart. The P2X4 receptor (P2X4R) is an important subunit of native cardiac P2X receptors. With transgenic mice with cardiac-specific overexpression of P2X4R (Tg) used as a model, the objectives here were to characterize the P2X receptor-mediated cellular contractile and Ca2+ transient effects and to determine the mechanism underlying the receptor-induced increase in myocyte contractility. In response to the agonist 2-methylthioATP (2-meSATP), Tg myocytes showed an increased intracellular Ca2+ transient, as defined by fura 2 fluorescence ratio, and an enhanced contraction shortening that were unaccompanied by cAMP accumulation or L-type Ca2+ channel activation. The increased Ca2+ transient was not associated with any alteration in action potential duration, resting membrane potential, or diastolic fluorescence ratio or rates of rise and decline of the Ca2+ transient. Simultaneous Ca2+ transient and contraction measurements did not show any agonist-mediated change in myofilament Ca2+ sensitivity. However, activation of the overexpressed P2X4 receptor caused an enhanced SR Ca2+ loading, as evidenced by a 2-meSATP-evoked increase in the caffeine-induced inward current and Ca2+ transient. Similar data were obtained in wild-type mouse ventricular myocytes. Thus an increased SR Ca2+ content, occurring in the absence of cAMP accumulation or L-type Ca2+ channel activation, is the principal mechanism by which cardiac P2X receptor mediates a stimulatory effect on cardiac myocyte contractility.

RECEPTORS FOR PURINE NUCLEOTIDES, known as P2 purinergic receptors, are activated by extracellular adenine nucleotides such as ATP and ADP (16, 18). The P2 receptor subfamily includes the ligand-gated receptor channel P2X receptor and the G protein-coupled P2Y receptor (1, 18, 26). Previous studies have shown that extracellular ATP can cause a nonselective cationic current in murine (21), rat (20), and guinea pig (17) cardiac ventricular myocytes. Evidence is accumulating to indicate that the cardiac myocyte P2X receptor mediates this ATP-evoked current. Of the P2X receptor family members, the P2X4 receptor is an important subunit of the native cardiac myocyte P2X receptor (21). Activation of the P2X receptor leads to the opening of its channel permeable to Na+, K+, and Ca2+ with reversal potential near 0 mV. ATP or the P2X receptor agonist 2-methylthioATP (2-meSATP) can also stimulate an increase in myocyte contractility and in intact heart contractile function (8, 15). Transgenic mice with cardiac-specific overexpression of the P2X4 receptor (Tg) showed an increased myocyte contractility caused by ATP or 2-meSATP and an enhanced contractile performance of the intact heart (8). However, the mechanism by which cardiac P2X receptor activation leads to this enhanced contractility and heart function is not known.

The objectives of the present study were to characterize the P2X receptor-mediated cellular contractile and Ca2+ transient effects and to determine the mechanism underlying the receptor-induced increase in myocyte contractility. Tg mice were used as a model for the current objectives. The data showed that activation of the cardiac P2X4 receptor led to an enhanced myocyte contraction shortening (CS) and Ca2+ transients. The P2X4 receptor-mediated increase in myocyte contractility occurred in the absence of any change in cAMP level or L-type Ca2+ channel activity and did not involve any change in the sensitivity to intracellular Ca2+. An increased sarcoplasmic reticulum (SR) Ca2+ store is the principal mechanism responsible for the enhanced contractile state induced by this receptor channel.

MATERIALS AND METHODS

Isolation of adult mouse cardiac ventricular myocytes from P2X4 receptor Tg mice. Ventricular myocytes were obtained from 3-mo-old P2X4R transgenic (Tg) or wild-type (WT) mice by an enzymatic dissociation procedure as described previously (21, 29). The study was approved by the IACUC at the University of Connecticut Health Center. The hearts were rapidly excised from mice that had been anesthetized with pentobarbital and treated with 1,000 U of heparin. The aorta was cannulated and the heart perfused in a Langendorff apparatus with oxygenated (95% O2-5% CO2) Ca2+-free solution (37°C) for 5 min at a rate of 2.5 ml/min. The solution composition was (in mM) 126 NaCl, 4.4 KCl, 1.0 MgCl2, 18 NaHCO3, 11 glucose, 4 HEPES, and 3 BDM (2,3-butanedione monoxime) (pH 7.3 adjusted with NaOH). Thereafter, the perfusing solution was changed to one containing 25 µM CaCl2 and liberase (70 µg/ml, Roche Molecular Biochemicals) for 8–10 min. Cells were then exposed to a stepwise increase in extracellular Ca2+ from 0.025, 0.2, and then 1.0 mM, allowed to settle for 30 min at room temperature, and finally suspended with Tyrode’s solution containing (in mM) 135 NaCl, 4.4 KCl, 1.0 MgCl2, 18 NaHCO3, 11 glucose, 4 HEPES, and 10 dextrose (pH 7.4 adjusted with NaOH). The experiments were carried out at room temperature (22–23°C) and were completed within 4–6 h after myocyte isolation.

Electrophysiological recordings. Action potentials were elicited at 0.5 Hz in current-clamp mode. Myocytes were superfused with normal Tyrode’s solution. The pipette was filled with a solution...
containing (in mM) 120 potassium aspartate, 30 KCl, 5 Na2ATP, 1 MgCl2, 5 HEPES, and 10 EGTA (pH 7.3 was adjusted with KOH). 

L-type Ca2+ current was measured by the whole cell patch-clamp technique. The patch electrodes were prepared from borosilicate glass pipette (1.2 mm ID) with a two-step pulling procedure and had a resistance of 2–4 MΩ when filled with a solution containing (in mM) 135 cesium aspartate, 5 NaCl, 5 Mg2ATP, 10 HEPES, and 10 EGTA (pH 7.3 adjusted with CsOH). As soon as electrical contact was established, the superfusion medium was changed to a modified Tyrode’s solution (5.4 mM KCl was omitted and 10 mM CsCl was added to Tyrode’s solution) to block K+ currents. Generation of voltage clamp protocols and acquisition of data were carried out with pCLAMP software (version 7; Axon Instruments). Sampling frequency was 10 kHz. The voltage-clamp protocol used to elicit the current-voltage relation was a series of 300-ms steps of increasing voltage, from −30 to +60 mV in 10-mV increments at a frequency of 0.2 Hz. Each step was preceded by a 300-ms step from a holding potential of −80 to −40 mV that inactivated the fast Na+ current and T-type Ca2+ current.

**Estimation of SR Ca2+ content.** The Ca2+ content of the SR was obtained from experiments with caffeine. The experimental conditions of recording Na+/Ca2+ exchange current (I_prot Ca) was similar to L-type Ca2+ current experiment except that EGTA in the pipette was replaced to 0.5 mM. The outlet of the rapid solution device (SF-77B, Warner Instrument) was brought within 50 μm of the cell. After recording conditions for the cell had stabilized, Tyrode’s solution, identical to the bath solution, was applied to the cell by using the rapid solution device. Three 300-ms conditioning stimuli (−80 to +50 mV) at 0.2 Hz were applied to cells. At 2 s after the last stimulus, the perfusion was rapidly changed to a solution containing 10 mM caffeine for 10 s. With the membrane held at −80 mV, the Ca2+ released by caffeine induced a large inward current via the Na/Ca exchanger; this current represented the basal control SR Ca2+ content (3). Integration of this inward current provides an estimate of SR Ca2+ content. The Ca2+ content of the SR was obtained from experiments with caffeine. The experimental conditions of recording Na+/Ca2+ exchange current (I_prot Ca) was similar to L-type Ca2+ current experiment except that EGTA in the pipette was replaced to 0.5 mM. The outlet of the rapid solution device (SF-77B, Warner Instrument) was brought within 50 μm of the cell. After recording conditions for the cell had stabilized, Tyrode’s solution, identical to the bath solution, was applied to the cell by using the rapid solution device. Three 300-ms conditioning stimuli (−80 to +50 mV) at 0.2 Hz were applied to cells. At 2 s after the last stimulus, the perfusion was rapidly changed to a solution containing 10 mM caffeine for 10 s. With the membrane held at −80 mV, the Ca2+ released by caffeine induced a large inward current via the Na/Ca exchanger; this current represented the basal control SR Ca2+ content (3). Integration of this inward current provides an estimate of SR Ca2+ content (25). Baseline of the current was defined as that measured at −2,500 ms from the peak current and was used for current transient integration. After a control test with caffeine, 2-meSATP (3 μM) was applied for 3 min. Caffeine was rapidly superfused again after three conditioning stimuli were delivered under conditions identical to those for the basal SR Ca2+ content determination. Data were taken only from those cells that could be held during the control period, in the presence of 2-meSATP and after washout. The caffeine-induced inward current via the Na/Ca exchanger has been used previously to estimate the SR Ca content in adult mouse ventricular myocytes (24, 30).

Another method to estimate the SR Ca2+ content is to measure the amplitude of the caffeine-induced Ca2+ transients (7, 9). In these experiments, Tg or WT myocytes were field stimulated at 0.2 Hz for 5 min and then 10 mM caffeine was rapidly applied for 10 s. The amplitude of Ca2+ transient was recorded as the basal SR Ca2+ content. Five minutes after washout of caffeine, 3 μM 2meSATP was applied for 5 min while the myocytes were continuously paced at 0.2 Hz. This was then followed by the same brief exposure to caffeine in the presence of 2-meSATP and the Ca2+ transients were recorded. The same procedure was repeated during washout of 2-meSATP.

**Determination of CS, cAMP, and Ca2+ transients in isolated cardiac myocytes.** Myocyte CS by changes in sarcomere length and Ca2+ transients were recorded simultaneously from single isolated myocytes with an epifluorescence inverted microscope with Ionoptix software and camera (Ionoptix, Milton, MA) as previously described (5, 6, 10, 12). Cells were superfused with Tyrode’s solution at 25°C and field stimulated as previously described (21). Ca2+ transients were measured with the ratiometric dye fura 2-AM. Myocytes were loaded with 2 μM of fura 2-AM for 20 min at 25°C. Ca2+ transients were recorded as the fluorescence ratio at 510 nm in response to excitation from 340 and 380 nm. Ca2+ transients were digitized and analyzed. Ca2+ transients and CS were measured simultaneously in the same myocytes. The background fluorescence, determined by measuring and averaging the autofluorescence of randomly selected 6–9 myocytes not loaded with fura 2, was subtracted. Steady-state recordings were measured under baseline control conditions and after exposure to 2-meSATP. Various CS and Ca2+ transient parameters such as amplitude, maximal rates of rise and fall, time to 50% peak, and time to 50% decline were measured with Ionoptix curve-fitting algorithm software as previously described (5, 6, 12, 13).

Cyclic AMP was determined with an enzymatic immunoassay kit according to manufacturer’s instructions, similar to the method used to estimate cAMP level in dispersed mammalian cardiac ventricular myocytes (28, 14). In brief, myocytes were isolated and plated in a 96-well plate at a density of 10^5 cells/well. Following a 1-h equilibration, myocytes were incubated with either vehicle or 2-meSATP (10 μM) or isoproterenol (0.1 μM) for 10 min and then lysed. cAMP measurements were made with the GE Healthcare cAMP Direct Biotak Enzyme Immuno Assay Kit according to manufacturer’s instructions.

**Statistical analysis.** Data were presented as means ± SE. The significance of differences between means was analyzed with appropriate analysis of variance and posttest comparison by Newman-Keuls test. Values with P < 0.05 were considered statistically significant. In other comparisons, a paired t-test was used.

**Drugs and solutions.** 2-meSATP and ATP were obtained from Sigma Chemical (St. Louis, MO). Fura 2-AM was obtained from Molecular Probes (Eugene, OR) and used according to manufacturer’s instructions as previously described (5, 6, 10, 12). CAMP EIA kit was from GE Healthcare (Piscataway, NJ). The P2X,R transgenic construct was generated and bred as previously described (8).

**RESULTS**

**Characteristics of cardiac P2X receptor-mediated increase in myocyte contraction shortening and intracellular Ca2+ transients.** Activation of the P2X receptor caused an increased contractile state of cardiac myocytes from the P2X4 receptor Tg animals. The basal CS amplitude in the Tg myocyte was similar to that of WT cardiac myocytes (8). Myocytes from the Tg heart were used as model to characterize the cardiac P2X receptor-mediated increase in CS and Ca2+ transient. The contractile parameters and Ca2+ transient at control and in response to the P2X agonist 2-meSATP were characterized in these myocytes. Data in Fig. 1, A and C, showed a simultaneous measurement of Ca2+ transient and CS in a Tg cardiac myocyte. 2-meSATP caused a concomitant increase in CS and Ca2+ transient. When the CS and Ca2+ transient were normalized to their maximal amplitudes, the normalized fluorescence ratio and CS obtained during control and 2-meSATP stimulation were superimposable (Fig. 1, B and D). Although 2-meSATP caused a significant increase in CS (P < 0.05), the maximal rates of contraction shortening and relaxation, the time to 50% peak, and the time to 50% relengthening were similar under control and 2-meSATP conditions (P > 0.1, n = 9 myocytes, Table 1). Similarly, the P2X agonist-stimulated increase in Ca2+ transient was not associated with any alteration in the maximal rates of rise or fall of the fluorescence ratio (P > 0.1, n = 9 myocytes, Table 2). The time to 50% peak and time to 50% decline of the fluorescence ratio under control conditions were the same as those obtained in the presence of 2-meSATP (P > 0.1, Table 2). The various CS and Ca2+ transient parameters were similar to those in WT cardiac myocytes reported by others (9–11, 23). In adult ventricular myocytes of WT mice, 2-meSATP was also able to cause an increase in the CS (basal: 0.071 μm ± 0.013 μm; 2-meSATP:...
Fig. 1. Characteristics of cardiac P2X receptor-mediated increase in CS and Ca2+ transient. Cardiac ventricular myocytes were prepared from hearts from transgenic (Tg) mice with cardiac-specific overexpression of P2X4R receptor. Contraction shortening (CS) and Ca2+ transients were measured as described in MATERIALS AND METHODS. In a typical Tg cardiac myocyte paced at 2 Hz, 2-methylthioATP (2-meSATP; 3 μM) caused an increase in both Ca2+ transient (A) and CS (C). The CS and Ca2+ transient obtained under control (CTR) conditions and during 2-meSATP stimulation were normalized to their respective peak amplitudes. The normalized fluorescence ratio (B) and the normalized CS (D) under CTR were superimposable on those obtained during 2-meSATP stimulation.

0.086 μm ± 0.013 μm, n = 4 myocytes, 0.2 Hz, P = 0.04, paired t-test) and Ca2+ transient amplitude (basal ratio: 0.058 ± 0.006; 2-meSATP: 0.067 ± 0.006, 0.2 Hz, P = 0.026, paired t-test). Similar to data obtained in Tg myocytes, the times to 50% peak and to 50% relengthening were similar in the presence or the absence of 2-meSATP (P > 0.1). The times to 50% peak and to 50% decline of fluorescence ratio under basal condition were also similar to those obtained in the presence of 2-meSATP (P > 0.1). The proportion of WT myocytes (4 of 17 myocytes from 10 mice) showing an increase in CS and Ca2+ transient was similar to that reported previously (21). These data indicate that the P2X receptor-induced stimulation of CS and Ca2+ transient was limited to only an increase in the amplitude, without any change in other characteristics of these two physiological parameters.

Cardiac P2X receptor-mediated increase in myocyte contractility is associated with a concomitant increase in intracellular Ca2+ transients. P2X4R Tg cardiac myocytes demonstrated a significant increase in the extent of contraction shortening (CS) in response to extracellular 2-meSATP at 0.2 and 1.0 Hz (Fig. 2A) at 0.2 Hz, basal = 0.087 ± 0.011 μm vs. 2-meSATP = 0.116 ± 0.011 μm, P < 0.01 paired t-test, n = 9 myocytes; at 1.0 Hz, basal = 0.038 ± 0.007 μm vs. 2-meSATP = 0.052 ± 0.01 μm, P < 0.05). The extent of CS was reduced under basal and 2-meSATP-stimulated conditions at higher stimulation rates (Fig. 2B). There was a negative force-frequency relationship within this range of stimulation frequencies. At 4 Hz, others have reported a greater Ca2+ transient or contraction compared with those obtained at 1 Hz in adult murine cardiac myocytes (2). In the present study, the fura 2 Ca2+ fluorescence and CS obtained at 2 Hz were similar to those obtained at 1 Hz, similar to those reported previously (2). In comparing the change in CS with that in Ca2+ fluorescence ratios, the P2X receptor-mediated increase in CS was associated with an increase in the intracellular Ca2+ transients, assessed as fluorescence ratios at various frequencies of pacing (Fig. 2, A and B) (at 0.2 Hz, basal fluorescence ratio = 0.066 ± 0.012 vs. ratio in presence of 2-meSATP = 0.081 ± 0.013, P < 0.01, paired t-test, 9 myocytes; at 1.0 Hz, basal = 0.045 ± 0.0081 vs. 2-meSATP = 0.051 ± 0.008, P < 0.05). The plot of change in Ca2+ fluorescence vs. that in CS in the absence of P2X agonist was superimposable on the plot obtained in the presence of the agonist (Fig. 2C). The slope without extracellular 2-meSATP (0.21 ± 0.036) was similar to that with 2-meSATP (0.20 ± 0.02, P > 0.1). The data indicate that the agonist-induced increase in myocyte contractility was not due to an increase in sensitivity to intracellular Ca2+.

Stimulatory effect of extracellular 2-meSATP on SR Ca2+ content in P2X4R Tg myocytes. The P2X receptor-mediated increase in Ca2+ transients may be the result of an enhanced SR Ca2+ store during receptor activation. To test this hypothesis, caffeine-induced inward I_NaCa was used to determine the SR Ca2+ content in the absence and the presence of P2X agonist. Fig. 3A showed that the presence of extracellular 2-meSATP caused a significant increase in the caffeine-induced inward current in 10 of 16 Tg myocytes (P < 0.05). The increase was significant in both the peak (Fig. 3A) and the integrated (Fig. 3B) I_NaCa inward current in these myocytes (P < 0.05 vs. the basal control level). Similarly, 2-meSATP was also able to induce significant increases in caffeine-evoked inward current in 5 of 13 WT cardiac myocytes (P < 0.05 vs. the basal control level) (Fig. 3, A and B). In general, more P2X4R Tg than WT myocytes showed a response to extracellular 2-meSATP (21). The proportions of 2-meSATP-responsive Tg and WT cells in the present study were similar to those reported previously (21). The P2X receptor agonist effect dissipated upon its washout in both WT and Tg myocytes. The increased caffeine-mediated inward I_NaCa by 2-meSATP in P2X4R Tg myocytes was not due to an increased density of the Na+/Ca2+ exchanger in the Tg myocyte since the peak and the integrated I_NaCa currents under basal agonist-free condition in

Table 1. Contractile parameters

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<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>2me-SATP</th>
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<tbody>
<tr>
<td>Amplitude, μm</td>
<td>0.087±0.011</td>
<td>0.116±0.011*</td>
</tr>
<tr>
<td>Max rate of shortening, μm/s</td>
<td>133.9±24.7</td>
<td>135.1±25.6</td>
</tr>
<tr>
<td>Max rate of relaxation, μm/s</td>
<td>93.7±48.5</td>
<td>91.9±48.4</td>
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<tr>
<td>Time to 50% peak, ms</td>
<td>45.5±7.5</td>
<td>43.9±10.5</td>
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<tr>
<td>Time to 50% baseline, ms</td>
<td>119.2±11.2</td>
<td>125.3±12.2</td>
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Values are means ± SE; 9 transgenic (Tg) cardiac myocytes were stimulated at 0.2 Hz, *P < 0.05 (paired t-test). The rates of rise and fall of fluorescence ratios were presented as fluorescence unit per second (U/s).

Table 2. Ca2+ transient

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>2me-SATP</th>
</tr>
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<tbody>
<tr>
<td>Diastolic ratio</td>
<td>0.96±0.031</td>
<td>0.97±0.030</td>
</tr>
<tr>
<td>Amplitude (systolic minus diastolic)</td>
<td>0.066±0.012</td>
<td>0.081±0.013*</td>
</tr>
<tr>
<td>Max rate of rise, U/s</td>
<td>13.3±2.4</td>
<td>13.2±1.7</td>
</tr>
<tr>
<td>Max rate of decline, U/s</td>
<td>3.4±0.38</td>
<td>3.3±0.58</td>
</tr>
<tr>
<td>Time to 50% peak, ms</td>
<td>14±0.8</td>
<td>13.4±1.09</td>
</tr>
<tr>
<td>Time to 50% decline, ms</td>
<td>195.8±20.6</td>
<td>193.7±22.5</td>
</tr>
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</table>

Values are means ± SE; 9 transgenic (Tg) cardiac myocytes were stimulated at 0.2 Hz, *P < 0.05 (paired t-test). The rates of rise and fall of fluorescence ratios were presented as fluorescence unit per second (U/s).
the Tg cells (−0.58 ± 0.09 pA/pF and −0.94 ± 0.23 pA·ms⁻¹·pF⁻¹, respectively, n = 10 myocytes) were similar to those in the WT cells (−0.54 ± 0.04 pA/pF and −0.84 ± 0.07 pA·ms⁻¹·pF⁻¹, n = 5 myocytes, P > 0.1 vs. Tg myocytes). Activation of native cardiac P2X receptors on WT myocytes was also able to increase caffeine-evoked inward current (Fig. 3, B and C). The data further support the concept that the caffeine-induced \( I_{\text{Na/Ca}} \) by P2X agonist is likely due to an enhanced loading of the SR with Ca\(^{2+}\).

The caffeine-induced Ca\(^{2+}\) transient amplitude was also used to estimate the SR Ca\(^{2+}\) content in both the Tg and the WT myocytes. Data summarized in Fig. 4 showed that under the control condition without any agonist, a brief caffeine exposure caused a large Ca\(^{2+}\) transient secondary to its release from the SR. The amplitude of Ca\(^{2+}\) transient represented the basal SR Ca\(^{2+}\) store in the Tg (Fig. 4, right) and WT (left) myocytes. However, in the presence of 2-meSATP, the caffeine-induced Ca\(^{2+}\) transient was significantly greater than that obtained under the basal control condition in both the Tg and the WT myocytes (Fig. 4) (\( P < 0.05 \), paired t-test), providing further evidence for a SR Ca\(^{2+}\) loading effect of cardiac P2X receptor.

The P2X receptor-mediated increase in myocyte contractility or Ca\(^{2+}\) transient occurred in the absence of any cAMP accumulation in the P2X\(_4\)R Tg myocyte. 2-meSATP did not cause any increase in the intracellular cAMP level (Fig. 5). The basal cAMP level was 3.97 ± 0.33 pmol/mg protein and was similar to that obtained in the presence of 2-meSATP at 10 \( \mu \)M (3.18 ± 0.45 pmol/mg, \( P > 0.1 \)). The cAMP level increased to 16.6 ± 1.53 pmol/mg in the presence of isoproterenol (\( P < 0.05 \) vs. basal or 2-meSATP, \( n = 12 \) determinations from myocytes of three Tg mice, ± SE). Additionally, 2-meSATP also did not increase cAMP accumulation in WT cardiac myocytes (basal: 1.89 ± 0.21 pmol/mg, ± SE, vs. 2-meSATP: 2.36 ± 0.36 pmol/mg, \( P > 0.1 \), \( n = 32 \) determinations from myocytes of six mice). Isoproterenol increased the cAMP level to 8.47 ± 1.15 pmol/mg in WT myocytes (\( P < 0.05 \) vs. basal or 2-meSATP). Given the known effect of cAMP on L-type Ca\(^{2+}\) channel activity, the lack of any stimulatory effect of 2-meSATP on cAMP accumulation is compatible with its lack...
of stimulation of the L-type Ca\textsuperscript{2+} channel, as reported previously (21).

Effects of 2-meSATP on action potential duration, resting membrane potential, and L-type Ca\textsuperscript{2+} current. To further characterize the effects of cardiac P2X receptor activation, Tg myocytes overexpressing P2X\textsubscript{4} receptors were exposed to 2-meSATP, and action potential duration at 50 and 90% of repolarization (APD\textsubscript{50} and APD\textsubscript{90}, respectively) and membrane potential were determined. The P2X agonist did not cause any change in the APD\textsubscript{50} or APD\textsubscript{90} (P > 0.1, paired t-test). Action potential Ca\textsuperscript{2+} current was measured as described in MATERIALS AND METHODS. The current at each voltage was plotted as mean and SE and was obtained in 5 myocytes (from 5 mice). The current determined in the presence of 2-meSATP was similar to that obtained in the absence of the agonist at each voltage (P > 0.1, paired t-test).

Table 3. Action potential characteristics

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<tr>
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<th>Control</th>
<th>2me-SATP</th>
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<tbody>
<tr>
<td>Resting potential</td>
<td>−72.6±1.47</td>
<td>−71.7±1.34</td>
</tr>
<tr>
<td>APD\textsubscript{50}, ms</td>
<td>18.8±2.19</td>
<td>19.1±2.48</td>
</tr>
<tr>
<td>APD\textsubscript{90}, ms</td>
<td>80.5±19.62</td>
<td>81.2±19.91</td>
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Resting potential and action potential duration at 50 and 90% of repolarization (APD\textsubscript{50} and APD\textsubscript{90}, respectively) were determined by using the current-clamp mode in 6 Tg cardiac myocytes as described in MATERIALS AND METHODS.
cardiac myocyte. Activation of the overexpressed P2X4 receptor did not change the L-type Ca\(^{2+}\) current at any of the voltages tested. The current-voltage relation of the L-type Ca\(^{2+}\) channel in the Tg myocyte was similar whether or not 2-meSATP was present (Fig. 6B).

**DISCUSSION**

Activation of cardiac P2X receptors is capable of enhancing the contractile state of the myocyte and intact heart (8, 15, 29). Little is known regarding the mechanism of P2X receptor-mediated increase in contractility. By using transgenic mice with cardiac-specific overexpression of P2X4 receptors as a model, the present data show that an increased SR Ca\(^{2+}\) loading, occurring independent of any change in the cAMP level or the activity of L-type Ca\(^{2+}\) channel, is the mechanism of P2X receptor-mediated increase in contractility.

Of the P2X receptor subfamily, the P2X4 receptor is an important subunit of the cardiac myocyte P2X receptor (21). Cardiac myocytes isolated from transgenic mice with cardiac-specific overexpression of the P2X4 receptor were developed as a model to investigate the mechanism of enhanced contractility. A number of lines of evidence indicate that the mechanism of this enhanced contractile state is the result of an increased SR Ca\(^{2+}\) loading via activation of this receptor channel by extracellular ATP. First, activation of the overexpressed P2X4 receptor by the P2X agonist 2-meSATP caused an increased intracellular Ca\(^{2+}\) transient. This increase in cellular Ca\(^{2+}\) transient was temporally associated with the increase in myocyte contractility. Second, activation of the receptor channel in P2X4R Tg myocytes increased the caffeine-induced \(I_{\text{Na/Ca}}\) inward current in the presence of extracellular 2-meSATP. This increase in \(I_{\text{Na/Ca}}\) current in the Tg myocyte was not due to an increased \(I_{\text{Na/Ca}}\) density in the Tg myocyte since the latter has a similar basal \(I_{\text{Na/Ca}}\) density as the WT myocytes. In WT cardiac myocytes, extracellular 2-meSATP induced a caffeine-evoked inward current, indicating that activation of the endogenous cardiac P2X receptor in WT myocyte can also increase SR Ca\(^{2+}\) store. Thus the increased \(I_{\text{Na/Ca}}\) current was the result of an increased SR Ca\(^{2+}\) store. Further evidence for a SR Ca\(^{2+}\) loading effect of the overexpressed P2X4 receptor was provided by significantly larger amplitude of caffeine-induced Ca\(^{2+}\) transient in the presence of P2X agonist than that obtained in the absence of the agonist in both the Tg and the WT myocytes. Third, the relationship between the change in CS and that of intracellular Ca\(^{2+}\) was the same before and after P2X agonist application. The slopes were virtually identical. Changes in myofilament Ca\(^{2+}\) sensitivity can manifest as alterations in the duration of contraction (23). If P2X receptor activation could alter myofilament Ca\(^{2+}\) sensitivity, the presence of 2-meSATP would have changed the duration of contraction. However, data summarized in Fig. 1D and Table 1 showed that this was not the case. Overall, the data suggest that activation of the overexpressed P2X4 receptor did not change sensitivity to cellular Ca\(^{2+}\), suggesting against the possibility that an enhanced Ca\(^{2+}\) sensitivity was the mechanism of receptor-induced contractility increase. Fourth, our previous study demonstrated that activation of the native or the overexpressed cardiac P2X receptors had no effect on the L-type Ca\(^{2+}\) channel current (21). In the present study, activation of the overexpressed P2X4 receptor had no effect on the L-type Ca\(^{2+}\) current. In the Tg myocyte, the L-type Ca\(^{2+}\) current-voltage relation in the presence of 2-meSATP was the same as that obtained in its absence. The data ruled out an increased L-type channel activity as a cause of the increased myocyte contractility via P2X receptors. That the agonist-induced increase in Tg myocyte contractility was not associated with any cAMP increase is consistent with a lack of effect of P2X receptor activation on the L-type Ca\(^{2+}\) channel. The increase in cardiac myocyte contractility induced by the overexpressed P2X4 receptor was not associated with any change in the time to peak, the maximal rate of shortening or of relaxation, or the time to 50% relengthening. Similarly, the receptor-mediated increase in Ca\(^{2+}\) transient also did not cause any alteration in the diastolic Ca\(^{2+}\), the maximal rates of rise or decline, the time to 50% peak, or the time to 50% decline of the fluorescence ratio. The lack of any effect on the rates of shortening and relaxation of CS or those of Ca\(^{2+}\) transient is consistent with an absence of an increase in cAMP level since cAMP is known to accelerate the rate of rise and relaxation with a shortening effect on the duration of both contraction and Ca\(^{2+}\) transients (11, 27). The P2X receptor-mediated increase in Ca\(^{2+}\) transient was not due to any change in the action potential duration or characteristics since 2-meSATP had no effect on the APD\(_{90}\), APD\(_{99}\), or the resting membrane potential of the Tg cardiac myocyte. That the P2X4 receptor Tg myocytes showed a similar SR Ca\(^{2+}\) ATPase activity and SERCA2a and phospholamban levels as the WT cardiac myocytes suggests against an increased SR Ca\(^{2+}\) uptake as the mechanism for the enhanced SR Ca\(^{2+}\) loading effect of P2X receptor. The exact mechanism by which activation of the overexpressed P2X4 receptor leads to an increase SR store is not known but deserves future investigation. The 2-meSATP-induced increase in the contractility of WT myocytes was also not accompanied by any change in the rates of rise and decline of contraction or Ca\(^{2+}\) transient or the duration of action potential. These data further support the validity of findings in the P2X4 receptor-overexpressing Tg myocytes. These Tg myocytes represent a useful model to study the cardiac P2X receptor action and mechanism.

The stimulatory effect of P2X receptor agonist on Ca\(^{2+}\) transient was confined to that on the systolic but not the diastolic level. The lack of any effect on the diastolic Ca\(^{2+}\) level should not result in any increase in contractile state during diastole. This property would avoid a negative lusitropic effect and should permit the manifestation of a greater \(-dP/dt\) in the intact heart contractility measurement that was observed in previous studies (8, 15). Overall, the cardiac myocyte P2X4 receptor represents a ligand-gated cell surface receptor channel that can induce an increase in the SR Ca\(^{2+}\) store and contractile performance independent of L-type Ca\(^{2+}\) channel activation or cAMP accumulation. The contractile characteristics of this receptor channel differ from those of the 1, 17- or 1, 18-adrenergic receptor pathways and represent a novel mechanism to augment myocardial SR Ca\(^{2+}\) store and contractility.

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