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A beneficial role of cardiac $P2X_4$ receptors in heart failure: rescue of the calsequestrin overexpression model of cardiomyopathy

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[1]*Pat and Jim Calhoun Cardiology Center, University of Connecticut Health Center, Farmington, Connecticut 06030;* [2]*Krannert Institute of Cardiology, Indiana University School of Medicine, Indiana 46202; and* [3]*University of Utah School of Medicine, Salt Lake City, Utah 84132*

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Yang, Alexander, Dimitry Sonin, Larry Jones, William H. Barry, and Bruce T. Liang. A beneficial role of cardiac $P2X_4$ receptors in heart failure: rescue of the calsequestrin overexpression model of cardiomyopathy. *Am J Physiol Heart Circ Physiol* 287: H1096–H1103, 2004. First published May 6, 2004; 10.1152/ajpheart.00079.2004.—The $P2X_4$ purinergic receptor ($P2X_4R$) is a ligand-gated ion channel. Its activation by extracellular ATP results in Ca^{2+} influx. Transgenic cardiac overexpression of the human $P2X_4$ receptor showed an in vitro phenotype of enhanced basal contractility. The objective here was to determine the in vivo cardiac physiological role of this receptor. Specifically, we tested the hypothesis that this receptor plays an important role in modulating heart failure progression. Transgenic cardiac overexpression of canine calsequestrin (CSQ) showed hypertrophy, heart failure, and premature death. Crossing the $P2X_4R$ mouse with the CSQ mouse more than doubled the lifespan (182 ± 91 days for the binary CSQ/$P2X_4R$ mouse, $n = 35$) of the CSQ mouse (71.3 ± 25.4 days, $n = 50$, $P < 0.0001$). The prolonged survival in the binary CSQ/$P2X_4R$ mouse was associated with an improved left ventricular weight-to-body weight ratio and a restored β-adrenergic responsiveness. The beneficial phenotype of the binary mouse was not associated with any downregulation of the CSQ level but correlated with improved left ventricular developed pressure and ±dP/d*t*. The enhanced cardiac performance was manifested in young binary animals and persisted in older animals. The increased contractility likely underlies the survival benefit from $P2X_4$ receptor overexpression. An increased expression or activation of this receptor may represent a new approach in the therapy of heart failure.

cardiac failure; contraction; isoproterenol; purines; adenine nucleotide

ATP HAS LONG BEEN DEMONSTRATED to stimulate cardiac myocyte contractility at submicromolar concentrations (3, 4, 5, 18, 23). Recent studies show that activation of the P2X receptor subtype of the extracellular ATP receptor family increases the contractility of both cardiac myocytes and the intact heart (3, 4, 5, 9, 17, 18, 23). P2X receptors are ligand-gated ion channels whose activation results in sodium and calcium entry (6, 10, 12, 19, 24). Transgenic overexpression of the $P2X_4$ subtype of this family of receptor channels exhibits a phenotype of enhanced basal cardiac contractility and output in a working heart model (9). While these data suggest that the P2X receptor subfamily may mediate the effect of ATP on cardiac contractility, the potential cardiac physiological role of this receptor channel is not known. Because of the enhanced cardiac contractile performance of the $P2X_4$ receptor ($P2X_4R$) transgenic mouse, it is possible that $P2X_4$ receptor overexpression will modulate the progression of heart failure. To test this hypothesis, the $P2X_4R$ mouse was crossed with the calsequestrin (CSQ) mouse model of cardiac hypertrophy and heart failure. The CSQ model of severe heart failure is generated by overexpressing the sarcoplasmic reticulum (SR) calcium-binding protein CSQ, which disrupts coordinated regulation of calcium release. The model showed a phenotype of hypertrophy that progress to dilated cardiomyopathy, failure, and premature cardiac death by 16 wk of age (2, 11). Here, we tested whether the cardiac $P2X_4R$ mediates a beneficial effect on the progression to heart failure in the CSQ-overexpressing mouse. We find that the binary CSQ/$P2X_4R$ transgenic mouse exhibits improved cardiac function, characterized by a reduced left ventricular (LV) weight-to-body weight ratio, a restored β-adrenergic responsiveness, and a marked prolongation in survival that is associated with significant augmentation of cardiac contractility.

MATERIALS AND METHODS

Methods

Physiological measurement in isolated mouse hearts. The use of mice under the present study was approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee. Various parameters of intact heart function, such as LV developed pressure (LVDP) and rates of contraction and relaxation (±dP/d*t*) were quantitatively determined using the working heart or the Langendorff preparations (9, 17). After the injection of heparin via a tail vein (500 U/kg iv) and anesthetization with Nembutal (150 mg/kg) intraperitoneally, the heart with all major vessels and lungs attached was excised. The aorta was then cannulated with a 20-gauge catheter, positioned about 2 mm above the coronary ostia. For the working heart model (9, 17), a column of Krebs-Henseleit solution (KHS) buffer produced a constant hydrostatic pressure of 55 mmHg. The opening of the pulmonary vein was connected via a polyethylene-50 catheter to a reservoir of KHS buffer that maintained a "venous return" flow into the left atrium of ~5 ml/min under the resting condition. The LVDP was the difference between LV systolic and diastolic pressures. The basal heart rate was determined in the absence of pacing.

For the Langendorff method, a water-filled latex balloon inflated to a constant diastolic pressure of 5 mmHg was inserted into the lumen of the LV via the left atrium according to previously described method (17). The retrograde perfusion via the aorta was carried out by a perfusion pump maintaining a column of KHS [composed of (in mM) 120 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.5 EDTA, 25 $NaHCO_3$, 2 pyruvate, and 11 glucose, pH 7.4, gassed with 95% O_2-5% CO_2 at 37°C] to provide a constant coronary perfusion pressure of 55 mmHg. The coronary perfusion pressure was confirmed

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by a pressure transducer connected via a side port to the aorta perfusion cannula. Drugs were infused into a side port of a reservoir of buffer that reached the heart via retrograde perfusion of the aorta. The duration of circulation of the drug-containing perfusate was ~2–2.5 min during which the various functional parameters were recorded. The pressure recordings were made using a fluid-filled system of pressure transducer, channeled from a precalibrated amplifier (Kent Scientific; Litchfield, CT), and the signals were digitized via a PCM-DAS 16S/330 interface board (Computer Boards; Mansfield, MA). Data were analyzed by computer software (WorkBench for Windows+, Kent Scientific). The amplified and digitized signals from the transducers were constantly displayed and analyzed.

Data points under each basal condition and during infusion of each drug concentration are summarized as means ± SE. For analysis between three or more groups, one-way ANOVA analysis and posttest (Newman-Keuls) comparison were carried out. Survival data were compared using the Kaplan-Meier survival curve with a log rank method of statistical analysis. To compare the effects between groups treated with two different agonists or under different conditions, an unpaired *t*-test was used.

Generation of the P2X$_4$R transgenic mice. The P2X$_4$R transgenic construct was generated by subcloning a 1.8-kb *Hin*d III fragment of human P2X$_4$R cDNA into the *Hin*dIII site of an α-myosin heavy chain (MyHC) expression vector and bred in B6SJL mice as previously described (9). The CSQ transgenic mouse overexpressing the canine CSQ was generated in DBA mice as previously described using the same α-MyHC overexpression promoter (11). The CSQ mice were then crossed with wild-type B6SJL mice and bred in the B6SJL background for 10 generations before being crossed with the P2X$_4$R mice.

Immunoblotting. Hearts from 3-mo-old CSQ, P2X$_4$R, CSQ/P2X$_4$R, and nontransgenic (NTG) mice were isolated, blotted dry, weighed, and homogenized in ice-cold buffer containing 0.25 M sucrose and 10 mM MOPS, pH 7.2 (16 ml/g wt), using a tissue homogenizer (PowerGen Model 125, Fisher Scientific; Pittsburgh, PA). After solublization in sample buffer, SDS-PAGE and immunoblotting were conducted as recently described (13). Twenty-five micrograms of homogenate protein were electrophoresed per gel lane using 8% polyacrylamide and transferred to nitrocellulose membranes. For detection of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA2a), CSQ, and phospholamban (PLB), monoclonal antibody 2A7-A1, affinity-purified rabbit polyclonal antiserum, and monoclonal antibody 2D12 were used, respectively (13). Antibodies bound to proteins were detected with ^{125}I-labeled protein A and quantified using a PhosphorImager (GS-250 Molecular Imager, Bio-Rad; Hercules, CA) (13). For detection of the P2X$_4$R, rabbit polyclonal antibody directed against an unique COOH-terminus sequence of the rat P2X$_4$R [Alomone; Jerusalem, Israel (15, 20)], which cross-reacted with both the human and mouse P2X$_4$Rs, was used (9). The membrane was incubated with peroxidase-coupled anti-rabbit Ig antibody (1:5,000) and developed with an ECL-Plus kit (Amersham). The level of the P2X$_4$R protein was quantified via a BioRad Geldoc 2000 using the Discovery Series Quantity One version 4.5.2 (Bio-Rad). The quantity of the band was obtained as the sum of intensity of all pixels within the band boundary multiplied by the area of each pixel. Equal amounts of proteins were loaded per gel lane, which was subsequently confirmed by Ponceau S staining of the blot (7, 14, 25) and by probing with an affinity-purified goat polyclonal antibody against the COOH terminus of a broad range of actin isoforms such as β- and α-actin (actin, 1–19: sc-1616) [identical in the human, rat, and mouse (1, 16)].

Ca^{2+}-ATPase assay. For the assay of Ca^{2+}-ATPase activity, 80 μg of homogenate protein were incubated at 37°C in 1 ml of buffer containing 50 mM histidine (pH 7.2), 3 mM $MgCl_2$, 50 μM $CaCl_2$, 3 mM ATP, 100 mM KCl, 5 mM NaN_3, and 3 μg A23187 (13). P_i release was measured colorimetrically. The Ca^{2+}-ATPase activity reported is that activity specifically inhibited by 3 μM thapsigargin. The units for Ca^{2+}-ATPase assay are micromoles of P_i per milligram of protein per hour.

Materials

ATP, α,β-methylene ATP, 2-meSATP, Ponceau S, and suramin were obtained from Sigma Chemical (St. Louis, MO). Liberase (type 2) was from Roche Molecular Biochemicals (Indianapolis, IN). Actin (1–19: sc-1616), which cross-reacts with a broad range of actin isoforms in the mouse, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The α-MyHC promoter was obtained from Dr. J. Robbins (University of Cincinnati), and the full-length cDNA encoding the human P2X$_4$R was supplied by Drs. W. Stumers and F. Soto (Max-Planck Institute for Experimental Medicine, Gottingen, Germany). The ECL-Plus kit was from Amersham (Piscataway, NJ). B6SJL/F1 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

RESULTS

The Binary CSQ/P2X$_4$R Mouse Showed Similar Levels of the Transgenes as the CSQ or P2X$_4$R Mouse

Previous studies have demonstrated an important role of the P2 purinergic receptor (3, 4, 5, 17, 18, 23), specifically, the P2X$_4$R (9, 10), in mediating the increased cardiac contractility induced by ATP. Using the working heart preparation to determine the in vitro cardiac function, we recently showed that cardiac transgenic overexpression of the human P2X$_4$R results in enhanced basal LVDP, ±dP/d*t*, and cardiac output compared with NTG littermates (9). In the present study, the increased cardiac contractile performance of the P2X$_4$R transgenic mouse was confirmed in the isolated perfused Langendorff heart model. Compared with the NTG animal, the P2X$_4$R mouse exhibited a higher basal level of LVDP (Fig. 1*A*), contractility (+dP/d*t*; Fig. 1*B*), and relaxation (−dP/d*t*, not shown) ($P < 0.001$, Student's *t*-test). As observed using the working heart model previously (9), there was no difference in the basal spontaneous heart rate between the transgenic and NTG animals ($P > 0.1$, Student's *t*-test) using the Langendorff model.

To examine a potential in vivo role of the P2X$_4$R, the P2X$_4$R mouse was crossed with the CSQ overexpression model of hypertrophy and heart failure. The CSQ transgenic mouse developed hypertrophy, followed by congestive dilated cardiomyopathy, and showed premature death with markedly reduced longevity (2, 8, 11). At comparable ages, the binary CSQ/P2X$_4$R mouse (age 85 ± 4.4 days, mean ± SD, $n = 6$) maintained overexpression of both transgenes, showing a level of overexpression of CSQ or of P2X$_4$R similar to that observed for the single transgenic mice for CSQ overexpression (age = 98.6 ± 17.4 days, ± SD, $n = 6$ hearts; Fig. 2) or for P2X$_4$R overexpression (age = 105 ± 21 days, ± SD, $n = 7$ hearts; not shown) mice, respectively. Quantitative immunoblotting revealed that CSQ was overexpressed 15-fold in myocardial homogenates from single and double transgenic mice, and P2X$_4$R was overexpressed 20-fold in single and double transgenic mouse homogenates.

P2X$_4$R Expression Prolonged the Longevity of the CSQ Mouse

Because CSQ mice died prematurely and P2X$_4$R mice have augmented cardiac contractility, we tested whether overexpressing the P2X$_4$R in the CSQ mouse can confer a survival benefit. The CSQ mouse and the binary CSQ/P2X$_4$R transgenic mouse were monitored for survival; the lifespans were then compared with that of NTG littermates as well as to that of the single P2X$_4$R transgenic mouse. Figure 3 shows the survival curves for the CSQ ($n = 50$), CSQ/P2X$_4$R ($n = 35$),

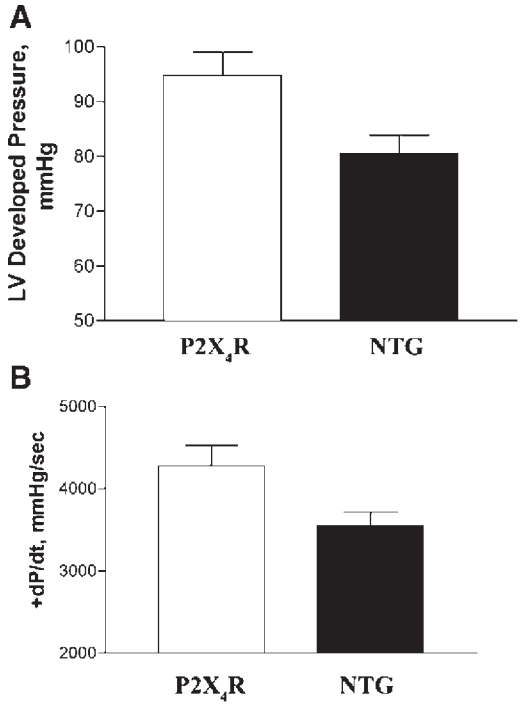

Fig. 1. Increased basal intact heart function of the $P2X_4$ receptor ($P2X_4R$) transgenic (TG) animals. The various functional parameters of cardiac performance, such as left ventricular (LV) developed pressure (LVDP), rate of contraction (+dP/d*t*), and rate of relaxation (−dP/d*t*), were quantified using the Langendorff heart model as described in MATERIALS AND METHODS. Data points under each basal condition are summarized as means ± SE from seven $P2X_4R$ TG and eight non-TG (NTG) animals, and the statistical differences were analyzed by Student's *t*-test (unpaired). The basal level of LVDP (*A*) was significantly greater, as was the basal level of ± dP/d*t* (*B*), in the $P2X_4R$ TG mouse than in the NTG animal ($P < 0.001$, Student's *t*-test).

$P2X_4R$ ($n = 44$), and NTG ($n = 44$) animals; these survival curves are significantly different from one another (log rank test, χ^2 of 235.1, $df = 3$, $P < 0.0001$). Whereas the CSQ mouse lived an average age of 71.3 ± 25.4 days, the double CSQ/

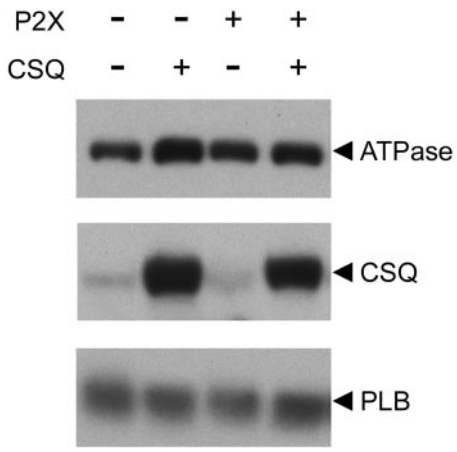

Fig. 2. Expression levels of sarco(endo)plasmic Ca^{2+}-ATPase (SERCA)2a, calsequestrin (CSQ), and phospholamban (PLB) in homogenates from TG hearts. Hearts of 3-mo-old NTG, CSQ, $P2X_4R$, and CSQ/$P2X_4R$ mice were homogenized, solublized, and immunoblotted as described in MATERIALS AND METHODS. Identical amounts of protein were loaded per lane for each antibody used. Antibodies were specific for SERCA2a, CSQ, and PLB. The autoradiograph was typical of four similar experiments.

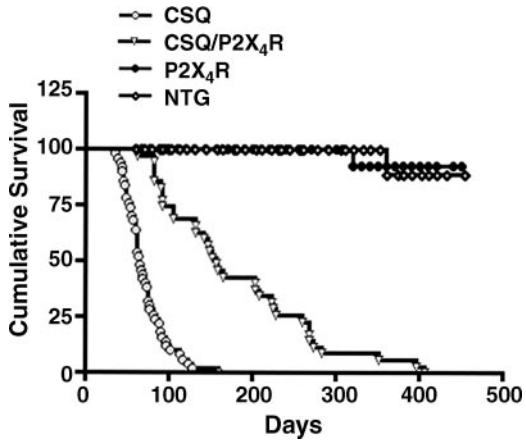

Fig. 3. Increased survival in the CSQ/$P2X_4R$ versus CSQ mice. Kaplan-Meier analysis was used to determine the survival probability in single CSQ, single $P2X_4R$, binary CSQ/$P2X_4R$, and NTG animals. The log rank test method was used to analyze the survival curves ($P < 0.0001$). The mean survival age of the CSQ mice was 71.3 ± 25.4 days and was significantly less than that of the binary CSQ/P2X4R mice, which was182 ± 91 days. NTG: $n = 44$ mice; $P2X_4R$: $n = 44$; CSQ: $n = 50$; CSQ/$P2X_4R$: $n = 35$.

$P2X_4R$ mouse had a more than doubled lifespan of 182 ± 91 days. These data indicate that $P2X_4R$ overexpression prolongs life in the CSQ mouse.

Cardiac Performance in the Binary CSQ/P2X4R Mouse

At comparable ages, the positive effect of $P2X_4R$ overexpression on the survival of the CSQ transgenic mouse is associated with a significant decrease in the LV weight-to-body weight ratio. Although the LV weight-to-body weight ratio in the CSQ/$P2X_4R$ mouse (9.22 ± 3.5 mg/g, $n = 6$ mice) was significantly greater than the LV weight-to-body weight ratio of the NTG animal (5.36 ± 0.49, $n = 8$ mice), it was less than that of the single CSQ mouse (15 ± 1.94, $n = 6$ mice) (means ± SD, one-way ANOVA and posttest comparison, $P < 0.01$; Fig. 4*A*). Similar to findings by others (8), we found that the response to β-adrenergic stimulation was impaired in the CSQ mouse. The stimulation of LVDP (Fig. 4*B*), +dP/d*t* (Fig. 4*C*), or −dP/d*t* (not shown) by isoproterenol was at least 50% less in the CSQ TG mouse compared with that of the NTG mouse ($P < 0.05$). The beneficial effect of $P2X_4R$ expression in the CSQ mouse was associated with a dramatic improvement of β-adrenergic responsiveness. In the CSQ/$P2X_4R$ mouse, the isoproterenol-induced stimulation of LVDP (%stimulation: 48 ± 25%; Fig. 4*B*) and +dP/d*t* (%stimulation: 87 ± 80%; Fig. 4*C*) was significantly greater than that found for the CSQ mouse (%stimulation for LVDP: 19 ± 23%; for +dP/d*t*: 31 ± 35%, $P < 0.05$) and was, in fact, similar to the extent of isoproterenol-stimulated response in the NTG animals (%stimulation for LVDP: 42 ± 19%; for +dP/d*t*: 102 ± 29%, $P > 0.05$, one-way ANOVA analysis and posttest comparison). Similar data were obtained using −dP/d*t* as the end point (not shown). These data demonstrate that the survival benefit from $P2X_4R$ overexpression is associated with a decreased LV weight-to-body weight ratio and restoration of β-adrenergic responsiveness to normal levels.

Effects of overexpressing the $P2X_4R$ on levels of SERCA2a and PLB were determined in the CSQ/$P2X_4R$ mice. SERCA2a and PLB are key proteins controlling SR filling and contractility

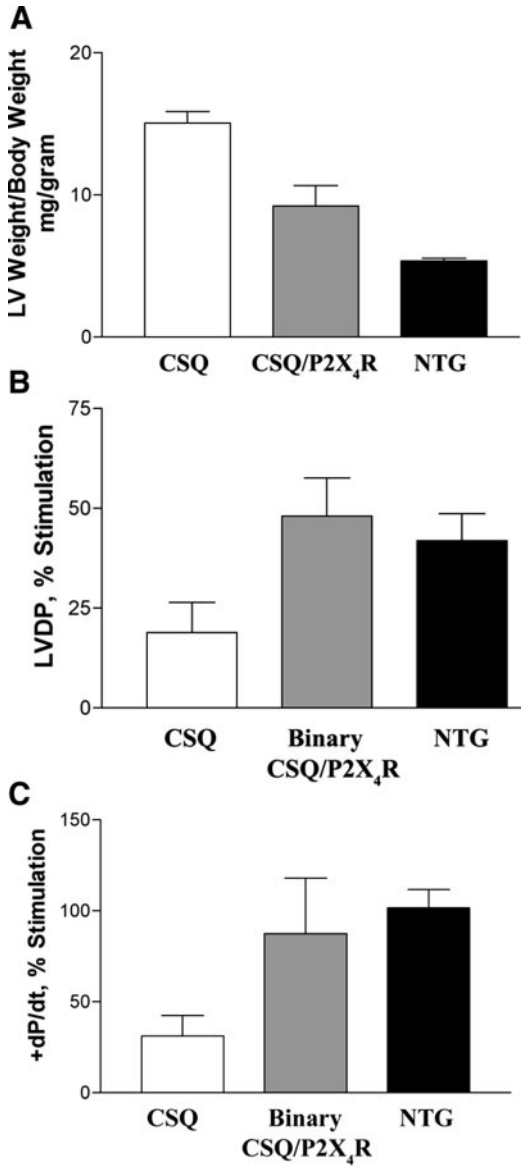

Fig. 4. Improved LV weight-to-body weight ratio and restored β-adrenergic responsiveness in CSQ/P2X$_4$R mice. *A*: LV weight, body weight, and LV weight-to-body weight ratios were determined for CSQ (6 animals, age of 98.6 ± 17.4 days, mean ± SD), CSQ/P2X$_4$R (6 animals, age of 85 ± 4.4 days, mean ± SD), and NTG (8 animals, age of 100 ± 15 days, mean ± SD) mice. One-way ANOVA analysis and Newman-Keuls posttest comparison showed significant difference between any two groups of mice ($P < 0.01$). *B* and *C*: retrograde and antegrade perfusions of the aorta and coronary artery in the isolated mouse heart were carried out using the Langendorff preparation as described in MATERIALS AND METHODS. The β-adrenergic receptor agonist isoproterenol (10 nM) was infused into a side port of a reservoir of buffer that reached the heart via retrograde perfusion of the aorta. LVDP and +dP/d*t* were determined before and after infusion of isoproterenol for each animal. Increases in LVDP (*B*) and +dP/d*t* (*C*) are shown as the percent stimulation above the predrug values and are represented as means ± SE of 10 CSQ (age of 92 ± 16 days), 7 CSQ/P2X$_4$R (age of 87 ± 7 days), and 8 NTG (100 ± 15 days) mice.

in mouse myocardium (21, 22). Expression levels of SERCA2a and PLB were quantified in homogenates of NTG, CSQ, P2X$_4$R, and CSQ/P2X$_4$R mouse hearts after the ^{125}I-labeled immunoblots were scanned in a PhosphorImager. Typical expression levels are summarized in Fig. 2. The levels of SERCA2a (one-way ANOVA analysis, $F = 2.5$, $P = 0.11$) and PLB ($F = 1.52$, $P = 0.26$) were similar in all four groups. Similarly, Ca^{2+}-ATPase enzymatic activity, determined as that activity specifically inhibited by thapsigargin, was also similar in NTG (5.01 ± 0.72), CSQ (5.58 ± 1.37), P2X$_4$R (5.14 ± 0.39), and CSQ/P2X$_4$R (6.38 ± 1.30) hearts (one-way ANOVA analysis, means ± SD, $n = 4$ hearts for each genotype, $F = 1.44$, $P = 0.28$). Thus the beneficial effect of P2X$_4$R overexpression is apparently not associated with any change in the ability of SR to actively transport Ca^{2+}.

Mechanism of Increased Longevity: Enhanced Cardiac Contractile Performance

Because the P2X$_4$ transgenic mouse showed augmented basal cardiac contractility and performance, we tested the hypothesis that an enhanced cardiac contractile performance in the binary

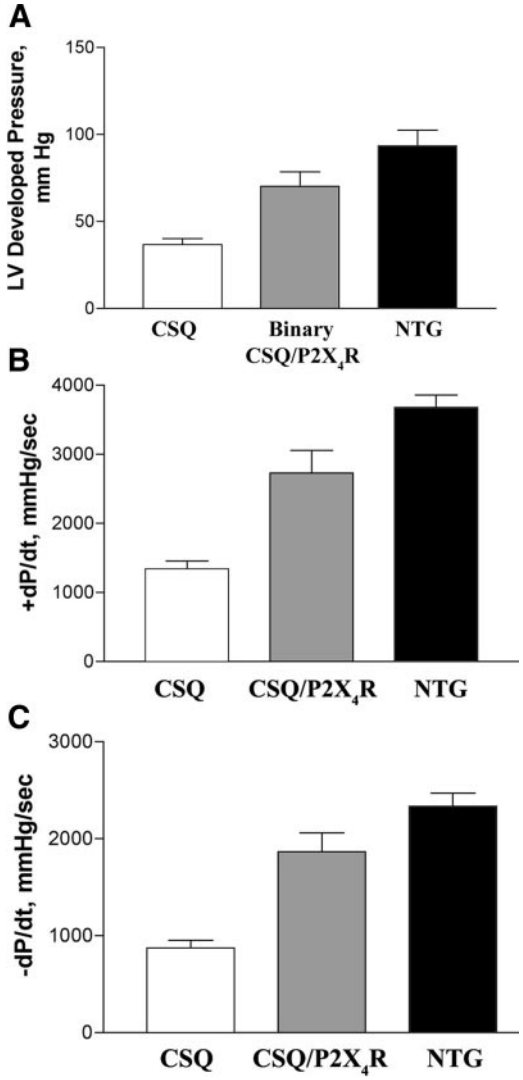

Fig. 5. P2X$_4$R overexpression reversed the depressed LVDP, +dP/d*t*, and −dP/d*t* of the CSQ mouse. Retrograde perfusion of the aorta and coronary artery in the isolated CSQ, CSQ/P2X$_4$R, and NTG hearts was carried out using the Langendorff model as described in MATERIALS AND METHODS. The basal values for LVDP (*A*), +dP/d*t* (*B*), −dP/d*t* (*C*) were summarized as means ± SE from 10 CSQ (age 92 ± 16 days), 7 CSQ/P2X$_4$R (age of 87 ± 7 days), and 9 NTG (age of 100 ± 15 days) mice. One-way ANOVA analysis and Newman-Keuls posttest comparison showed significant difference between any two groups of mice ($P < 0.05$).

CSQ/P2X$_4$R mouse is a mechanism by which the P2X$_4$R overexpression improved the lifespan and rescued the heart failure phenotype of the CSQ mouse. The heart function of the binary mouse was determined using the isolated Langendorff model and was compared with that of the single CSQ transgenic mouse. The binary CSQ/P2X$_4$R mice had significantly higher LVDP (70 ± 24 mmHg; Fig. 5*A*), +d*P*/d*t* (2,729 ± 921 mmHg/s; Fig. 5*B*), and −d*P*/d*t* (1,866 ± 577 mmHg/s; Fig. 5*C*) than did the age-matched CSQ mouse (LVDP: 37 ± 9 mmHg; +d*P*/d*t*: 1,340 ± 285 mmHg/s; −d*P*/d*t*: 874 ± 198 mmHg/s, $P < 0.05$). The cardiac physiological indexes of the binary animals were lower than those in NTG mice, which had LVDP of 93 ± 29 mmHg, +d*P*/d*t* of 3,674 ± 524 mmHg/s, and −d*P*/d*t* of 2,334 ± 385 mmHg/s (Fig. 5; one-way ANOVA analysis and posttest comparison, $P < 0.05$). Similar improvements of LVDP and ±d*P*/d*t* were also found in the CSQ/P2X$_4$R versus CSQ comparison when the working heart model was used to determine the cardiac contractile function (not shown).

The improvement in LVDP and ±d*P*/d*t* as a result of P2X$_4$R overexpression in the CSQ model was not associated with any change in the basal heart rate in vitro. The CSQ model of severe heart failure showed a depressed basal heart rate in the isolated Langendorff heart preparation (319 ± 40 beats/min, $n = 6$ mice) compared with the NTG animal (361 ± 38 beats/min, $n = 11$ mice, $P < 0.05$). The binary CSQ/P2X$_4$R mouse showed a basal heart rate (314 ± 37 beats/min, $n = 9$ mice) that was the same as the CSQ mouse (one-way ANOVA and posttest comparison, $P > 0.05$). Thus overexpression of the P2X$_4$R conferred a favorable inotropic effect selectively in the CSQ mice, having no effect on the depressed chronotropic state of the cardiomyopathic animals.

Histological examination of the hearts showed individual myocyte hypertrophy (Fig. 6*D*) and focal areas of increased collagen deposits (Fig. 7*D*) in the CSQ hearts compared with the NTG or P2X$_4$R hearts ($n = 4$ for each group). The binary heart (Fig. 7*C*) showed fewer collagen deposits than the CSQ heart, whereas the binary myocyte was similarly enlarged as the CSQ cardiac myocytes.

Evidence for Early Restoration of Cardiac Function in the Binary CSQ/P2X$_4$R Mouse

At a young age (53 ± 5.3 days), the CSQ mouse already exhibited decreased cardiac LVDP and ±d*P*/d*t* (Table 1). In fact, basal LVDP and ±d*P*/d*t* were similarly depressed in young and older CSQ mice ($P > 0.1$, *t*-test). Overexpressing the P2X$_4$R in the CSQ animals restored the cardiac contractility with an increased LVDP and ±dP/dt in the young binary CSQ/P2X$_4$R mouse (Table 1). The improved cardiac contractility of the CSQ/P2X$_4$R mouse was associated with a lower LV weight-to-body weight ratio in the binary compared with CSQ mouse (Table 1). Compared with the older CSQ mice (98.6 ± 17.4 days), the young CSQ animals had a lower LV weight-to-body weight ratio ($P < 0.05$, *t*-test). On other hand, the ratio did not increase with age in the binary CSQ/P2X$_4$R animals ($P > 0.1$). Thus the rescuing effect of cardiac P2X$_4$R overexpression was correlated with a restoration of cardiac contractility at a young age, before progression to the severe cardiomyopathy. The beneficial effects on cardiac contractility and LV weight-to-body weight ratio were sustained even as the binary mice matured into older adulthood.

DISCUSSION

The P2X$_4$R is a ligand-gated ion channel (6, 10, 12, 19, 24). It is a member of the extracellular ATP receptor family. Activation of the P2X$_4$R by ATP causes Ca^{2+} influx and

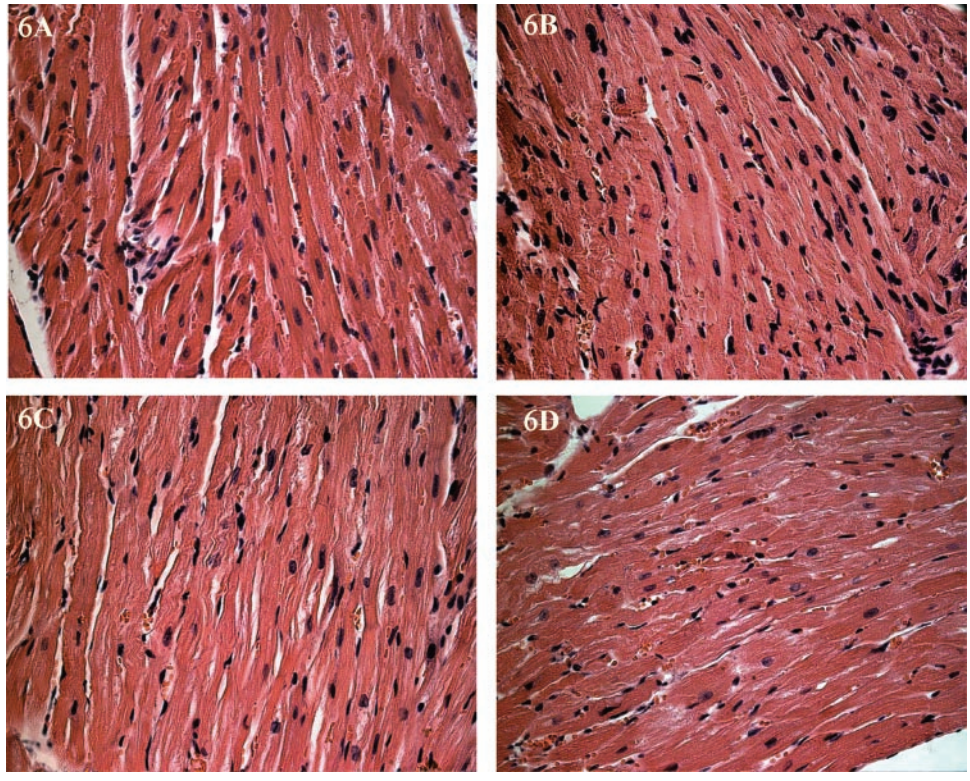

Fig. 6. Heart histology in NTG, P2X$_4$R, CSQ, and CSQ/P2X$_4$R mice. Hematoxylin-eosin-stained sections from NTG (*A*), P2X$_4$R (*B*), CSQ (*C*), and CSQ/P2X$_4$R (*D*) mice were obtained. The stained sections were typical of four mice from each group. Areas of collagen deposits were present in the CSQ hearts.

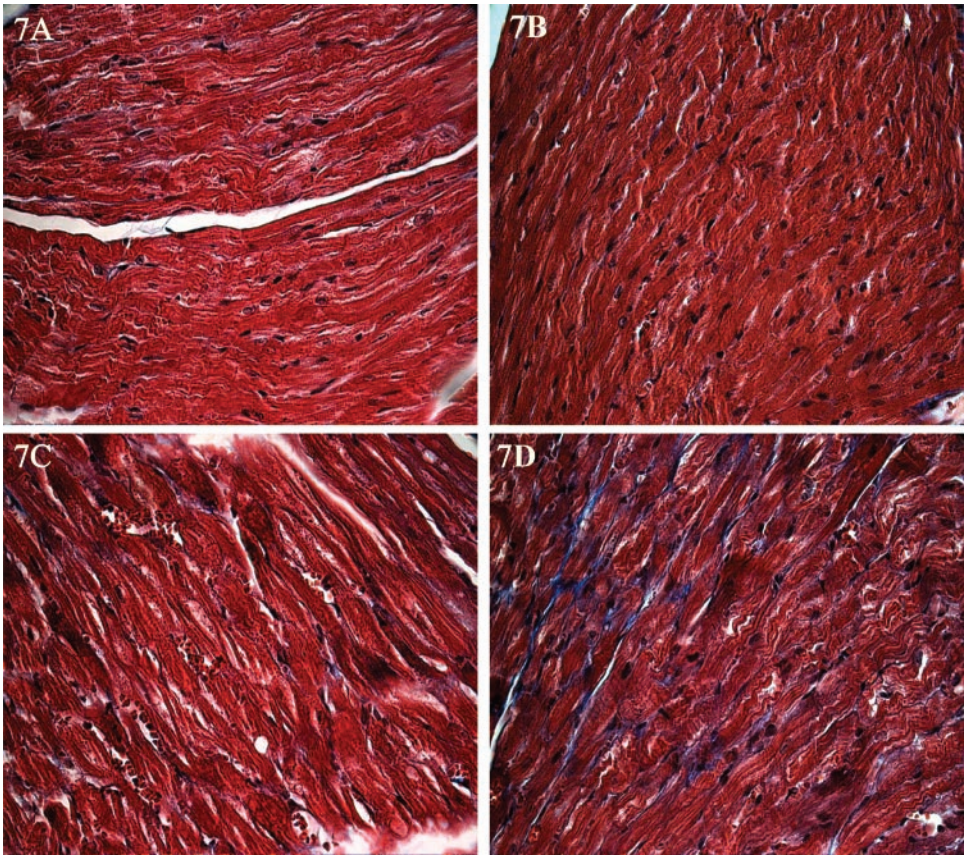

Fig. 7. Heart histology in NTG, P2X$_4$R, CSQ, and CSQ/P2X$_4$R mice. Trichrome-stained sections from NTG (*A*), P2X$_4$R (*B*), CSQ (*C*), and CSQ/P2X$_4$R (*D*) mice were obtained. The stained sections were typical of four mice from each group. Areas of collagen deposits were present in the CSQ hearts.

stimulates the contractions of both isolated cardiac myocytes and the intact heart (3–5, 9, 17, 18, 23). Transgenic overexpression of the human P2X$_4$R showed enhanced basal contractility and cardiac performance in vitro (9). Although the in vivo cardiac biological function of the P2X$_4$R is not known, evidence has accumulated suggesting that it has an important biological role. Because overexpression of the P2X$_4$R enhances basal cardiac contractility, the objective of the present study was to test the hypothesis that P2X$_4$R overexpression can confer a beneficial effect on the progression of heart failure. To test this hypothesis, P2X$_4$R mice were mated with CSQ-overexpressing mice, which exhibit a well-documented syndrome of cardiac hypertrophy progressing to heart failure and premature death at a very early age. The aggressive cardiomyopathy of the CSQ overexpression mouse was significantly attenuated by overexpression of the P2X$_4$R. The data demonstrated a more than doubled lifespan of the binary CSQ/P2X$_4$R animal, which showed a markedly improved LV weight-to-body weight ratio as well as β-adrenergic responsiveness.

Table 1. *Early restoration of cardiac function in the binary CSQ/P2X$_4$R mouse*

	CSQ	CSQ/P2X$_4$R	NTG
Age, days	53±5.3	56±5.4	56±5
LV weight/body weight, mg/g	11.9±2.8*	9.2±1.2†	4.9±0.3
LVDP, mmHg	35.9±22.8*	61.4±9	65.5±4.6
+dP/d*t*, mmHg/s	1,491±1,107*	2,907±671	3,534±434
−dP/d*t*, −mmHg/s	894±519*	1,683±127	1,997±260

Values are means ± SD; $n = 7$ calsequestrin (CSQ), 5 CSQ/P2X$_4$ receptor (P2X$_4$R) and 8 non transgenic (NTG) mice. Retrograde perfusion of the aorta and coronary artery in the isolated CSQ, CSQ/P2X$_4$R, and NTG hearts was carried out using the Langendorff model as described in MATERIALS AND METHODS. One-way ANOVA analysis and Newman-Keuls posttest comparison were carried out. LV, left ventricular; LVDP, LV developed pressure. *Significantly different from either of the other two types of mice ($P < 0.05$); †significantly different from the NTG animals.

The cardiac CSQ overexpression mouse is a well-studied model of hypertrophy and heart failure. The canine CSQ model is characterized by severe heart failure with depressed cardiac contractile function and premature death by 16 wk (2, 8, 11). In an earlier study, heart failure in the mice was rescued by overexpression of a cardiac β-adrenergic receptor kinase (βARK) inhibitor (8). Here, we used the same CSQ model to test whether overexpression of the cardiac P2X$_4$R could also rescue heart failure in these mice. A beneficial role of the P2X$_4$R in modulating heart failure progression was supported by a number of lines of evidence. First, and perhaps most important, transgenic overexpression of the P2X$_4$R in the CSQ model of severe heart failure dramatically increased animal survivability. The lifespan of the binary CSQ/P2X$_4$R mouse was increased nearly 2.5-fold. Second, the P2X$_4$R overexpression reduced the hypertrophy in the CSQ/P2X$_4$R mice as evidenced by a reduced LV weight-to-body weight ratio compared with that of the age-matched CSQ mice. Histological examination using trichrome staining of heart sections showed fewer collagen deposits in the binary mouse than the CSQ mouse. Third, while impaired β-adrenergic responsiveness is a marker for heart failure in the CSQ and other models (8), P2X$_4$R expression restored the β-adrenergic response in the CSQ mice. The isoproterenol-induced stimulation of LVDP

and ±dP/d*t* was significantly greater in the binary CSQ/P2X$_4$R mice than in the CSQ mice. Overexpression of the P2X$_4$R did not affect the extent of overexpression of CSQ in the binary CSQ/P2X$_4$R animals. The rescue from premature death in the CSQ animals was therefore not due to any decrease in the extent of CSQ overexpression in the binary transgenic mice. The two key SR proteins that regulate calcium uptake into the SR, the Ca^{2+} pump (SERCA2a) and PLB, showed no change in their levels in the binary CSQ/P2X$_4$R hearts versus the CSQ hearts. The Ca^{2+}-ATPase activity also showed no alteration in the CSQ/P2X$_4$R hearts compared with CSQ or any other genotype hearts. The absence of change in SERCA2a, PLB, or Ca^{2+}-ATPase activity suggested that the rescuing effect of P2X$_4$R expression was not due to any beneficial alteration of SR calcium uptake. On the other hand, the increased longevity in the CSQ/P2X$_4$R mouse is clearly associated with an enhanced cardiac contractile performance in the binary animal. The CSQ/P2X$_4$R mouse showed significantly higher basal levels of LVDP, +dP/d*t*, and −dP/d*t* than did the CSQ animals. The rescuing effect of P2X$_4$R overexpression became manifested early on, during young adulthood of the binary animals. The basal LVDP, ±dP/d*t*, and LV weight-to-body weight ratio of the binary animals were significantly improved as early as 7–8 wk old compared with the CSQ mice of comparable age. This increased cardiac contractility and improved heart weight-to-body weight ratio in the binary mice persisted into older adulthood. The basal cardiac contractile indexes and the heart weight-to-body weight ratios of the older binary mice were similar to those of the young binary mice. These data further supported the concept that an enhanced cardiac performance is a mechanism by which P2X$_4$R overexpression rescued the severe heart failure phenotype of the CSQ mice. It is likely that the rescuing effect delayed the progression of severe heart failure, which ultimately caused death due to pump failure.

The basal heart rate, as determined in the isolated Langendorff model, remained similarly depressed in both the CSQ/P2X$_4$R and CSQ animals. The actual heart rate in vivo, which is dependent on the intrinsic sinus rate and sympathetic and vagal activities, is not known. The lack of any difference in the in vitro heart rate between CSQ and binary mice suggested that P2X$_4$R overexpression did not affect the intrinsic sinus rate of the CSQ mice, separate from any potential effect on sympathetic or vagal activities. Taken together, P2X$_4$R expression did not rescue the depressed chronotropic state of the CSQ mice but rather selectively restored their basal cardiac contractility. The data suggest that increased Ca^{2+} influx at the cardiac sarcolemma may at least in part account for the beneficial effects of P2X$_4$R overexpression. Although cardiac βARK inhibition also prolongs survival in the canine CSQ overexpression model of heart failure (8), the survival benefit induced by βARK inhibition in the canine CSQ overexpression model is not associated with a reversal of the depressed cardiac contractility. The mechanism of heart failure rescue by βARK inhibitor overexpression appears to be different from the mechanism by which P2X$_4$R overexpression rescues CSQ cardiomyopathic animals.

Overall, the present study indicates a novel physiological role for the cardiac P2X$_4$R: that of a beneficial life-prolonging role in heart failure. Increased expression or activation of these ATP-stimulated receptor channels may represent a new therapeutic approach for the treatment of heart failure.

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