

## P2X<sub>1</sub> receptors are closely associated with connexin 43 in human ventricular myocardium

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### Abstract

**Background:** It has been suggested that gap-junctional conductance between cardiomyocytes is regulated through a specific ligand-receptor interaction between ATP and connexins. In this study we examined the localization of P2X<sub>1</sub> ionotropic receptors and their relation to connexin43 in gap junctions in human left ventricles. **Methods and results:** Using immunohistochemistry, we detected P2X<sub>1</sub> expression predominantly in the intercalated discs. Labelling of the P2X<sub>1</sub> receptor and the gap junction protein connexin43 showed close association in some gap junctions, while in others the two proteins often appeared to be spatially discrete. Western blotting detected four major bands at 45, 60, 95 and 120 kDa in the protein extracts from human left ventricles corresponding to equivalent bands from rat vas deferens. The most prominent band in human left ventricles was at 95 kDa, possibly a dimer of the native P2X<sub>1</sub> receptor, whereas in rat vas deferens it was at 60 kDa. After preincubation of the antibody with its epitope peptide, the 45 and 60 kDa bands almost disappeared and the 95 and 120 kDa bands were significantly attenuated. **Conclusions:** P2X<sub>1</sub> receptors in human myocardium are densely localized in gap junctions at intercalated discs between muscle cells. Close association of P2X<sub>1</sub> receptors and connexin 43 occurred in some regions of some gap junctions, but in others they were spatially separate. Little difference in the pattern of distribution of P2X<sub>1</sub> receptors was found in failing left ventricles of patients with dilated cardiomyopathy, although Western blots showed an enhancement of P2X<sub>1</sub> receptor protein.

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**Keywords:** P2X<sub>1</sub> receptor; Cardiomyocyte; Gap junctions; Cardiomyopathy

### 1. Introduction

P2 purinergic receptors are expressed in the plasma membranes of a wide range of tissues [1]. ATP is released from hypoxic cardiomyocytes [2,3] and is postulated to exert potent inotropic effects mediated by P2 receptors or after ectoenzymatic breakdown to adenosine, via P1 receptors [2,4,5]. The presence of P2 receptors in cardiomyocytes has been confirmed by the expression of mRNA in foetal human heart [6] and rat heart [7]. The level of mRNA for the P2X<sub>1</sub> receptor was increased by 2.7-fold in left ventricle (LV) in a congestive heart failure rat model [7]. Immunohistochemistry has demonstrated the distribution of P2X<sub>1</sub> receptors in vascular smooth muscle cells and cardiac

myocytes [8–11]. A low density of P2X<sub>1</sub> expression was found on cardiomyocytes in the rat heart [12]. Two major forms of P2X<sub>1</sub> receptor (45 and 60 kDa) have been reported using photoaffinity binding [13,14] and Western blotting [15,16], and the 60 kDa protein is believed to be the glycosylated form of the 45 kDa native form.

In the myocardium, neighbouring cardiomyocytes are joined by intercalated discs, the specialised portions of plasma membrane consisting of gap junctions, fascia adherens, and desmosomes [17]. The fascia adherens and desmosomes are forms of anchoring junction, responsible for attachment of the myofibrils and the desmin cytoskeleton to the adjacent plasma membrane. Gap junctions are ion channels responsible for cell-to-cell rapid conduction of action potentials and direct transmission of chemical signals [18]. In mammalian hearts, the gap junction is built of membrane-spanning proteins, mainly connexin43 [19]. It has been suggested that gap-junctional conductance is

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regulated through a specific ligand-receptor interaction between ATP and connexins [20–23].

In this study we investigate the expression and localization of P2X<sub>1</sub> receptors in normal human heart and in failing hearts of dilated cardiomyopathy (DCM) patients [24,25]. In particular, we examine the localization of P2X<sub>1</sub> receptors in relation to the intercalated discs, especially the gap junctions.

## 2. Materials and methods

### 2.1. Tissue samples

Samples of the anterior free wall of LV were dissected and preserved in liquid nitrogen in the operating theatres of St. Vincent's Hospital, Sydney, Australia, during heart transplantation. The project was approved by the University of Sydney Human Ethics Committee (K03/1–9/3/2786) and the St. Vincent's Hospital Ethics Committee (H91/048/1). The investigation conforms with the principles outlined in the Declaration of Helsinki. Eight human failing hearts with DCM and five human donor hearts as normal control were used in this study. The failing hearts were dissected immediately after removal. The donor hearts (confirmed free of cardiovascular diseases) were flushed with cold cardioplegic solution (119 mM NaCl, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 4 mM KCl, 25 mM NaHCO<sub>3</sub>, 1.2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, and 11 mM glucose) and transported on ice to the transplant theatre. The donor samples used in this study were from whole hearts obtained after cancellation of a scheduled transplantation. Samples of the failing hearts were usually dissected within 10–30 min of cross-clamp (loss of coronary circulation) and the donor samples within 30–120 min of removal.

Clinical data on the DCM patients is summarised in Table 1. Among the eight DCM patients only one was

female which is consistent with the prevalence of this disease. The average age of the patients was  $52 \pm 14$  years ( $n=8$ , range 26 and 65 years) and average duration of the disorder was  $7.0 \pm 6.6$  years ( $n=8$ , range 1 and 18 years). At the time of transplantation, the average left ventricular ejection fraction (LVEF) was  $0.23 \pm 0.10$  ( $n=8$ ) compared to the normal value typically 0.6–0.7. The average fractional shortening was  $9.5 \pm 3.9\%$  ( $n=8$ ) compared to the normal range 28–38%. The average LV diastolic dimension was  $78 \pm 11$  mm ( $n=8$ ) indicating significant dilatation compared to the normal value of 35–56 mm. Taken together, these features are consistent with severe heart failure.

### 2.2. Immunohistochemistry

Frozen sections (10  $\mu$ m) were treated in 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min and fixed in 4% paraformaldehyde for 2 min. The sections were then treated with blocking reagents comprising 1% BSA and 20% horse serum in PBS. Primary polyclonal antibodies against P2X<sub>1</sub> were obtained from two sources: Roche Bioscience, Palo Alto, CA and Alomone Laboratories, Jerusalem, Israel. Both antibodies were designed against an epitope at the C-terminus of the protein. The epitope sequence for the Roche antibody was P2X<sub>1</sub> 385–399 (ATSSTLGLQENMRTS). Specificity of the antibody was demonstrated by Oglesby et al. [26]. The Alomone epitope sequence was slightly longer comprising P2X<sub>1</sub> 382–399 plus additional N-terminal cysteine and serine (CSDPVATSSTLGLQENMRTS). Monoclonal antibody against connexin43, a cardiac specific gap junction protein, was a gift from Dr. David Becker of the Department of Anatomy and Development, University College London. Primary antibody was applied at an appropriate dilution determined by titration and followed by a biotinylated secondary antibody (anti-rabbit IgG for P2X<sub>1</sub> antibody and anti-mouse IgG for connexin43 antibody). For light microscopy, ExtrAvidin-horseradish peroxidase was applied, followed by a nickel intensified DAB chromogenic reaction. For fluorescence or confocal microscopy, StreptAvidin-FITC and/or StreptAvidin-Cy3 (Sapphire Bioscience, Alexandria, NSW, Australia or Pierce, Rockford, CT) were used to label P2X<sub>1</sub> receptor and/or connexin43. Negative controls were achieved by: (i) omitting the primary antibody; and (ii) preabsorption of the corresponding epitope-peptide with the antibody. The images were observed using a Zeiss fluorescence microscope (Zeiss, Oberkochen, Germany) or the FITC-TRITC channel of a Leica TCS NT confocal microscope (Leica, Heidelberg, Germany). For confocal microscopy, the whole thickness of the section was scanned before any image was taken. Series z-sections were not routinely taken but when they were, 20 optical sections within a 10- $\mu$ m thick tissue were used. Channel cross-talk was limited to a very low level in our system by setting the filter detector for emission by FITC in the range of 500–550 nm and by TRITC at 585–615 nm.

Table 1  
Clinical parameters of DCM patients and donor hearts

Sample	Duration of disorder (years)	NYHA <sup>a</sup> class	Ejection fraction	Fractional shortening %	LVEDD <sup>b</sup> (mm)	Age (years) and gender
DCM 1 <sup>c</sup>	14	III	0.40	15	79	63 F
DCM 2	6	III	0.15	7	100	26 M
DCM 3	1	III	0.15	7	81	50 M
DCM 4	11	IV	0.15	3	81	63 M
DCM 5	1	III	0.17	9	75	36 M
DCM 6	4	IV	0.25	11	67	55 M
DCM 7	18	III	0.30	14	69	56 M
DCM 8	1	IV	0.23	10	70	65 M
Donor 1						23 M
Donor 2						55 F
Donor 3						34 F
Donor 4						36 M
Donor 5						N/A

<sup>a</sup> NYHA: New York Heart Association.

<sup>b</sup> LVEDD: left ventricular end diastolic dimension.

<sup>c</sup> DCM: dilated cardiomyopathy.

Images were digitally processed using Photoshop 5.0 software (Adobe Systems, San Jose, CA).

### 2.3. Western blotting

Western blotting was performed as described previously [27]. Proteins were extracted from homogenized tissues in a buffer containing 5 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 0.1 mM EDTA, 0.4 mM dithiothreitol (DTT), 5 μl/ml phenylmethylsulfonyl fluoride and 5 μl/ml protease inhibitor cocktail including chymostatin and leupeptin. The homogenates were supplemented with sodium dodecyl sulfate (SDS) to a final concentration of 1% and boiled for 5 min. After centrifugation, the supernatants were stored in aliquots at –80 °C. Protein concentration was determined by the Bradford method (Bio-Rad Protein Assay, Hercules, CA). The proteins were then subjected to a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) with a constant voltage of 150 V. Protein loadings were determined by titration and adjusted to 50 μg/lane. Prestained protein molecular weight (MW) markers (Life Technologies, Gaithersburg, MD) were used in the gel. After transferring the proteins from the gel onto a PVDF membrane (Amersham Pharmacia Biotech Australia, Castle Hill, NSW, Australia) in a semi-dry transferring apparatus (BioRad), the membrane was blocked with 1% BSA and 10% horse serum albumin in PBS for 30 min before the application of the primary antibody which was dissolved in the blocking buffer solution at a dilution of 1:1000 for 1 h. The secondary biotinylated anti-rabbit IgG was then applied at a dilution of 1:2000 for 30 min. The chromogenic procedure was carried out using a BLAST kit for Western Blotting (NEN Life Science). The volume densities of the bands were quantified using a Densitometer with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

### 2.4. Statistical analysis of data

Data are presented as means ± SEM (*n*) unless otherwise indicated. *n* refers to the number of experiments carried out

for each individual sample. Student's *t*-tests were performed using StatView software (Abacus Concepts, Berkeley, CA, USA) to analyse the significance of differences observed between experimental means. A probability *p* < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Immunohistochemistry of P2X<sub>1</sub> receptors

The expression and localization of P2X<sub>1</sub> receptors in the human LV were examined by immunohistochemistry using the antibodies from Roche and Alomone Labs (see Methods), both of which showed the typical positive staining pattern at intercalated discs. A light microscopic image (using Roche antibody) with nickel-intensified DAB staining is shown in Fig. 1A,B. P2X<sub>1</sub> labelling indicated by solid arrows is concentrated in the region of intercalated discs although some labelling appeared in other regions of cardiomyocytes. The blood vessels also showed dense P2X<sub>1</sub> labelling (open arrow), associated with the smooth muscle cells. Higher magnification images of intercalated discs are shown in Fig. 1B. In Fig. 2A, P2X<sub>1</sub> antibody was labelled with fluorescence probe FITC and the image was observed using a Zeiss fluorescence microscope. Distinct staining of P2X<sub>1</sub> at intercalated discs (Fig. 2A) was lost (Fig. 2B) after preincubation with the epitope peptide, although substantial yellow and green autofluorescence remained.

### 3.2. Double labelling of P2X<sub>1</sub> and connexin43

A more detailed localization of P2X<sub>1</sub> at the intercalated discs was achieved by coincident staining of LV sections with antibodies to P2X<sub>1</sub> and connexin43 (Fig. 2C,D,E). Connexin43 is a known cardiac specific protein which distributes at the gap junctions [28]. Fig. 2C is a confocal image of a section labelled with both P2X<sub>1</sub> (green) and

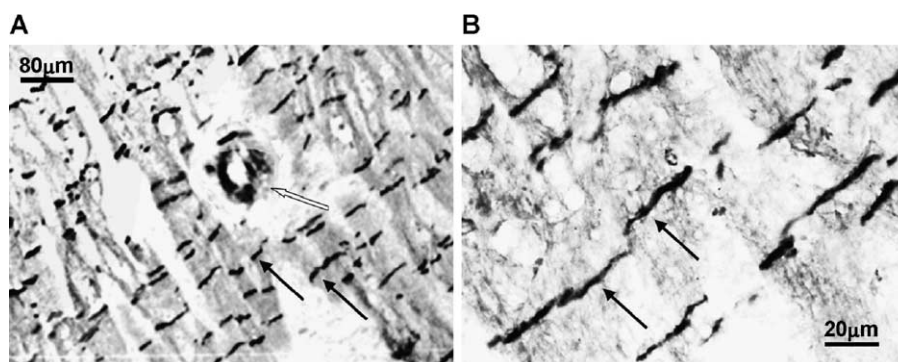


Fig. 1. Immunohistochemical staining of P2X<sub>1</sub> receptors in human LV. (A) Light microscopic image shows the P2X<sub>1</sub> receptors concentrated in two main regions: the tunica media of blood vessels (open arrow); and the intercalated discs (solid arrows) recognisable by their typical morphology. (B) High magnification of P2X<sub>1</sub> immunostaining. Scale bar in A = 80 μm and in B = 20 μm.

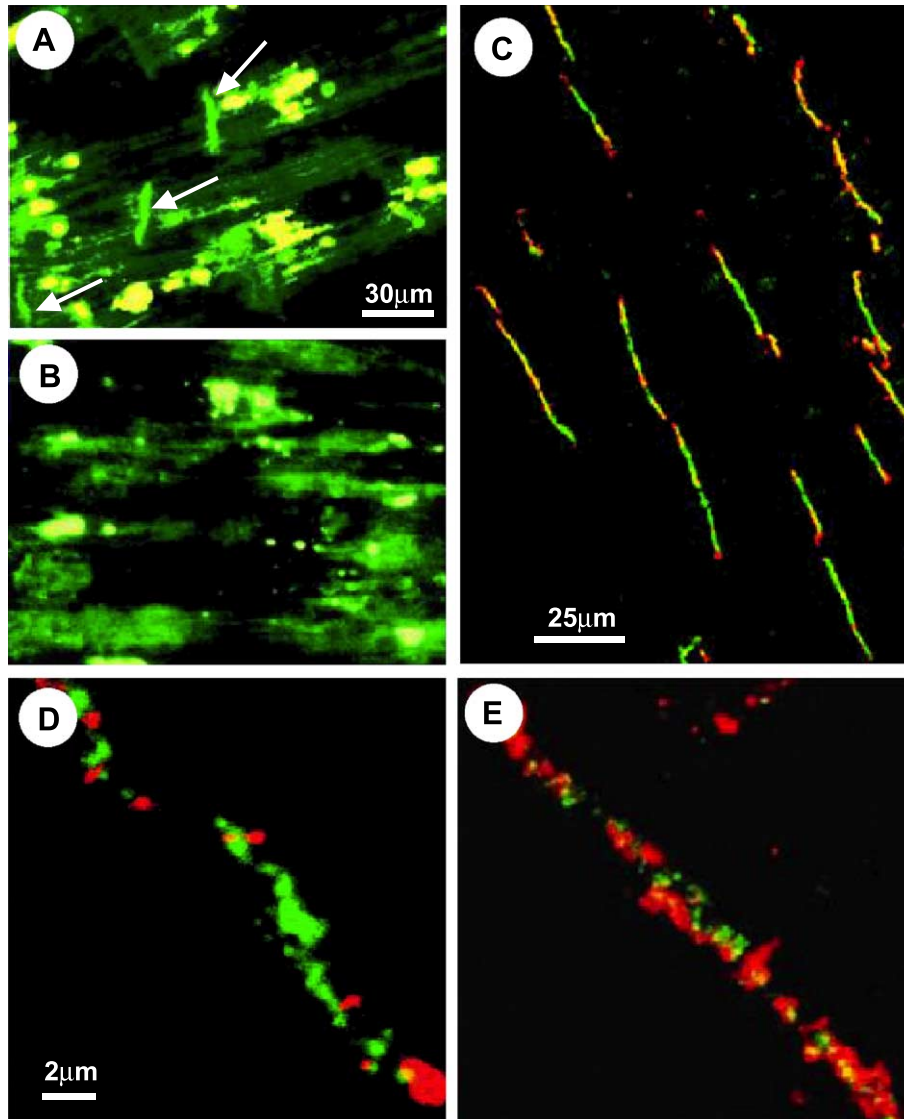


Fig. 2. Immunohistochemical staining of P2X<sub>1</sub> receptors in human LV and co-localization with connexin43. (A) P2X<sub>1</sub> staining is shown (green, FITC) with arrows indicating the intercalated discs. Scale bar = 30  $\mu$ m. (B) After pre-incubation of the antibody with epitope peptide, positive P2X<sub>1</sub> staining at intercalated discs preferentially disappears, although some green and yellow auto-fluorescence remains (probably due to the highly fluorescent protein lipofuscin). Scale bar as in A. (C) Double labelling of P2X<sub>1</sub> receptor and connexin43 in human LV. Sections were double-labelled with anti-P2X<sub>1</sub> (green, FITC) and anti-connexin43 (red, Cy3). The images were obtained by confocal microscopy. Both P2X<sub>1</sub> and the gap junction protein connexin43 are localized in the intercalated discs. Note the variable degree of double labelling (yellow) in different discs. Scale bar = 25  $\mu$ m. (D) and (E) are higher magnification micrographs showing the variable amount of double labelling of P2X<sub>1</sub> receptors and connexin43 in two different gap junctions. Scale bar in D and E = 2  $\mu$ m.

connexin43 (red) demonstrating both proteins were localised at the intercalated discs, while yellow staining indicates close association, i.e. the two molecules are in close proximity but not bound to each other. Cardiomyocyte sarcolemma was not labelled. Analysis of the distribution of the P2X<sub>1</sub> receptors and connexin43 showed considerable variability in the pattern of staining in different intercalated discs (Fig. 2C) as can be clearly seen at higher magnification (Fig. 2D and E). Some gap junctions showed close association of the P2X<sub>1</sub> receptor and connexin 43 (Fig. 2E), while in others there was largely separation of the two proteins (Fig. 2D). In general, P2X<sub>1</sub> receptors were distributed across the central region of the intercalated discs,

whereas connexin43 labelling was scattered and was more dense at the borders of the discs. The pattern and distribution of P2X<sub>1</sub> receptor-staining in the LV of failing hearts, in DCM, showed no obvious differences (images not shown).

### 3.3. Western blotting analysis of the P2X<sub>1</sub> receptor in the human heart

In failing hearts, the protein expression of P2X<sub>1</sub> was significantly elevated compared with normal hearts, as demonstrated by Western blotting (Fig. 3). Four major bands were detected in the protein samples extracted from rat vas deferens, which constitutively express the P2X<sub>1</sub> receptor

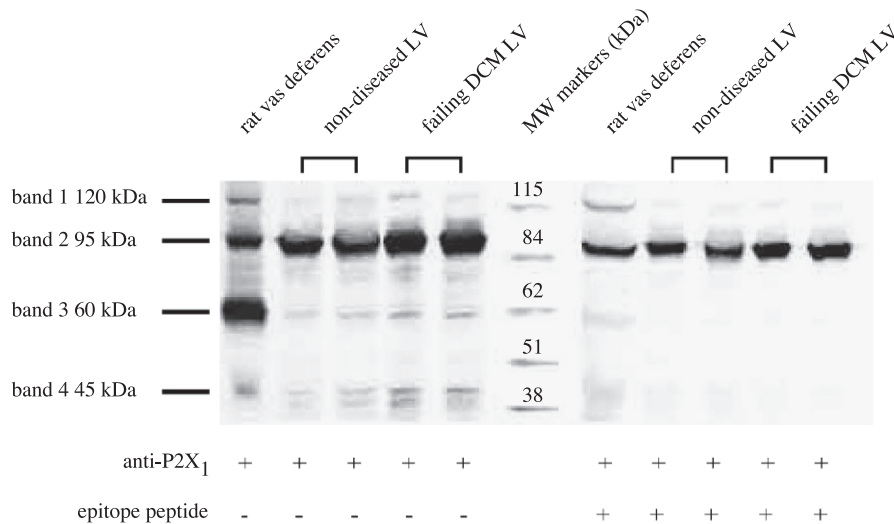


Fig. 3. Western blots of P2X<sub>1</sub> receptor expression in the human DCM failing LV and in non-diseased control samples. The blots were probed with anti-P2X<sub>1</sub> in the absence (left to the MW markers) and presence (right to the MW markers) of its epitope peptide. Four major bands are detected in the protein sample extracted from rat vas deferens: 120, 95, 60 and 45 kDa. These bands are significantly attenuated when the epitope peptide is present except that the most prominent band migrates at 95 kDa. The same bands are observed in the LV samples.

[29]. The major band at the apparent molecular weight of 60 kDa represents the glycosylated form of P2X<sub>1</sub>, while its native form is at 45 kDa. Other two bands at 95 and 120 kDa may be the polymerised form of the receptor. These bands were also detected in the samples of human heart muscle extracts. Among them, the 45 kDa band showed the most significant alteration with the volume density for the normal samples measured  $1884.4 \pm 185.5$  ( $n=5$ ) and  $7070.0 \pm 213.6$  ( $n=8$ ,  $p<0.01$ ) for the failing samples.

The increased expression of this band in the failing samples was 3.8-fold. The 60 kDa band was also changed with the volume density for the normal samples  $1942.5 \pm 44.9$  ( $n=5$ ) and  $3700.0 \pm 352.1$  ( $n=8$ ,  $p<0.05$ ) for the failing samples, with a 1.9-fold elevation in the latter. However, unlike rat vas deferens, the most densely expressed band detected in the human samples was at 95 kDa which also showed alteration with the volume density for the normal samples  $48843 \pm 4844$  ( $n=5$ ) and  $81091 \pm 3549$  ( $n=8$ ,  $p<0.05$ ), representing an increase of 1.7-fold. No significant change was detected for the band at 120 kDa with the volume density for normal samples  $1869.5 \pm 321.1$  ( $n=5$ ) and  $2177.0 \pm 290.4$  ( $n=8$ ,  $p=0.65$ ) for the failing samples.

#### 4. Discussion

ATP is released as a co-transmitter following stimulation of sympathetic nerves and from endothelial cells during shear stress or hypoxia to elicit vasoconstriction or vasodilatation of vascular smooth muscles [30]. ATP acts on P2X receptors to produce vasoconstriction and on P2Y receptors to produce vasodilatation [31,32]. Cardiomyocytes have a high concentration (5 mM) of ATP and are probably a source of ATP release (in addition to vascular endothelial

cells) during hypoxia associated with ventricular arrhythmias [33]. Increased expression of P2X<sub>1</sub> receptors was detected in the atria of human failing hearts using Western blotting analysis [34,35]. [<sup>35</sup>S]ATPγS binding studies have shown that P2 receptors are present in cardiac sarcolemma and are susceptible to modification by oxidative stress under pathophysiological conditions including myocardial ischemia–reperfusion injury [36].

We showed that P2X<sub>1</sub> receptors are expressed in human cardiomyocytes and are mainly distributed in the regions of intercalated discs. Both P2X<sub>1</sub> receptors and connexin43 are localized in this region; there is some close association of the two proteins in most gap junctions, but there is clear separation in many regions. The specificity of the antibody staining was demonstrated by: the use of antibodies against the same target from different sources (since the immunostaining pattern was similar), the use of epitope peptides as blocking reagents to eliminate the positive staining pattern, previous studies of specificity reported for the Roche antibody [26] and the use of positive control rat vas deferens in Western blots.

We also report here that the major forms of the P2X<sub>1</sub> receptor in human LV are the native form (45 kDa) and its dimers. It is known that P2X<sub>1</sub> receptors exist in tissues in different forms such as dimers and glycosylated forms [15]. We detected four major forms of P2X<sub>1</sub> in the human LV by Western blotting, with the 95 kDa band being the predominant one, probably the dimer of the native form of the P2X<sub>1</sub> receptor. The other bands detected were at 60 kDa (a glycosylated form of the P2X<sub>1</sub> receptor) and at 120 kDa (probably a dimer of the former). The positive identification of these bands was also supported by epitope peptide preincubation. It should be noted, however, that although the 60 and 45 kDa bands were almost abolished indicating

specific labeling, the 95 kDa band was only partially diminished implying some non-specific labeling including the P2X<sub>1</sub> receptor dimer. Since the 95 kDa band is the predominant band in Western blots, this staining pattern could result from the labeling of both P2X<sub>1</sub> receptors and the other proteins at 95 kDa. Nevertheless, this doubt does not affect the specific labeling in immunohistochemistry of P2X<sub>1</sub> receptors at the intercalated discs as evidenced by the complete removal of the staining in this region after epitope peptide preincubation.

The authors are aware of the use of ‘house-keeping’ proteins to normalize the loading on Western blots and the most common of these in the heart muscle is actin. However, in a separate study in our laboratory using a canine heart failure model [37], we found that the expression of proteins in myocardium were significantly changed in the failing heart compared to the normal control. These proteins included house-keeping proteins such as actin. Similar findings were reported in human dilated cardiomyopathy [38]. Therefore, we believe it is not appropriate to use a “house-keeping” protein to normalize Western blotting signals in the case of heart muscle, either in pathological or induced-pathological conditions.

Although the protein extracts used for Western blots were from heart muscle that predominantly contains cardiomyocytes, other minor cell types are also present in the tissue. However, we do not believe that the altered level of P2X<sub>1</sub> expression detected is an artefact due to an increased percentage of other cell types in the infarct areas (e.g., fibroblasts) that is usually associated with ischemic hearts. In fact, in non-infarct areas the mass of myocytes in failing myocardium was found to be increased because of myocyte hypertrophy [39]. In this study, we used DCM failing hearts only and they are generally free of infarct areas.

In addition, the Western blot band profiles for P2X<sub>1</sub> were not different in that all four bands were present in both vas deferens and human myocardium (Fig. 3). However, as we have noted there was a difference in the relative intensity of the bands. This may reflect species-specific differences in the receptor protein expression or a difference in the processing of proteins in different tissue types.

A previous report by Berry et al. [40] indicated that there was no difference in the expression of P2X<sub>1</sub> receptors in the failing human LV compared to non-diseased samples, although this group did find an elevated expression of P2X<sub>1</sub> receptors in human failing atria using the same methods [34,35]. Berry et al. used a polyclonal antibody designed against a peptide in the N-terminus of the P2X<sub>1</sub> receptor that recognised a single band in their Western blots. The difference between these observations may be due to several factors including differences in the specificities of the antibodies. Both of our polyclonal antibodies (Roche and Alomone Laboratories) identified four major bands in human LV samples which were qualitatively consistent with bands observed in rat vas deferens, although there were clear quantitative differences. Our major finding is that

P2X<sub>1</sub> receptors are localized at the intercalated discs, with variable coincidence with connexin43.

The function of P2X<sub>1</sub> receptors in the intercalated discs of myocardium is not yet fully understood. However, there is evidence that ATP is involved with connexins in gap junction formation permselectivity [41–46]. Intercalated discs join neighbouring cardiomyocytes and provide mechanical adhesion as well as electrical and metabolic coupling [47]. Protein changes in these junctions, particularly in gap junctions, are known to accompany pathological conditions [48,49]. It has been suggested that dysregulated signalling in gap junctions contributes to altered impulse propagation and arrhythmia in the myopathic heart [50].

The DCM failing hearts used in this study had significantly enlarged left ventricular end diastolic dimensions and therefore the cardiomyocytes were mechanically stretched but there were no obvious differences in the distribution of P2X<sub>1</sub> receptors in gap junctions, seen with immunostaining, although Western blots did show an increase in P2X receptor expression. The presence of P2X<sub>1</sub> receptors at the intercalated discs suggests their role in the restoration of cardiomyocyte contractility, but further study is needed to prove this.

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