ATP increases $[\text{Ca}^{2+}]_i$ and activates a $\text{Ca}^{2+}$-dependent $\text{Cl}^-$ current in rat ventricular fibroblasts

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Abstract
Effects of ATP on enzymatically isolated rat ventricular fibroblasts maintained in short-term (36–72 h) cell culture were examined. Immunocytochemical staining of these cells revealed that a fibroblast, as opposed to a myofibroblast, phenotype was predominant. ATP, ADP or uridine 5’-triphosphate (UTP) all produced large increases in $[\text{Ca}^{2+}]_i$. Voltage-clamp studies (amphotericin-perforated patch) showed that ATP (1–100 μM) activated an outwardly rectifying current, with a reversal potential very close to the Nernst potential for $\text{Cl}^-$. In contrast, ADP was much less effective, and UTP produced no detectable current. The non-selective $\text{Cl}^-$ channel blockers niflumic acid, DIDS and NPPB (each at 100 μM), blocked the responses to 100 μM ATP. An agonist for P2Y purinoceptors, 2-MTATP, activated a very similar outwardly rectifying $\text{Cl}^-$ current. The P2Y receptor antagonists, suramin and PPADS (100 μM each), significantly inhibited the $\text{Cl}^-$ current produced by 100 μM ATP. ATP was able to activate this $\text{Cl}^-$ current when $[\text{Ca}^{2+}]_o$ was removed, but not when $[\text{Ca}^{2+}]_o$ was buffered with BAPTA-AM. In the presence of the phospholipase C inhibitor U73122, this $\text{Cl}^-$ current could not be activated. PCR analysis revealed strong signals for a number of P2Y purinoceptors and for the $\text{Ca}^{2+}$-activated $\text{Cl}^-$ channel, TMEM16F (also denoted ANO6). In summary, these results demonstrate that activation of P2Y receptors by ATP causes a phospholipase C-dependent increase in $[\text{Ca}^{2+}]_i$, followed by activation of a $\text{Ca}^{2+}$-dependent $\text{Cl}^-$ current in rat ventricular fibroblasts.

KEYWORDS
ATP, $\text{Ca}^{2+}$-activated $\text{Cl}^-$ current, purinergic receptors, ventricular fibroblasts, voltage clamp

1 | INTRODUCTION

ATP has significant physiological effects, either as a cotransmitter or as a paracrine substance, when it is present in the extracellular space of many mammalian tissues (cf. Burnstock, 2006, 2007, 2012). These effector sites include the central and peripheral nervous systems, the digestive tract and the cardiovascular system. The main purpose of this study was to identify and begin to define the major electrophysiological effects of ATP, acting as a purinergic receptor agonist on isolated fibroblasts isolated from the mammalian (adult rat) ventricle.

In the mammalian heart, significant electrophysiological and mechanical effects of ATP have been reported in studies of both atria and ventricles (Christie, Sharma, & Sheu, 1992; Gurung, Kalin, Grace, & Huang, 2009; Parker & Scarpa, 1995; Shen, Pappano, & Liang, 2006, 2007; Vassort, 2001) and the sino-atrial node (Musa et al., 2009). Although these effects are somewhat diverse, most are attributable to selective ATP/purinergic receptor interactions within signalling complexes that are localized to the surface membrane or sarcotendine of these myocytes. In myocytes themselves, this primarily P2X$_4$-mediated action of ATP can result in a depolarization and induction of rhythm disturbances (Gurung et al., 2009). It is also known that ectonucleotidases, specific catabolic enzymes for ATP, that are located on the surface of many mammalian cells can quickly and effectively hydrolyse ATP to adenosine (Communi, Janssens, Suarez-Huerta, Robaye, & Boeynaems, 2000; Lu & Insel, 2013; Montalbetti et al., 2011; Yegutkin, 2008). Adenosine acting through its distinct and selective receptors produces marked chronotropic and inotropic effects in the mammalian heart (Corriden & Insel, 2010; Kuzmin, Lakomkin, Kapelko, & Vassort, 1998) and can also strongly modulate cell migration and proliferation (Elliott et al., 2009).
What is the central question of this study?
Although electrophysiological and biophysical characteristics of heart fibroblasts have been studied in detail, their responses to prominent paracrine agents in the myocardium have not been addressed adequately. Our experiments characterize changes in cellular electrophysiology and intracellular calcium in response to ATP.

What is the main finding and its importance?
In rat ventricular fibroblasts maintained in cell culture, we find that ATP activates a specific subset of $\text{Ca}^{2+}$-activated $\text{Cl}^-$ channels as a consequence of binding to P2Y purinoceptors and then activating phospholipase C. This response is not dependent on $[\text{Ca}^{2+}]_i$ but requires an increase in $[\text{Ca}^{2+}]_o$ and is modulated by the type of nucleotide that is the purinergic agonist.

New Findings

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Within the past 8–10 years, microanatomical, molecular and electrophysiological studies of mammalian heart tissues have revealed important new roles for cardiac fibroblasts in both electrophysiological and endocrine/paracrine settings (Baudino, Carver, Giles, & Borg, 2006; Brown, Ambler, Mitchell, & Long, 2005; Camelliti, Borg, & Kohl, 2005; Hinz et al., 2007; Porter & Turner, 2009). Four of these significant new findings are as follows.

(i) Fibroblasts and myocytes in both atria and ventricles can interact electronically via connexin-mediated intercellular current flow.

(ii) Fibroblasts express functional receptors for a variety of transmitters, cotransmitters and paracrine agents; these include noradrenaline, ATP, adenosine, natriuretic peptides and sphingosine-1-phosphate (Rose & Giles, 2008).

(iii) ATP can be released from fibroblasts, and this release is mediated by either connexin- or pannexin-based hemi-channels (Lu, Soleymani, Madakshire, & Insel, 2012; Samuels, Lipitz, Dahl, & Muller, 2010).

(iv) Activation of distinct subtypes of purinergic receptors in the P2Y family can produce large and receptor subtype-specific changes in the amplitude and time course of $[\text{Ca}^{2+}]_i$, while also altering fibroblast/myofibroblast proliferation (Certa et al., 2015, 2016).

This background and the extensive literature on purinergic neurotransmission and related paracrine effects in the cardiovascular system provided the basis for investigating a number of fundamental issues concerning the electrophysiological responses and molecular pharmacological signalling in ventricular fibroblasts in response to ATP. Important unanswered questions include the following.

(i) Does application of extracellular ATP at physiological concentrations result in reproducible electrophysiological responses in ventricular fibroblasts?

(ii) Can these responses be described/rationalized in terms of present knowledge of purinergic molecular pharmacology in the heart?

(iii) What is the nature of the ligand (ATP)-gated ion channel associated with the ATP-mediated response in terms of the ion channel selectivity/permeability and (b) the predominant ion transfer or I-V characteristics?

(iv) Is an increase in $[\text{Ca}^{2+}]_i$, an obligatory step in this purinergic signal transduction cascade?

(v) Is the ATP-dependent activation of the fibroblast mediated by the well-known intracellular second messenger pathway via phospholipase C activation?

(vi) Are the actions of ATP on ventricular myocytes and fibroblasts initiated by the same subtypes of purinergic receptors or are they cell-type specific?

Our study addresses each of these questions. Significant new information concerning the electrophysiological, biophysical and pharmacological effects of ATP in the adult ventricular myocardium is reported. These results advance present understanding of purinergic receptor function in the mammalian heart by identifying new sites of action that are localized to the fibroblast. With these new perspectives, the actions of ATP in both physiological and pathophysiological conditions can be reconsidered. A rationale for new fibroblast-specific drug targets emerges (Williams & Jarvis, 2000; Wynn, 2007), which might be relevant to the initiation or maintenance of rhythm disturbances (Du et al., 2010; Knollmann & Roden, 2008).

2 | METHODS

2.1 | Ethical procedures

All animals received care in compliance with the Canadian Council on Animal Care (CCAC) guidelines. All surgical procedures were performed in accordance with the CCAC guidelines, with approval from the University of Calgary Animal Care Committee (protocol BI 2007–42). All procedures used conform to the principles and regulations as described by the guidelines given (Grundy, 2015).

2.2 | Isolation and short-term two-dimensional (2-D) of culture of ventricular fibroblasts

These studies were done on fibroblasts isolated from the ventricles of male Sprague–Dawley rats weighing 200 ± 15 g. These animals were obtained from Charles River Canada Ltd. A normal diet (pellets) and fresh water were provided ad libitum.

Fibroblasts were isolated from the ventricles of adult rats after minor modifications of our published methods (Chilton et al., 2005). Briefly, Sprague–Dawley male rats (150–200 g) were anaesthetized by inhalation of isoflurane at 5% and the hearts excised into a modified Tyrode solution containing (mM): 140 NaCl, 5.4 KCl, 1 CaCl$_2$, 1 MgCl$_2$, 10 Hepes and 5.5 glucose. The pH was adjusted to 7.4 with NaOH. Ventricular tissue was minced and digested in a 37°C shaker bath for 40 min. During this time, the Tyrode solution was modified as follows: 10 $\mu$M CaCl$_2$, 1 mg ml$^{-1}$ collagenase II ( Worthington Biochemical), 0.15 mg ml$^{-1}$ protease XIV ( Sigma-Aldrich) and 0.5% bovine serum.
albunin (Sigma-Aldrich). To obtain a predominantly fibroblast cell population, debris and myocytes were removed by centrifugation. The supernatant was then collected and resuspended in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco) medium supplemented with 10% fetal calf serum (Gibco), penicillin-streptomycin (20 units ml\(^{-1}\), Gibco), gentamicin (50 \(\mu\)g ml\(^{-1}\); Gibco) and fungizone (0.0125 \(\mu\)g ml\(^{-1}\); Gibco). Before each experiment, the fibroblasts were seeded on small pieces of coverslip (\(~3\) mm \(\times\) 3 mm) in culture dishes and incubated at 37°C in a humidified incubator equilibrated with 5% \(\text{CO}_2\). After allowing a 3 h period for attachment of the cells to these uncoated pieces of coverslip, the unattached cells and superfusate were removed by rinsing with phosphate-buffered saline (PBS; Gibco). Patch clamp and fura-2 experiments were performed within 36–72 h after this initial culture procedure.

The procedures for enzymatic isolation of rat ventricular myocytes and for recording from them with patch electrodes have been described in our previous publication (Bouchard, Clark, Juhasz, & Giles, 2004).

### 2.3 Immunocytochemistry

Immunocytochemistry was performed by using a modification of previously published methods (Hatano, Ohya, & Imaizumi, 2002). In brief, either acutely isolated or cultured cardiac fibroblasts were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS for 30 min at room temperature. The fixed cells were rinsed twice with PBS before being incubated overnight with 0.2% Triton X-100 (Wako), 1% normal goat serum (Sigma-Aldrich) and the selected primary antibodies from Sigma-Aldrich: vimentin, 1:200; \(\alpha\)-smooth muscle actin, 1:800; or desmin, 1:100 in PBS at 4°C. These cells were then washed twice with PBS and incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies (Molecular Probes, Invitrogen) for 1 h at room temperature. In experiments in which fibroblasts were double-stained with vimentin and an additional antibody, a second incubation with Alexa Fluor 568-conjugated goat anti-mouse IgM antibodies (Molecular Probes, Invitrogen) was performed for 1 h at room temperature. The resulting immunofluorescence staining patterns were examined using a confocal fluorescence microscope (Zeiss LSM510). Excitation light wavelengths were 488 and 543 nm from an argon ion laser and a helium ion laser, respectively, and emission wavelengths of 503–530 nm and >560 nm were selected.

### 2.4 Electrophysiology

Membrane currents from single ventricular myocytes from single ventricular fibroblasts were recorded using whole-cell voltage-clamp methods at room temperature (22 ± 1°C). Patch pipettes were made from borosilicate glass (World Precision Instruments) with a P-87 Flaming/Browning pipette puller (Sutter Instruments). Pipette tips were heat polished on a microforge (Narishige Scientific Instruments). These pipettes had resistances of 3–8 M\(\Omega\) when filled with the pipette solution. For electrophysiological recordings, Heps-buffered solution was used as the superfusate. It contained (mm): 137 NaCl, 5.4 TEA-Cl, 2.2 CaCl\(_2\), 1.2 MgCl\(_2\), 10 glucose and 10 Heps. The pH was adjusted to 7.4 with NaOH.

When the perforated patch method was used, the pipette solution contained (mm): 110 caesium aspartate, 30 CsCl, 4 MgCl\(_2\), 10 Heps and 300 \(\mu\)g ml\(^{-1}\) amphotericin B. The pH was adjusted to 7.2 with CsOH. A correction of \(-10\) mV was applied to recorded membrane potentials to account for the liquid junction potential between pipette and bath solutions.

Membrane currents were monitored continuously and the data stored as previously reported (Chilton et al., 2005; Shibukawa et al., 2005). Voltage-clamp command waveforms and the resulting capacitative and ionic current changes were acquired using an Axoclamp 200B amplifier (Molecular Devices), a Digidata 1322A digitizer (Molecular Devices) and Clampex version 8.1 software (Molecular Devices). Data sets were analysed with Clampfit (Molecular Devices) and Origin version 6.1 (OriginLab Corp.).

In some experiments, membrane currents were recorded from voltage-clamped single cardiac fibroblasts using perforated patch recording methods, which minimized disruption of intracellular signalling cascades. These single, isolated cardiac fibroblasts had an average membrane capacitance of 16.9 ± 0.5 pF (\(n = 174\)). As described previously, these fibroblasts expressed small voltage-dependent K\(^+\) currents when using bath and pipette solutions containing normal concentrations of K\(^+\) (Chilton et al., 2005; Shibukawa et al., 2005). No voltage-dependent Na\(^+\) and Ca\(^{2+}\) currents were observed (data not shown).

Cells were exposed to ATP at each selected concentration for 2–4 min to obtain the peak response. When the effects of Cl\(^-\) channel blockers, P2Y receptor blockers, Ca\(^{2+}\)-free solution, 1-(6-(17\(\beta\)-3-methoxyester-1,3,5(10)-trien-17-yl)amino)-hexyl)-1H-pyrrrole-2,5-dione (U73343), and 1-{6-[17\(\beta\)-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino}hexyl-2,5-pyrrolidine-dione (U73343) on ATP-induced responses were examined, cells were pretreated with these drugs for 2–4 min. A change in the solution was achieved within 30 s. The voltage-clamp command waveform used in these experiments was a 400 ms voltage ramp from \(-110\) to +90 mV, applied at 0.1 Hz.

### 2.5 \([\text{Ca}^{2+}]\text{I}\) measurements

Populations of cultured rat ventricular fibroblasts that had adhered to small pieces of coverslip were loaded with 5 \(\mu\)M fura-2 acetoxyethyl ester (fura-2 AM) in Heps-buffered solution for 30 min at 37°C. Measurements of fura-2 fluorescence signals made using the Argus/Hisa imaging system (Hamamatsu Photonics Ltd). Images were acquired at 0.2 Hz. Relative changes in \([\text{Ca}^{2+}]\text{I}\) are expressed in terms of the fura-2 fluorescence intensity ratio at 340 nm/380 nm wavelengths.

### 2.6 Quantitative real-time PCR

Total RNA was extracted from cardiac fibroblasts that had been cultured for 72 h and reverse transcribed as reported previously (Shibukawa, Chilton, MacCannell, Clark, & Giles, 2005). The PCR amplification protocol was as follows: 15 s at 95°C and 60 s at 60°C for 1 min, according to AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
primers were used to confirm that the products generated were representative of RNA. Each amplified product was sequenced by the chain termination method with an ABI PRISM 3100 genetic analyser (Applied Biosystems). Real-time quantitative PCR was performed with the use of SYBR Green chemistry on an ABI 7000 sequence detector (Applied Biosystems). Experimental quantities were calculated relative to the standard curve for each selected set of primers, yielding transcriptional quantification of gene products relative to the endogenous standard (GAPDH).

The following PCR primers were used: P2Y_1 (GenBank accession no. NM_012800, 515–615), amplicon = 101 bp; P2Y_2 (GenBank accession no. BC061754, 222–322), amplicon = 101 bp; P2Y_4 (GenBank accession no. NM_031680, 531–631), amplicon = 101 bp; P2Y_6 (GenBank accession no. NM_057124, 227–377), amplicon = 151 bp; P2Y_12 (GenBank accession no. NM_022800, 416–516), amplicon = 101 bp; P2Y_13 (GenBank accession no. BC083545, 65–165), amplicon = 101 bp; TMEM16A (GenBank accession no. NM_012800, 515–615), amplicon = 121 bp; TMEM16B (GenBank accession no. BC083545, 65–165), amplicon = 121 bp; TMEM16F (GenBank accession no. NM_001108108, 711–811), amplicon = 101 bp; and GAPDH (GenBank accession no. NM_017008, 1533–1636), amplicon = 104 bp.

2.7 Chemicals and cell culture materials

Collagenase type II was obtained from Worthington Biochemicals Ltd. The DMEM/F12, fetal calf serum, fungizone, gentamicin and penicillin-streptomycin solution were purchased from Gibco. Fura-2 AM, Alexa Fluor 488-conjugated goat anti-mouse IgG antibodies and Alexa Fluor 568-conjugated goat anti-mouse IgM antibodies were from Molecular Probes (Invitrogen). Triton X-100 was purchased from Wako Ltd. All other chemicals were obtained from Sigma-Aldrich.

2.8 Statistical analysis

Pooled data were expressed as means ± S.D. Statistical significance was examined using either Student’s t test for two groups, or Dunnett’s or Tukey’s test for multiple groups, respectively. In all text figures, statistical significance is indicated as follows: *P < 0.05 or **P < 0.01.

3 RESULTS

3.1 Immunocytochemical characterization of short-term cultured ventricular fibroblasts

Most previous studies of cardiac fibroblasts have been done using preparations that have been held in conventional 2-D culture systems for relatively long times; for example, after two to five cell-passage procedures and several weeks. It is known that one consequence of this is a significant change in phenotype; often the fibroblasts are transformed and become predominantly myofibroblasts (Banyasz et al., 2008; Chilton et al., 2005; Ivey & Talquist, 2016; Rohr, 2011). For this reason, our electrophysiological recordings and measurements of [Ca^{2+}]_{i} were made only on short-term cultured rat ventricular fibroblasts (36–72 h after initial isolation) to minimize associated phenotype changes (Baudino et al., 2006; Camelliti et al., 2005; Hinz et al., 2007). The expression levels of several marker proteins for fibroblasts in these 2-D cultured preparations were assessed. This was done using standard immunocytochemistry techniques based on detection of antibodies against the following: (i) vimentin (a fibroblast marker); (ii) desmin (a smooth muscle marker); and (iii) α smooth muscle actin (α-SMA; a fibroblast/myofibroblast transition marker). These immunocytochemical criteria were applied to three separate rat ventricular fibroblast populations: (i) acutely isolated; (ii) short-term cultured (36–72 h after isolation); and (iii) long-term cultured (passage 3). As shown in Figure 1, short-term cultured fibroblasts (middle row) are approximately spindle-shaped cells as opposed to the round morphology of acutely isolated fibroblasts (top row). These cells stained positive for vimentin, but negative for desmin. The staining patterns for these two markers were similar in long-term cultured cardiac fibroblast/myofibroblasts. However, and as expected, the expression level of α-SMA in short-term cultured fibroblasts was lower than that in long-term cultured fibroblasts, and stress fibres were not observed in short-term cultured fibroblasts (compare data in the middle versus the bottom row of Figure 1). Taken together, these findings indicate that the cultured ventricular fibroblasts used in this study were very similar to those in previous studies (Camelliti et al., 2005; Wang, Thampatty, Lin, & Im, 2007) and that the predominant phenotype is that of a cardiac fibroblast, as opposed to a myofibroblast.

3.2 ATP and related purinergic agonists increase [Ca^{2+}]_{i} in ventricular fibroblasts in short-term 2-D culture

Extracellular ATP, when functioning as a neurotransmitter (or cotransmitter) can activate a variety of purinergic receptors. The resulting intracellular signal transduction pathways can have diverse effects depending on the tissue and cell types (cf. Burnstock, 2006, 2007, 2012). To test for functional purinergic receptor expression in our short-term cultured ventricular fibroblasts, fura-2-based Ca^{2+} imaging was used. The resulting pattern of [Ca^{2+}]_{i} changes in response to ATP superfusion at physiological levels and/or selected P2 purinergic receptor agonists or blockers (Erlinge & Burnstock, 2008) is summarized in Figure 2.

ATP, when added to the superfusate, consistently increased [Ca^{2+}]_{i} in these fibroblasts. As shown in Figure 2a, this effect was concentration dependent, with the 50% effective concentration (EC_{50}) being ~3.8 μM (n = 11–18). Two related purinergic receptor agonists, ADP or uridine 5’-triphosphate (UTP), also increased [Ca^{2+}]_{i} in a similar, concentration-dependent manner (Figure 2b,c). The EC_{50} values for ADP and UTP were 2.1 μM (n = 9–19) and 4.9 μM (n = 6–21), respectively. The increases in [Ca^{2+}]_{i} produced by 100 μM ATP, ADP or UTP were not significantly different in size or time course (Figure 2d)
FIGURE 1  Cell biology marker analysis for short- versus long-term cultured ventricular fibroblasts (CFs) from adult rat hearts. The expression of these selected marker proteins was assessed in freshly isolated (top row), short-term cultured (36–72 h after isolation; middle row) and long-term cultured (36–72 h; bottom row) ventricular fibroblasts using standard immunocytochemical techniques, described in detail in the Methods. Cells double-labelled with antibodies against vimentin, a fibroblast marker (red), and desmin, a smooth muscle marker (green), are illustrated in the two left-hand columns, whereas cells stained with vimentin or α-SMA, a putative myofibroblast marker (green), are shown in the two right-hand columns. The general pattern of results (i.e. stronger vimentin than desmin or α-SMA staining) indicates but does not prove that these cells maintain a predominantly fibroblast phenotype within the time period of this study. Abbreviations: P = 1, initial seeding in culture; P = 3 cells have been passaged twice

![Images of vimentin and desmin staining in different cultured conditions](image)

FIGURE 2  Effects of ATP and other related purinergic agonists on [Ca^{2+}]_i in short-term cultured ventricular fibroblasts. Fibroblasts were loaded with fura-2 AM, and fluorescence was monitored as described in the Methods. (a) Summarized data illustrating the ATP concentration–response relationship. The increase in fluorescence produced by selected concentrations of ATP (0.3–30 µM) was normalized to that induced by 100 µM ATP in the same cells (n = 11–18). The calculated EC_{50} for ATP was 3.8 µM. (b) Concentration–response relationship for increases in [Ca^{2+}]_i as a function of [ADP] (n = 9–19), yielding an EC_{50} of 2.1 µM. (c) Concentration–response relationship for UTP (n = 6–21), yielding an EC_{50} of 4.9 µM. (d) Mean changes in the fluorescence intensity ratio 340 nm/380 nm produced by 100 µM of ATP, ADP and UTP. (e) Quantitative RT-PCR analysis of P2Y receptor subtypes, relative to GAPDH from the same RNA preparation of short-term cultured cardiac fibroblasts. Results are expressed as means ± SD
3.3 | Messenger RNAs for P2Y receptors are expressed in cultured ventricular fibroblasts

As a first step towards identifying the purinergic receptor subtypes in these ventricular fibroblasts, total RNA was isolated from ventricular fibroblasts that had been maintained in 2-D culture for 72 h. These RNA samples were probed for the P2Y receptor subtype signals. Primers developed against the known rat gene sequences yielded the pattern of results shown in Figure 2e. As shown, this real-time quantitative PCR analysis revealed abundant expression of P2Y<sub>2</sub> receptor. Relative to GAPDH, this value was 0.0255 ± 0.0021 (n = 4).

The expression level of P2Y<sub>6</sub> receptor subtype (cf. Nishida et al., 2008) was also relatively high, whereas the expression levels of P2Y<sub>1</sub> and P2Y<sub>4</sub> were very low, <0.001 (n = 4). Expression of other P2Y receptor transcripts (P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>), could not be detected (data not shown). This pattern of responses differs markedly from results obtained with isolated myocytes, in which P2X<sub>4</sub> receptor subtype expression is most prominent (cf. Banfi et al., 2005; Shen, Shutt, Pappano, & Liang, 2007, 2014).

3.4 | ATP activates an outwardly rectifying current in short-term cultured ventricular fibroblasts

Effects of ATP on membrane currents in these ventricular fibroblasts were examined using perforated-patch techniques to ensure that intracellular signalling pathways were not significantly changed/blunted (Bouchard et al., 2004). The voltage-clamp command waveform used in these experiments was a 400 ms voltage ramp from −110 to +90 mV, applied at 0.1 Hz. The holding potential, −60 mV, was in the range of the resting membrane potential of acutely isolated rat ventricular fibroblasts (Shibukawa et al., 2005). Application of 100 μM ATP activated a small inward membrane current at the holding potential and produced a significant increase in the outward current during the depolarizing voltage ramp (Figure 3a). A representative I–V relationship (Figure 3b) shows that this ATP-induced current exhibited distinct outward rectification and reversed at −38 ± 4 mV (n = 12). This is very close to the calculated electrochemical equilibrium potential (E<sub>Cl</sub>) of −34.3 mV. The summary data set in Figure 3c shows that 100 μM ATP consistently activated an outwardly rectifying current compared with the baseline or control current, when measured at both +80 and −80 mV (control, 3.08 ± 1.84 and −0.75 ± 0.58 pA pF<sup>−1</sup>; 100 μM ATP, 10.74 ± 6.91 pA pF<sup>−1</sup>, P < 0.01 versus control, and −1.42 ± 1.06 pA pF<sup>−1</sup>, P < 0.01 versus control, n = 12).

ATP produced an increase in this current that was concentration dependent (Figure 3d). The ATP-induced current plotted in Figure 3d was obtained by subtracting currents recorded in the absence versus the presence of ATP at selected concentrations (1–100 μM). The approximate threshold for this ATP-induced current change was between 1 and 3 μM, and the maximal response occurred at [ATP] >30 μM. Based on these results, the EC<sub>50</sub> was calculated to be 3.5 μM (n = 5–12). It is of interest that these electrophysiological measurements yielded an EC<sub>50</sub> value for ATP that was similar to the EC<sub>50</sub> for the increase in [Ca<sup>2+</sup>]<sub>i</sub> produced by ATP in these of rat ventricular fibroblasts.

3.5 | Ion selectivity and pharmacological profile of ATP-induced currents

ATP can activate Cl<sup>−</sup> currents in diverse cell types (Gurung et al., 2009; Ma, Zhou, Liang, Saxena, & Warnock, 2004). As shown in Figure 3, the reversal potential of ATP-induced current in our rat ventricular fibroblasts is close to the calculated Nernst potential for Cl<sup>−</sup>, suggesting that this ATP-induced current is carried mainly by Cl<sup>−</sup>.

To establish this important point, Cl<sup>−</sup> substitution experiments were conducted, and changes in this ATP-induced current were recorded. In the first such test, extracellular [Cl<sup>−</sup>] was decreased from 149.7 to 32 mM by equimolar substitution of Cl<sup>−</sup> with methanesulfonate as shown in Figure 4a–c. When [Cl<sup>−</sup>] was 32 mM, the reversal potential of ATP-induced current shifted to −3.5 ± 4.4 mV (n = 7). This depolarizing shift, 34.5 mV, of the reversal potential for the ATP-induced current is very close to that expected for an ‘ideal’ Cl<sup>−</sup> channel (38.6 mV). This finding strongly supports the hypothesis that the ATP-induced current is carried primarily by Cl<sup>−</sup>. These primary current records also show that this ATP-induced current exhibited much less outward rectification in low-[Cl<sup>−</sup>] solution (Figure 4b,c) compared with normal [Cl<sup>−</sup>] solution (Figure 3b,c).

To identify additional functional properties of this ATP-induced Cl<sup>−</sup> current, effects of selected Cl<sup>−</sup> channel blockers [niflumic acid, DIDS, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and antracene-9-carboxylic acid (9-AC)] were examined (Figure 4d). In these experiments, single fibroblasts voltage clamped at −60 mV were superfused with 100 μM ATP before, during and after washout of purinergic receptor-selective compounds. These included: niflumic acid (control, 1.48 ± 0.37 pA pF<sup>−1</sup>; ATP, 1.75 ± 0.45 pA pF<sup>−1</sup>, n = 4, P > 0.05 versus control), DIDS (control, 2.04 ± 0.50 pA pF<sup>−1</sup>; ATP, 3.16 ± 1.62 pA pF<sup>−1</sup>, n = 4, P > 0.05 versus control) and NPPB (control, 2.99 ± 1.15 pA pF<sup>−1</sup>; ATP, 3.30 ± 1.03 pA pF<sup>−1</sup>, n = 4, P > 0.05 versus control); each completely inhibited the ATP-induced current at +80 mV. In contrast, 9-AC (control, 3.15 ± 0.90 pA pF<sup>−1</sup>; ATP, 5.67 ± 2.28 pA pF<sup>−1</sup>, n = 6, P < 0.05 versus control) was not an effective inhibitor of this ATP-induced Cl<sup>−</sup> current.

3.6 | Effects of P2 receptor agonists and/or antagonists on fibroblast Cl<sup>−</sup> currents

As shown in Figure 2, [Ca<sup>2+</sup>]<sub>i</sub> levels in short-term cultured cardiac fibroblasts increased significantly in response to ADP, UTP and ATP. To investigate whether ADP and UTP also could activate this Cl<sup>−</sup> current, these compounds were applied in perforated patch clamp conditions using the same voltage-clamp protocol as described in Figure 3. The effects of two other P2Y receptor agonists, 2-methylthio-ATP (2-MTATP) and a,b-methylene ATP (a,b-meATP), were also evaluated (Erlinge & Burnstock, 2008; Webb, Boluyt, & Barnard, 1996).

As shown in Figure 5a, 2-MTATP can activate the Cl<sup>−</sup> current almost as effectively as ATP; in contrast, both ADP and UTP produced much smaller Cl<sup>−</sup> currents. The apparent rank order for the potency of this Cl<sup>−</sup> current activation in these fibroblasts was that ATP was approximately equivalent to 2-MTATP, and both of these compounds were more potent than ADP or UTP. In the next experiments, the P2Y
FIGURE 3  Effects of extracellular ATP on membrane current in short-term cultured rat ventricular fibroblasts. ATP (100 μM) was added to the superfusate, and whole-cell current was recorded in perforated patch clamp conditions. (a) A 0.4 s ramp voltage-clamp waveform, from −110 to +90 mV, was applied at 0.1 Hz. The holding potential was −60 mV. The plot shows currents measured at −110 mV (filled circles) and +90 mV (open circles) during successive voltage ramps. (b) Examples of membrane currents produced by voltage ramps in control conditions and at end of application of 100 μM ATP; same cell as in (a). Note that ATP increased both inward and outward current, and both effects were reversible after removal of ATP. (c) Summary of magnitude of inward (at −80 mV) and outward (at +80 mV) currents during ramp voltages, in control conditions and after 100 μM ATP. n = 12 cells. (d) Plot of the relationship between net ATP-induced current amplitude (Δoutward current density) and the applied concentration of ATP (n = 5–12). The response threshold was ∼1 μM, and the maximal response occurred at concentrations of ATP >30 μM. The calculated EC<sub>50</sub> was 3.5 μM. Bars indicate means ± SD.

FIGURE 4  Study of the selectivity or ionic basis of ATP-induced ion transfer mechanism in rat ventricular fibroblasts. (a) Effects of reducing extracellular Cl<sup>−</sup> on ATP-induced currents. These currents were activated by 100 μM ATP in a modified external solution, in which [Cl<sup>−</sup>]<sub>o</sub> was reduced to 32 mM (21.4% of normal). (b) Current records shown in (a) were subtracted to obtain the I–V relationship of net ATP-induced current. Note that at a [Cl<sup>−</sup>]<sub>o</sub> of 32 mM, the reversal potential of ATP-induced current was close to 0 mV. (c) Summary of maximal inward (at −80 mV) and outward (at +80 mV) components of the ATP-induced currents in low-Cl<sup>−</sup> solution (control, 2.07 ± 0.75 and −0.74 ± 0.74 pA pF<sup>−1</sup>; ATP, 4.13 ± 2.20 pA pF<sup>−1</sup>, P < 0.05 versus control, and −1.71 ± 0.68 pA pF<sup>−1</sup>, P < 0.01 versus control, n = 7). As shown, in these conditions, the outward rectification of the current decreased when [Cl<sup>−</sup>]<sub>o</sub> was reduced. (d) Effect of selected Cl<sup>−</sup> channel blockers on ATP-induced current. Fibroblasts held at −60 mV were superfused continuously with 100 μM ATP. In separate experiments, 100 μM niflumic acid (NifA; n = 4), DIDS (n = 4), NPPB (n = 4) or 9-AC (n = 6) was applied after ATP. As shown, three of these agents significantly blocked this ATP-induced current, but 9-AC was not effective.
receptor antagonists, suramin and pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), were applied to test whether this ATP-induced Cl\(^{-}\) current was mediated by activation of P2Y receptors. The results in Figure 5b show that both suramin (100 μM) and PPADS (100 μM) significantly inhibited the Cl\(^{-}\) current activated by 100 μM ATP. The numerical values that document this conclusion are as follows: ATP, 8.51 ± 3.31 pA pF\(^{-1}\); suramin plus ATP, 2.09 ± 1.12 pA pF\(^{-1}\) (P < 0.01 versus ATP, n = 4); and PPADS plus ATP, 4.64 ± 1.37 pA pF\(^{-1}\) (P < 0.05 versus ATP, n = 6). This pattern of results strongly suggests that ATP activates this Cl\(^{-}\) current after binding to P2Y receptors. These findings, in conjunction with our PCR findings, implicate the P2Y\(_2\) receptor subtype as an essential mediator in these electrophysiological responses.

We have also thoroughly investigated whether activation of this ATP-dependent Cl\(^{-}\) current requires concurrent Ca\(^{2+}\) influx. One possibility is that this Ca\(^{2+}\) influx takes place through the cation channels that are coupled to P2X receptors (Shen, Yang, Pappano, A, & Liang, 2014), as is the case in mammalian ventricular myocytes. Accordingly, we examined whether the P2X receptor agonist aβ\(\text{-}\)meATP is an effective agonist for the ventricular fibroblast Cl\(^{-}\) current. As shown in Figure 5c, application of 100 μM aβ\(\text{-}\)meATP did not activate any detectable Cl\(^{-}\) current. A second possibility is that in the myocardium ATP is hydrolysed to adenosine and that adenosine then activates a Ca\(^{2+}\) influx. This appears not to be the case, because the A1 adenosine receptor agonist NECA [5′-(N-ethylcarboxamido) adenosine; 100 μM] also failed to activate the Cl\(^{-}\) current, even though ATP (100 μM) was an effective agonist in this same batch of fibroblasts (Figure 5d).

The next experiments were carried out to evaluate the functional importance of changes in [Ca\(^{2+}\)]\(_{o}\). Initial measurements were made

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**FIGURE 5** Effects of selected purinergic P2 agonists and antagonists on ATP-evoked Cl\(^{-}\) currents. Each P2 receptor agonist or antagonist was applied in perforated patch conditions at a holding potential of −60 mV. A ramp voltage waveform from −110 to +90 mV was applied at 0.1 Hz. (a) Effects of 100 μM 2-MTATP, ADP and UTP. Note that 2-MTATP activated a large Cl\(^{-}\) current (control, 2.70 ± 0.38 pA pF\(^{-1}\); 2-MTATP, 10.33 ± 3.02 pA pF\(^{-1}\), P < 0.01 versus control, n = 6). The current produced by ADP was much smaller but significantly different from control (control, 3.67 ± 2.28 pA pF\(^{-1}\); ADP, 6.36 ± 3.49 pA pF\(^{-1}\), P < 0.05 versus control, n = 10). UTP did not activate detectable Cl\(^{-}\) current (control, 2.78 ± 1.00 pA pF\(^{-1}\); UTP, 4.07 ± 2.15 pA pF\(^{-1}\), P > 0.05 versus control, n = 10). (b) Effects of the P2Y receptor antagonists, suramin and PPADS, on ATP-induced current. Cells held at −60 mV were superfused with 100 μM ATP before, during and after washout of 100 μM suramin or PPADS. In the presence of suramin, ATP did not activate any measureable Cl\(^{-}\) currents. PPADS also partly inhibited ATP-induced Cl\(^{-}\) currents. (c) Effects of aβ\(\text{-}\)meATP, a P2X agonist, on membrane current. Note that aβ\(\text{-}\)meATP (100 μM) did not activate a detectable Cl\(^{-}\) current (control, 3.68 ± 2.11 and −0.82 ± 0.64 pA pF\(^{-1}\); aβ\(\text{-}\)meATP, 4.21 ± 2.23 pA pF\(^{-1}\), P > 0.05 versus control, −0.79 ± 0.62 pA pF\(^{-1}\), P > 0.05 versus control; ATP, 11.58 ± 4.54 pA pF\(^{-1}\), P < 0.01 versus control, −1.70 ± 0.76 pA pF\(^{-1}\), P < 0.05 versus control, n = 7). (d) Comparison of membrane currents produced by ATP (100 μM) and the P1 adenosine receptor agonist NECA (100 μM). As shown, NECA failed to increase Cl\(^{-}\) currents above control levels. Data sets are expressed as means ± SD.
when the extracellular solution was effectively Ca\(^{2+}\) free (Ca\(^{2+}\) was chelated with 1 mM EGTA and no Ca\(^{2+}\) was added to the superfusate). As shown in Figure 6a, in this Ca\(^{2+}\)-free extracellular solution, 100 \(\mu M\) ATP still activated large Cl\(^{-}\) currents. These values were as follows: control, 4.06 ± 1.05 and −0.84 ± 0.18 pA pF\(^{-1}\); ATP, 16.46 ± 6.26 (P < 0.01 versus control) and −3.09 ± 0.82 pA pF\(^{-1}\) (P < 0.01 versus control, n = 5). In summary, these Cl\(^{-}\) currents were very similar in magnitude to those recorded in 2.2 mM [Ca\(^{2+}\)]\(_o\) (Figure 3c). Thus, activation of Cl\(^{-}\) current by ATP does not require or involve transmembrane Ca\(^{2+}\) influx, either through P2X-gated channels or by other mechanisms.

We have also addressed the possibility that an increase in [Ca\(^{2+}\)]\(_o\) is the principal or perhaps even essential trigger for this ATP-sensitive Cl\(^{-}\) current. In these studies, changes in [Ca\(^{2+}\)]\(_o\) were minimized or eliminated by first adding (pretreatment) the Ca\(^{2+}\) chelator BAPTA-AM to the superfusate. After incubation with 20 \(\mu M\) BAPTA-AM for 20 min, 100 \(\mu M\) ATP was applied to cardiac fibroblasts in perforated patch recording conditions. As shown in Figure 6b, ATP failed to activate Cl\(^{-}\) current in fibroblasts pretreated with BAPTA-AM (n = 6).

3.7 Molecular identity of Ca\(^{2+}\)-activated Cl\(^{-}\) current

Ca\(^{2+}\)-activated Cl\(^{-}\) channels have been identified in a number of different mammalian cells and tissues (Duran et al., 2011, 2012; Jentsch, 2008). Recent advances in studies of the molecular basis for this current have shown that the transcripts TMEM1 or TMEM2 (also denoted ANO1 or ANO2) are responsible for this current, although this family is known to include 10–12 additional transcripts.

The quantitative PCR results shown in Figure 7 address the question: Which TMEM family member is the most likely candidate for the ATP-induced Ca\(^{2+}\)-dependent Cl\(^{-}\) current in rat ventricular fibroblasts? Perhaps somewhat surprisingly, the answer appears to be TMEM16A or ANO6. The available literature would suggest that this is a plausible explanation or candidate, although this particular transcript has not been thoroughly studied as a surface membrane ion channel (see Discussion).

3.8 Initial signalling events for Cl\(^{-}\) current activation by ATP in ventricular fibroblasts

Taken together, the data in Figures 2, 5 and 6 indicate that although an increase in [Ca\(^{2+}\)]\(_o\) is necessary for activation of Cl\(^{-}\) current by purinergic agonists in these ventricular fibroblasts, this change alone is not sufficient. Thus, UTP consistently produces a large increase in [Ca\(^{2+}\)]\(_o\) (Figure 2c), but does not activate Cl\(^{-}\) currents (Figure 5a). It is well known that ATP activation of P2Y receptors can induce inositol phosphate production through activation of the holoenzyme phospholipase C (PLC), and it is likely that the P2Y receptor is G protein coupled. Normally, this functional link is to Gq (Meszaros...
et al., 2000). In an attempt to identify an initial step in this signalling cascade, the effects of a PLC inhibitor, U73122, on the ATP-induced Cl\(^{-}\) current were examined. Ventricular fibroblasts were superfused with 30 \(\mu\text{M}\) ATP before, during and after washout of either 3 \(\mu\text{M}\) U73122 (Figure 8a) or 3 \(\mu\text{M}\) U73343 (the non-functional analogue of U73122; Figure 8b). No detectable ATP-sensitive Cl\(^{-}\) current was present after U73122 pretreatment (Figure 8c). The measured current densities were as follows: control, 3.43 ± 1.03 pA pF\(^{-1}\); and ATP, 2.99 ± 1.42 pA pF\(^{-1}\) (\(P > 0.05\) versus control, \(n = 6\)). In contrast, the inactive analogue, U73343 was without effect, as follows: control, 3.04 ± 1.41 pA pF\(^{-1}\); and ATP, 9.23 ± 4.48 pA pF\(^{-1}\) (\(P < 0.05\) versus control, \(n = 5\)).

Taken together, these data show that Cl\(^{-}\) current activation by ATP in cultured ventricular fibroblasts requires an increase of [Ca\(^{2+}\)], and activation of PLC. However, additional signalling steps/factors must be involved, because ADP and UTP, both of which produced increases in [Ca\(^{2+}\)], that were very similar to those in response to ATP, either failed to activate Cl\(^{-}\) current (UTP) or produced a much smaller current (ADP; see Figure 5a). It is known that both G protein- and IP\(_3\)-mediated signalling can be highly compartmentalized in mammalian cells. This may contribute to the different pattern of results that we have observed in experiments involving measurements of Ca\(^{2+}\)-activated Cl\(^{-}\) current versus recordings of [Ca\(^{2+}\)], in rat ventricular fibroblasts (see Discussion).

Our experimental work has identified two distinct patterns of cell physiological responses to purinergic agonists in rat ventricular fibroblasts as judged by changes in [Ca\(^{2+}\)], versus activation of a Ca\(^{2+}\)-dependent Cl\(^{-}\) current. In addition to the marked differences of these two responses to ADP and UDP (Figure 3), their tendency to show desensitization or tachyphylaxis was also a distinguishing feature. These experiments, which sought to elicit and then separate these two responses, were based on the principle that the kinetics of tachyphylaxis in response to repeated applications of a near-maximal concentration of ATP may differ significantly. When 100 \(\mu\text{M}\) ATP was applied twice, in rapid succession, to the same cell there were marked differences in the two patterns of responses. As illustrated in Figure 9a,b, the ATP-induced increases in [Ca\(^{2+}\)], showed no tachyphylaxis. In contrast (Figure 9c,d), the 100 \(\mu\text{M}\) ATP-induced increase in Cl\(^{-}\) current was markedly reduced in response to the second application of ATP. It is apparent, therefore, that these two responses triggered by physiological levels of extracellular ATP are mediated by distinct signalling pathways.

4 DISCUSSION

4.1 New findings

Our results identify two significant physiological responses to ATP in rat ventricular fibroblasts that have been held in 2-D culture conditions. These are: (i) a large increase in [Ca\(^{2+}\)] in response to ATP (or ADP or UTP); and (ii) activation of an outwardly rectifying transmembrane current carried mainly by Cl\(^{-}\), a response that is much more sensitive to ATP than to UTP. Both responses were observed in virtually all of the ventricular fibroblasts in this study. PCR analysis suggests that this current in ventricular fibroblasts is generated by the TMEM16F variant of the family of Ca\(^{2+}\)-activated Cl\(^{-}\) currents (Acheson, 2016; Picollo et al. 2015; Scudieri et al., 2015; Shimizu et al., 2013). Large and complex patterns of increases in [Ca\(^{2+}\)], have been reported previously (Chen et al. 2010) when myofibroblasts respond to purinergic agonists. Chen et al. (2010) and Certo et al. (2015) have shown that when UTP is the agonist, this response is mediated by P2Y\(_{11}\) receptors; and Certo et al. (2015), have suggested the interesting possibility that cardiac fibrosis could be regulated through P2Y\(_{11}\)-mediated effects.

Our identification of P2Y- and PLC-mediated Ca\(^{2+}\)-dependent Cl\(^{-}\) currents in rat fibroblasts is novel. Moreover, our results showing that in distinction to ventricular fibroblasts, the main response to ATP in rat ventricular myocytes does not depend on changes in [Ca\(^{2+}\)], demonstrates clear-cut and cell type-specific differences in ATP responsiveness in the ventricular syncytium.

4.2 Effects of ATP on the mammalian myocardium

In the mammalian cardiovascular system, ATP is released from sympathetic nerve endings, platelets, neutrophils, red blood cells and both fibroblasts/myofibroblasts and myocytes themselves. In pathological conditions, it is likely that there are additional sources of ATP; these include ischaemic myocytes, ‘inflammatory’ cells (emigrated neutrophils or T lymphocytes) and endothelial cells. Not surprisingly, therefore, within localized extracellular regions in cardiovascular tissues (including the heart), relatively high concentrations of ATP (e.g. 100 \(\mu\text{M}\)) have been detected (Corrède & Insel, 2010; Elliott et al., 2009; Ostrom, Gregorian, & Insel, 2000).

In baseline conditions, ATP application results in positive inotropic effects in the mammalian heart in vitro and consistently produces augmented cell shortening in ventricular myocytes (Vassort, 2001). However, ATP often also increases heart rate, and this can also be pro-arrhythmic in isolated myocytes (Gurung et al., 2009). ATP can also stimulate gene expression and modulate cell proliferation in cardiac fibroblasts/myofibroblasts (cf. Wynn, 2007; Zheng, Christie, Levy, & Scarpa, 1992, 1998), therefore sometimes contributing to cardiac hypertrophy (Manabe, Shindo, & Nagai, 2002) and/or fibrosis (Nishida et al., 2008).

Cardiac fibroblasts make an important contribution to structural, biochemical, mechanical and electrical properties of the myocardium (cf. Travers, Kamal, Robbins, Yutzey, & Blaxall, 2016). Fibroblasts can also modulate cardiac electrophysiological properties via electrotonic currents through gap junctions (Baudino et al., 2006) flowing among myocytes and fibroblasts/myofibroblasts. Given that the resting potential of isolated fibroblasts (−40 to −60 mV) is significantly different from that of the ventricular myocytes (−80 to −85 mV), one would expect that the fibroblast would exhibit a depolarizing influence on the myocyte. However, the extent of this is uncertain for the following reasons: (i) the ratio of the number of fibroblasts to myocytes is unknown; (ii) the respective sizes of these two types of cells are very different; and (iii) the extent to which each fibroblast is functionally coupled to myocytes is unknown. Certainly, in the presence of ATP the...
**FIGURE 8** Phospholipase C (PLC)-mediated intracellular signalling is essential for activation of ATP-induced Cl\(^-\) current in short-term cultured ventricular fibroblasts. As shown in (a) and (b), fibroblasts voltage clamped at \(-60\) mV were superfused with 30 \(\mu\)M ATP before, during and after application of either the PLC inhibitor U73122 (3 \(\mu\)M) or its inactive analogue, U73343 (3 \(\mu\)M). The I–V curves were generated by application of the same ramp voltage-clamp protocol described in previous figure legends. (c) Summary of the data, demonstrating that inhibition of PLC strongly reduces the ATP-induced Cl\(^-\) current (U73122; \(n = 6\)) and that this effect is selective, because the inactive analogue (U73343; \(n = 5\)) has no inhibitory effect.

**FIGURE 9** Evaluation of any tachyphylaxis or desensitization of ATP-induced (i) changes in \([Ca^{2+}]_i\) or (ii) activation of previously \(Ca^{2+}\)-dependent Cl\(^-\) current. (a) Sequential applications of 100 \(\mu\)M ATP to cardiac fibroblasts that were loaded with fura-2. As shown, both applications of ATP, separated by 14 min, increased \([Ca^{2+}]_i\) approximately equally. The open circles show average responses from four separate cells. (b) Summary data showing no significant difference between the peak increase of fura-2 fluorescence ratio produced by the first and second applications of ATP. (c) In contrast, the ATP-activated Cl\(^-\) currents in response to sequential applications of 100 \(\mu\)M ATP showed significant differences. The two plots show currents at \(-110\) and \(+90\) mV during voltage ramps. Note that the first application (left) of ATP produced large Cl\(^-\) currents, but a second application (right) after a 15 min washout of ATP resulted in no detectable Cl\(^-\) response. (d) Summary of effects of two sequential applications of ATP on current at \(+80\) mV during ramps (first application of ATP, 7.07 \(\pm\) 4.32 pA pF\(^{-1}\); second application of ATP, 1.52 \(\pm\) 1.91 pA pF\(^{-1}\), \(P < 0.01\) versus first application, \(n = 7\)). Data are expressed as means \(\pm\) SD.
resulting Cl⁻ conductance will drive the membrane potential towards $E_C$ (see section 4.6 Concluding perspectives).

Electromechanical interactions among myocytes and fibroblasts are now well known (Camelliti et al., 2005). Stretch/mechanical perturbation of cultured adult fibroblasts can result in ATP release, mediated in part by connexin hemichannels (Dolmatova et al., 2012; Lu et al., 2012).

### 4.3 Relationship of our results to previous findings in mammalian hearts

Our findings, when considered in conjunction with previous work on purinergic responses in mammalian cardiac myocytes, provide new insights into the complex multifactorial purinergic signalling system in the ventricular myocardiun (Vassort, 2001). It is well established that ATP can exhibit positive inotropic effects in the heart, both in control conditions and in the setting of experimental cardiomyopathy (Vassort, 2001; Yang et al., 2014). Although extensive electrophysiological studies have been done on a variety of different isolated single myocyte preparations, the explanation for this consistent positive inotropic effect remains to be determined. In part, this is because the electrophysiological actions of ATP seem to be variable and perhaps also species-specific (Vassort, 2001). However, there is general agreement that ATP initially binds to specific subsets of P2X receptors (cf. Suprenant & North, 2009) localized on the sarcolemma of the myocyte. This receptor occupancy is linked to a cation-selective and voltage-independent conductance, apparently resulting in Na⁺ and Ca²⁺ influx (Banfi et al., 2005; Gurung et al., 2009; Parker & Scarpa, 1995; Zheng et al., 1992). In guinea-pig ventricular myocytes, ATP application can produce release of intracellular Ca²⁺ (Christie et al., 1992). However, there have been no detailed studies of the functional linkage between these Ca²⁺ changes and the increase in time-independent or background conductance in these myocytes.

### 4.4 Pharmacological properties of the ventricular fibroblast responses to ATP

ATP, ADP and UTP, when applied at 100 µM, all consistently elicited very similar increases in [Ca²⁺], in the rat ventricular fibroblasts that we have studied. This pattern of results strongly suggests an essential role for the P2Y family of purinergic receptors (Burnstock, 2012; von Kugelgen, 2006). These findings also indicate that more than one subtype of P2Y receptor is expressed in these short-term cultured ventricular fibroblasts. Published RT-PCR data from rat hearts have provided evidence for the expression of P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ transcripts (Certal et al., 2015, 2016; Lu et al., 2012; Webb et al., 1996).

Our electrophysiological results complement and extend this information concerning the functional expression of P2Y purinergic receptors in rat ventricular fibroblasts. As shown in Figure 5, both ATP and 2-methylthio-ATP are potent agonists for activation of a Ca²⁺-sensitive Cl⁻ current. ADP is less potent, and neither UTP nor αβ-methylene-ATP was an effective agonist. In combination, these results suggest that the ATP-activated Cl⁻ current in our fibroblasts is initiated by purinergic receptors having some of the properties of the P2Y₂, P2Y₄ and P2Y₆ subsets. Our results also show that this Cl⁻ current is not mediated mainly by P2Y₁, because ATP is a much more potent agonist than ADP (Martinez-Pinna et al., 2005).

The data at hand concerning the rank order potency of purinergic agonists and expression of purinergic receptor transcripts in ventricular fibroblasts/myofibroblasts suggest that another plausible purinoceptor candidate is P2Y₁₁ (Certal et al., 2015). The P2Y₁₁ receptor has been detected in human hearts (von Kugelgen, 2006), and this gene product has recently been detected in rat myofibroblasts (Certal et al., 2015). Additional evidence for this possibility is the finding that in 1321N1 cells in which the human P2Y₁₁ receptor had been expressed, both ATP and UTP caused a significant increase in [Ca²⁺], (von Kugelgen, 2006). Importantly, the recent comprehensive study carried out by Certal et al. (2015) clearly demonstrated that a significant component of the increase in [Ca²⁺], in response to UTP was mediated by the P2Y₁₁ purinergic receptor. Certal et al. (2015) also reported that suramin is a potent (but not entirely selective) antagonist for the P2Y₁₁-mediated increase in [Ca²⁺]. Interestingly, a follow-up study from this group has shown that these rat ventricular myofibroblasts respond to ADP by using a signalling pathway apparently initiated mainly by P2Y₄ activation. In fact, the response to ADP marks an increase [Ca²⁺], that is derived from an intracellular organelle (endoplasmic reticulum) and initiates a Ca²⁺-depletion signal, which then activates the so-called iCRAC, thus providing an additional Ca²⁺-selective channel in these cells (Certal et al., 2016).

### 4.5 ATP activates a [Ca²⁺]-sensitive Cl⁻ current via phospholipase C

Our results show that although the activation of this Cl⁻ current requires an increase in [Ca²⁺], this process is not dependent on a net transmembrane Ca²⁺ influx. Thus, as shown in Figure 6, when [Ca²⁺] is removed, this ATP-sensitive Cl⁻ current is not reduced. In contrast, when increases in [Ca²⁺] are prevented by inclusion of the rapid [Ca²⁺] buffer, this Cl⁻ current fails to show any significant activation, even in response to near-maximal concentrations of ATP or ADP.

Recently, very significant progress has been made in defining the molecular features of a large family of Ca²⁺-activated Cl⁻ currents (Duran & Hartzel, 2011; cf. Dutta et al., 2011; Yang et al., 2012; Pedemonte & Galietta, 2004). Based mainly on work done in epithelial tissues, these Ca²⁺-activated Cl⁻ channels are denoted TMEM116; although the equivalent descriptors anoctamin or ANO1–ANO10 are also often used. Additional study/analysis is required before we can be certain of the Ca²⁺-activated Cl⁻ subtypes in ventricular fibroblasts. However, our PCR data (Figure 7), somewhat surprisingly, suggest that the predominant subtype is TMEM16F, also denoted ANO6. The general features of this ATP-sensitive Ca²⁺-activated Cl⁻ current include: (i) functional coupling mainly to the P2Y subclass of purinergic receptors; and (ii) exhibiting a distinctive outwardly rectifying ion transfer or I–V relationship. Both of these are prominent features of our results.
FIGURE 10 Electrophysiological effects of ATP on acutely isolated rat ventricular myocytes. (a) Shortly after ATP (1 μM) is added to the superfusate, there is a depolarization (~5 mV) of the resting potential, and the action potential height is progressively reduced until the myocyte becomes unexcitable. The records on the lower row of (a) show that these effects are almost completely reversible when ATP is ‘washed out’. The records on the right side of (a) show that both of these effects (depolarization of the resting potential and reduction in action potential height) are reduced when the effects of ATP (0.5 μM) are challenged/inhibited by addition of suramin (50 μM) to the superfusate. The superimposed I–V curves in (b) (left) clearly illustrate an inwardly rectifying component of this ATP-induced current. These I–V curves also show that this 10 μM ATP-activated current has a reversal potential at approximately −40 mV. The bar graph in (b) (right) were constructed from multiple measurements of control versus ATP-induced current measured at −120 and +50 mV. (c) The raw current records (right) and time-course data (left) (from a different myocyte) show that suramin at 100 μM almost completely blocks the effects of ATP (10 μM).

By combining results from electrophysiological, molecular and Ca^{2+} imaging experiments, we have also obtained important new information concerning elements of the major intracellular signalling pathway for these ‘purinergic’ responses in ventricular fibroblasts. Both the ATP-induced Cl\(^-\) current and the increase in [Ca^{2+}]\(_i\) are dependent on an intracellular PLC-mediated signalling pathway. We suggest that ATP binds mainly to P2Y\(_2\) receptors and results in activation of PLC, producing an increase in IP\(_3\), and this causes release of Ca\(^{2+}\) from the fibroblast endoplasmic reticulum. This P2Y subtype is known to couple to Gq proteins and then stimulate PLC. Our results are consistent with this being an essential early step in this signal transduction, and it effectively blocks the ATP-induced activation of the Cl\(^-\).

The resultant increase in IP\(_3\) triggers a substantial release of Ca\(^{2+}\) from the endoplasmic reticulum, which we have measured as an increase in [Ca\(^{2+}\)]\(_i\).

4.6 Concluding perspectives

Activation of the ATP-sensitive Cl\(^-\) current that we have identified would be expected to depolarize the fibroblast by producing an inward current at membrane potentials that are negative to \(E_{\text{Cl}}\) (approximatley −40 mV). However, it is important to note that the apparent resting potential of the fibroblast is approximately −40 mV (Baudino et al., 2006). For this reason, activation of this Cl\(^-\) current...
may predominantly change the input resistance as opposed to the resting membrane potential of these cells. This may result in the fibroblast exhibiting a ‘shunted’ or reduced response to exposure to other paracrine agents or to cyclic stretch. In other preparations, similar effects of activation of time-independent or background Cl− conductance are known to produce this type of ‘differential shunting’ (Häusser, Major, & Stuart, 2001). However, as mentioned previously, when fibroblasts couple with ventricular myocytes, the electrotonic influence of the myocyte results in the fibroblast being hyperpolarized to approximately −60 mV. In this circumstance ATP, by enhancing a Cl− conductance, would depolarize the fibroblast and also exert a depolarizing influence on any electrotonically coupled myocytes. Paradoxically, however, any such small depolarization (5–10 mV) of the ventricular myocyte would move the membrane potential of the myocyte into a region where the input resistance would increase significantly owing to the non-linearity (negative slope region) of the background K+ conductance in these cells (Geukes Foppen, van Mil, & Siegenbeek van Heukelom, 2002).

It is clear, however, that a more complete understanding of the intrinsic electrophysiological properties of cardiac fibroblasts and myofibroblasts is needed (Zeigler, Richardson, Holmes, & Saucerman, 2016). One reason is that the fibroblast is often featured as an essential cellular ‘element’ in efforts to restore heart function using Regenerative Medicine techniques (cf. Barallobre-Barreiro et al., 2012; Srivastava & Ieda, 2012; Travers et al., 2016). Further progress in this challenging endeavour will require detailed understanding of the intrinsic and ligand-gated electrophysiological properties of fibroblasts and their phenotypic stability. Some of the additional experiments that will be needed include the following: (i) identifying other functionally important ion channel families, e.g. TRP channels (cf. Du et al., 2010; Rose & Giles, 2008), in these fibroblasts; (ii) increasing our understanding of cell-to-cell electrotonic communication (Chilton et al., 2005; Quinn et al., 2016); and (iii) relating these new results to fibroblast differentiation (Thodeti, Paruchuri, & Meszaros, 2013) and migration and remodelling (Porter & Turner, 2009).

Mechanistic insights into the physiological and pathophysiological role(s) of the actions of ATP in the mammalian myocardium will also require more detailed information concerning the similarities and differences in patterns of responses generated by each of the two principal cell types in the ventricular myocardium, namely fibroblasts and myocytes. Preliminary findings of this type are shown in Figure 10a. Note that when even 10 μM ATP is added to the superfusing solution of acutely isolated rat ventricular myocytes, the action potential upstroke is ‘shunted’ significantly. This is probably because there is a small (but significant) depolarization of the resting potential. Corresponding voltage-clamp analysis (Figure 10b,c) reveals that the ATP-activated non-selective cation current has a reversal potential near −40 mV. Although this current can be blocked by suramin, it differs substantially from the ATP-activated current in the fibroblast. Thus, the current in the myocyte has ion transfer or I−V characteristics that are different from the (much more linear) current activated by ATP in the fibroblast. In addition, the ATP-activated current in the myocyte is not strongly dependent on an increase in [Ca2+]i, because in these experiments increases in [Ca2+]i were significantly reduced or prevented by the inclusion of EGTA (1 mM) in the patch recording pipette.

The suramin sensitivity of these ATP-induced changes in resting potential and ATP-induced current are somewhat similar to those reported by Gurung et al. (2009) in their studies of Langendorff-perfused mouse hearts and isolated ventricular myocytes. Gurung and colleagues also observed that these responses were activated (in order of potency) by ATP, ADP and UTP and were blocked by
both suramin and PPADs. They suggested that P2X_4 receptors on the ventricular myocytes can mediate these effects. At present, we do not fully understand the functional role or significance of this ligand (ATP)-gated conductance in the adult rat ventricular myocyte. However, it is reasonable to propose that it will directly regulate or modulate ‘subthreshold electrical properties’ and indirectly alter excitability as a similar conductance in skeletal muscle does (Geukes Foppen et al., 2002; Pedersen, Huang, & Fraser, 2011). Given that ATP is rapidly broken down to adenosine in the cardiovascular system, additional studies of the effects of ATP on fibrosis will also need to explore and account for the related but distinct effects of adenosine (Corriden & Insel, 2012; Cronstein, 2011; Jacobson et al, 2012).

COMPETING INTERESTS
None declared.

AUTHOR CONTRIBUTIONS
All authors were involved in conception and design of experiments. N.H. carried out most of the experiments and did the primary data analysis. W.R.G. took final responsibility for writing this manuscript, and all authors contributed. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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