Inhibition of Thromboxane A₂-Induced Arrhythmias and Intracellular Calcium Changes in Cardiac Myocytes by Blockade of the Inositol Trisphosphate Pathway

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ABSTRACT

We have recently reported that left atrial injections of the thromboxane A₂ (TXA₂) mimetic, (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid (U46619), induced ventricular arrhythmias in the anesthetized rabbit. Data from this study led us to hypothesize that TXA₂ may be inducing direct actions on the myocardium to induce these arrhythmias. The aim of this study was to further elucidate the mechanism responsible for these arrhythmias. We report that TXA₂R is expressed at both the gene and protein levels in atrial and ventricular samples of adult rabbits. In addition, TXA₂R mRNA was identified in single, isolated ventricular cardiac myocytes. Furthermore, treatment of isolated cardiac myocytes with U46619 increased intracellular calcium in a dose-dependent manner and these increases were blocked by the specific TXA₂R antagonist, 7-(3-((2-((phenylamino)carbonyl)-

hydrazino)methyl)-7-oxabicyclo[2.2.1]hept-2-yl)-5-heptenoic acid (SQ29548). Pretreatment of myocytes with an inhibitor of inositol trisphosphate (IP₃) formation, gentamicin, or with an inhibitor of IP₃ receptors, 2-aminoethoxydiphenylborate (2-APB), blocked the increase in intracellular calcium. In vivo pretreatment of anesthetized rabbits with either gentamicin or 2-APB subsequently inhibited the formation of ventricular arrhythmias elicited by U46619. These data support the hypothesis that TXA₂ can induce arrhythmias via a direct action on cardiac myocytes. Furthermore, these arrhythmogenic actions were blocked by inhibitors of the IP₃ pathway. In summary, this study provides novel evidence for direct TXA₂-induced cardiac arrhythmias and provides a rationale for IP₃ as a potential target for the treatment of TXA₂-mediated arrhythmias.

Thromboxane A₂ (TXA₂) is a member of the prostaglandin family and is formed by the conversion of arachidonic acid to prostaglandin G₂ by cyclooxygenase, which is subsequently converted to TXA₂ by TXA₂ synthase. The well-known actions of TXA₂ include platelet aggregation and vasoconstriction (Hamberg et al., 1975; Moncada and Vane, 1978). Be-

cause of these actions, results of clinical trials have suggested nonsteroidal anti-inflammatory agents, such as aspirin, as a protective therapy to reduce the risk of cardiovascular events (Patrono et al., 2004). There is little doubt that TXA₂-mediated vasoconstriction and platelet aggregation can contribute to heart disease and arrhythmias in an *indirect* manner via the induction of platelet aggregation and coronary artery vasoconstriction. What has not been well characterized, however, are the potential *direct* arrhythmogenic effects of TXA₂ on the heart via stimulation of cardiac TXA₂ receptors.

Our research group has previously been interested in the ability of the TXA₂ mimetic, U46619, to stimulate neurons involved in the autonomic nervous system (Wacker et al., 2002). During the course of these in vivo experiments in the anesthetized adult rabbit, it was noted that left atrial injec-

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ABBREVIATIONS: TXA₂, thromboxane A₂; TXA₂R, thromboxane A₂ receptor; IP₃, inositol trisphosphate; IP₃R, inositol trisphosphate receptor; TNT, troponin T; 2-APB, 2-aminoethoxydiphenyl borate; TBST, Tris-buffered saline with Tween 20; HBSS, Hanks' buffered salt solution; MABP, mean arterial blood pressure; ECG, electrocardiogram; HR, heart rate; RT-PCR, reverse transcriptase polymerase chain reaction; U46619, (5Z)-7-[(1R,4S,5S,6R)-6-[(1E,3S)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid; SQ29548, 7-(3-((2-((phenylamino)carbonyl)hydrazino)methyl)-7-oxabicyclo[2.2.1]hept-2-yl)-5-heptenoic acid.

tions of U46619 also induced arrhythmias. These U46619-induced arrhythmias were documented in a subsequent study and found to be ventricular in origin and were inhibited by pretreatment with SQ29548, a specific inhibitor of the TXA₂ receptor (TXA₂R also known as TP) (Wacker et al., 2006). Coronary blood flow was measured during these experiments and was not significantly altered by the injections of U46619, and there were no ST segment changes in the ECG recordings. This would indicate that significant vasoconstriction or myocardial ischemia did not play a role in the genesis of these arrhythmias. Furthermore, we demonstrated that the number of arrhythmias induced by U46619 was not statistically altered by blockade of β -adrenergic receptors; thus U46619 did not augment β -adrenergic signaling to the heart to induce arrhythmias. Therefore, we hypothesized that direct activation of TXA₂Rs on cardiac myocytes may alter calcium dynamics, leading to these arrhythmias.

There is a strong rationale for this hypothesis. Previous studies have shown that there are binding sites for TXA₂Rs in the heart of various species (Lasserre et al., 1992; Bowling et al., 1994) and that TXA₂ can induce changes in intracellular calcium in neonatal rat cardiac myocytes (Hoffmann et al., 1993; Dogan et al., 1997). Therefore, the purpose of this study was to determine the mechanism by which activation of TXA₂Rs could induce changes in intracellular calcium in vitro and arrhythmias in vivo. TXA₂R is a G-protein-coupled receptor that has been well characterized to activate phospholipase C and induce increases in inositol trisphosphate (IP₃) (Baldassare et al., 1993; Dorn and Becker, 1993; Walsh et al., 2000). IP₃ is a well-known by-product from the enzymatic cleavage of phosphatidylinositol 4,5-bisphosphate, acts as an intracellular signaling molecule that binds to IP₃ receptors (IP₃R), and releases calcium from intracellular stores. It is noteworthy that the role of IP₃ in inducing arrhythmias and other cardiac pathologies has become an increasingly important research area in cardiac muscle physiology (Kocksämper et al., 2008). Therefore, we wanted to investigate whether IP₃ and IP₃Rs play a role in TXA₂R-mediated ventricular arrhythmias.

To test our hypothesis, the current study builds on the previous in vivo model of TXA₂-induced ventricular arrhythmias that we have established (Wacker et al., 2006) and uses in vitro calcium-imaging experiments with primary cardiac myocytes. Gentamicin and 2-aminoethoxydiphenyl borate (2-APB) have previously been used to inhibit the formation of IP₃ and block IP₃Rs, respectively, in other models and are suitable for use for in vivo studies. Therefore, we used these inhibitors of the IP₃ pathway to test the role of IP₃ in actions of U46619 in our experiments. We found that both gentamicin and 2-APB inhibited the U46619-induced increases in intracellular calcium in vitro and the U46619-mediated arrhythmias in vivo. Thus, our data support the hypothesis that TXA₂ can induce arrhythmias via direct stimulation of cardiac myocytes via a mechanism involving IP₃. This is a potentially novel mechanism of arrhythmogenesis and may provide a new therapeutic target for the treatment of arrhythmias.

Materials and Methods

RT-PCR. All experimental protocols and procedures using animals in this investigation were reviewed and approved by the Insti-

tutional Animal Care and Use Committee and carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Samples were taken from 4-kg, euthanized, male, New Zealand White rabbits. RNA from atria and ventricles of three rabbits were extracted by use of the RNeasy Fibrous Tissue Kit (QIAGEN; Valencia, CA). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on mRNA isolated from 20 mg of tissue following the protocol of the Superscript III RT-PCR kit (Invitrogen; Carlsbad, CA). TXA₂R primer sets were as follows: GCTGGTGCTCAACACCGTGA (forward) and CGTCAGCGCGATGAAGAC (reverse). These primers have been used previously by our laboratory, were designed to span an exon-exon junction, and are expected to yield a product size of 277 bp based on previous sequencing data (Wacker et al., 2005).

Western Blot. Clamp-frozen atria and ventricular muscles from three rabbits were homogenized in a 12:1 (volume/weight) ratio of ice-cold cell extraction buffer (Invitrogen) with protease inhibitor cocktail (500 μ l; Invitrogen), sodium fluoride (200 mM), sodium orthovanadate (200 mM), and phenylmethane sulfonylfluoride (200 mM) added. Homogenized samples were rotated for 30 min and then centrifuged for 20 min at 3000g at 4°C. Samples were diluted (1:1800) and total protein concentration of the samples was determined by use of the micro bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Samples were prepared in 5 $\times$ Laemmli buffer containing 100 mM dithiothreitol and heated at 85°C for 5 min. Next, 60 μ g of protein was subjected to SDS-polyacrylamide gel electrophoresis (12% gel) followed by a wet transfer to a polyvinylidene difluoride membrane for 90 min (200 mA/tank). Membranes were blocked for 1 h at room temperature in Tris-buffered saline, 0.1% Tween 20 (TBST)-5% nonfat dry milk followed by an overnight incubation at 4°C with the primary antibody (P-20, sc-31260; Santa Cruz Biochemicals, Santa Cruz, CA) at a concentration of 1:250 in TBST-1% milk. After three 5-min rinses in TBST, blots were incubated in TBST-1% nonfat dry milk with a Cy3 donkey anti-goat secondary antibody (705-165-147; Jackson ImmunoResearch Laboratories, West Grove, PA) at a concentration of 1:250 for 1 h at room temperature. Visualization of the protein bands was performed by use of a fluorescent imager (GE Healthcare Bio-Sciences Corp., Little Chalfont, Buckinghamshire, UK).

Isolation of Primary, Ventricular Cardiac Myocytes. Rabbit hearts were isolated and immediately attached to a Langendorf perfusion apparatus. Four hundred milliliters of calcium-free Hanks' buffered salt solution (HBSS; Invitrogen) with heparin was pumped through the heart. HBSS with collagenase type II (2 mg/ml; Worthington Biochemical; Lakewood, NJ), penicillin, streptomycin, and L-glutamine was then pumped through the heart for 25 min or until the heart seemed soft. The heart was then sliced into pieces of approximately 2 to 3 mm³ and placed in wash buffer composed of HBSS, penicillin, and streptomycin. If needed, additional digestion of heart pieces in collagenase was carried out. An underlay of 5% bovine serum albumin, 47.5% Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO), and 47.5% HBSS without calcium was placed below the wash solution and cells were allowed to gravity filtrate for 10 min. The underlay containing myocytes was then gently pipetted on to laminin-pretreated plates. After 2 h of incubation (37°C, 5% CO₂) myocytes were washed and plated in HBSS with calcium for 30 min for use in single-cell RT-PCR or calcium imaging.

Single-Cell RT-PCR. Protocols for isolating single cardiac myocytes were similar to the protocols that we followed for single-cell isolation of neurons (Wacker et al., 2005). Five individual cardiac myocytes were separately placed in five tubes containing SSIII RT-PCR buffer, which had primer sets for cardiac troponin T: GAGGAGGAGAGCTGGTTTC (forward) and CGCCTCCAGGTTATAGATGC (reverse) and TXA₂R: (forward previously listed) and CAAGATCTGTTCAGGTGGC (reverse). The RT step was carried out at 55°C for 20 min, and 35 cycles of PCR were conducted. One microliter was then removed from the reaction and placed into two tubes containing

PCR buffer for a second round of PCR. One tube contained nested primers for TNT: CAAGGACAGGATCGAGAAGC (forward) and CCTTCTCCCTCAGCTGATCTT (reverse) and the other tube contained nested primers for TXA₂R: ACGCTCTGCCGCTCTACCA (forward) and CCACGCGGAGGTAGATGAG (reverse). The expected product size for TNT was 323 bp and for TXA₂R, it was 229 bp. PCR products were run on a 1.8% ethidium bromide agarose gel and visualized by use of a commercially available UV system (Eastman Kodak; Rochester, NY). This protocol was repeated in three separate hearts.

Drugs. The stable TXA₂R agonist, U46619 (C₂₁H₃₄O₄; Cayman Chemical, Ann Arbor, MI), was dissolved in ethanol to a concentration of 7.13 mM. SQ29548 (C₂₁H₂₉N₃O₄; Cayman), was dissolved in ethanol to a concentration of 6.45 mM. Gentamicin (C₂₁H₄₃N₅O₇; Sigma-Aldrich) was prepared in deionized water at a concentration of 104.7 mM. 2-APB (C₁₄H₁₆BNO; Cayman) was dissolved in ethanol to a concentration of 22.21 mM. For in vitro cell-imaging experiments, U46619, gentamicin, and 2-APB stock solutions were further diluted in HBSS with calcium on the day of the experiment to make working stock solutions at lower concentrations.

Calcium Imaging. Cardiac myocytes were loaded with the calcium indicator dye fluo-4 AM (2 μ M) for 30 min. Plates were then washed twice with HBSS and fluo-4 was allowed to de-esterify for 15 min at room temperature. Intracellular calcium levels were measured at 37°C with use of an environmental chamber surrounding an inverted microscope with fluorescent imaging capabilities [Olympus IX51, Olympus, Melville, NY; Hamamatsu Orca-ERGA CCD camera (Hamamatsu, Bridgewater, NJ); Semrock Bright Line filter set (Semrock, Rochester, NY), EXFO X-cite metal halide light source (EXFO, Mississauga, ON, Canada), and Slidebook ratiometric software (Intelligent Imaging Innovations Inc., Denver, CO)]. In one series, plates were treated with various doses of U46619. In another series, plates were treated with 10 μ M of inhibitors (SQ29548, gentamicin, or 2-APB) 10 min before application of 5 μ M U46619. All data were presented as the peak increase in fluorescence (F) after U46619 application divided by the initial fluorescence before U46619 application (F₀). A F/F₀ of 1 indicates no change in fluorescence from the baseline. Myocyte viability was tested with KCl (80 mM) application at the end of the study, and was only included in the data if the response was greater than a 20% increase from baseline fluorescence. All diluted drugs were carefully pipetted by bolus injection at the same position (on the proximal side) of the plates. Cells for imaging were selected on the distal side of the plate to allow for drug dilution and diffusion. Nevertheless, because cells may not be equal distances from the drug delivery site, this may contribute to some of the variability in cell responses. Cells tested from each animal were averaged, and these averages were used for data analysis.

Statistical Analysis of Calcium Imaging. For the in vitro calcium-imaging studies, we used a one-way analysis of variance to detect differences among U46619 dosages and pretreatments with the F/F₀ ratio changes. If analysis of variance was statistically significant ($p < 0.05$), follow-up comparisons were made with unpaired *t* tests with use of a Holms-Bonferroni adjustment to control for type 1 error.

In Vivo Studies. Rabbits were prepared and anesthetized as described in our previous studies (Wacker et al., 2002, 2003, 2006). Arterial blood pressure was measured via a right femoral artery catheter connected to a pressure transducer and analyzed with PowerLab software (ADInstruments; Colorado Springs, CO). Heart rate (HR) was measured using software analysis of both arterial blood pressure and ECG. Protocols for ECG analysis and drug injections were followed as described previously (Wacker et al., 2006). All drugs were administered into the left atrium via a left atrial catheter and a syringe filled with the appropriate amount of drug solution. This drug solution was infused over a period of 3 to 4 s, followed by a flush with 0.5 ml of saline. Bolus injections of either 2-APB (at 5, 0.5, or 0.05 mg; $n = 9, 9$, and 9 , respectively) or gentamicin (at 225, 22.5, 2.25, or 0.225 mg; $n = 4, 6, 4$, and 6 , respectively) were given 10 min

before U46619 treatment. Gentamicin and 2-APB pretreatment groups were compared with a control group ($n = 28$) that received no pretreatment before U46619 injections, which has been documented previously by our group (Wacker et al., 2006). U46619 was administered in triplicate in each animal of each pretreatment group with incremental increases in dose (10, 20, and 30 μ g) with 5 min between injections as documented previously (Wacker et al., 2006). A group of eight animals received ethanol pretreatment at the same concentration used with U46619 as a vehicle control. Both the no pretreatment and ethanol pretreatment groups followed the same timeline as the gentamicin and 2-APB pretreatment groups.

The types of ventricular arrhythmias that occurred were documented by use of the Lambeth Convention guidelines (Walker et al., 1988). We calculated an "Arrhythmia Severity Score" based on previously used systems (Curtis and Walker, 1988). We used scores from 0 to 4 for each response to U46619 with 0 = no arrhythmias, 1 = ventricular premature beats, 2 = bigeminy, 3 = salvos, and 4 = ventricular tachycardia. The response to U46619 was assigned a score based on the most severe type of arrhythmia demonstrated.

Statistical Analysis of Arrhythmia Study. Our analysis accounts for two different components of the response: whether an animal had any arrhythmias, and then differences in the severity of the response. For the former, we scored responses dichotomously: 0, if the animal experienced no arrhythmias across all three U46619 doses, versus 1, if the animal experienced one or more. We applied logistic regression to determine whether treatment (dosage of 2-APB or gentamicin) significantly reduced the probability of arrhythmia. For 2-APB, the independent variable (dosage) ranged from 0 to 3 (for 0, 0.05, 0.5, and 5 mg, respectively). For gentamicin, the range was 0 to 4 (for 0, 0.225, 2.25, 22.5, and 225 mg, respectively). A second, complementary statistical analysis determined the effect of 2-APB and gentamicin dosage on the Arrhythmia Severity Score. Here, we applied standard linear regression with the average score for an animal across 10-, 20-, and 30- μ g doses of U46619 as the dependent variable and dosage as the independent variable. Both linear and logistic regressions were conducted by use of Minitab 14 (Minitab Inc., State College, PA).

Results

Gene and Protein Expression. Figure 1 demonstrates positive detection of TXA₂R mRNA and protein in both rabbit atria and ventricle. The primer sequences have been used previously to recognize rabbit TXA₂R, and PCR product sequences were verified by restriction enzyme digests (Wacker et al., 2005). Positive expression of the mRNA and protein was found in all three hearts tested. TXA₂R protein expression was verified by two additional antibodies that recognized different regions of TXA₂R and still displayed a band of the same molecular weight after Western blot (data not shown). We also conducted single-cell RT-PCR on isolated,

Fig. 1. TXA₂R mRNA and protein are present in the heart. A, RT-PCR of RNA isolated from tissue of the atria and ventricle with use of primers designed for rabbit TXA₂R (expected size, 172 bp). There was no amplification in the no-template control (NTC) or the no RT (no reverse transcriptase, Taq polymerase only) samples. B, Western blot with use of a TXA₂R antibody and total protein isolated from rabbit atria and ventricle. These findings were verified in three hearts.

Fig. 2. Successful detection of TXA₂R mRNA from a single, isolated, primary rabbit ventricular cardiac myocyte. A, isolation of a single cardiac myocyte by use of a micropipette controlled by a micromanipulator for use in single-cell RT-PCR. B, gel electrophoresis image of PCR products for troponin T (TNT) and TXA₂R from RT-PCR conducted on an isolated cardiac myocyte. The first RT-PCR step was conducted by use of primers for both TNT and TXA₂R. A 1- μ l aliquot was then removed, and a second round of PCR was conducted in separate tubes with use of nested primers for TNT (expected size, 323 bp) and TXA₂R (expected size, 229 bp). A control sample was taken in which a similar volume of medium was removed to ensure that there was no mRNA contamination in the media. These findings were verified by selecting 15 cells from three different hearts.

ventricular cardiac myocytes (Fig. 2A). Cardiac myocytes were identified and selected by visual morphology (rod-shaped with striations) under bright-field and phase-contrast microscopy. The selected cells also tested positive for the cardiac myocyte marker, troponin T, which was included in the RT-PCR experiments as a positive control. As demonstrated in Fig. 2B, TXA₂R was detected in the isolated cardiac myocytes, but not in the media samples that were taken from the same area as the myocytes. Results were verified in three hearts with five myocytes collected from each heart.

Intracellular Calcium Measurements. Calcium imaging was conducted on isolated, ventricular cardiac myocytes. Figure 3A displays a representative response of a cardiac

myocyte to U46619. This myocyte displayed spontaneous calcium oscillations before treatment, and after U46619 there was an increase in frequency of oscillations, and the cell ultimately overloaded with calcium. In cells that did not have spontaneous calcium oscillations, U46619 induced similar intracellular calcium responses that were also sometimes preceded by the induction of calcium waves. A dose response to U46619 was conducted in a separate series of experiments that is shown in Fig. 3B. There was a larger average increase in intracellular calcium with increasing doses of U46619 ($p < 0.05$, comparing 0.5 μ M with 50 μ M U46619). The average time of a response to U46619 was 86 ± 24 s. The average response time to KCl was 17 ± 4 s, and the average F/F_0 response to KCl was 2.16 ± 0.21 .

To ensure that the responses were receptor-specific, myocytes were pretreated with the TXA₂R antagonist, SQ29548 (10 μ M). Myocytes were tested with the dose of U46619 that provided the largest increase in intracellular calcium (50 μ M). There was no response to U46619 after pretreatment with SQ29548 (Fig. 3B; $p < 0.05$). SQ29548 did not affect the excitability of the myocytes as the average F/F_0 to KCl was 2.08 ± 0.27 . For the dose-response and SQ29548 experiments, the average number of hearts used per treatment was 6.0 ± 2.0 , and the average number of cells tested per heart was 4.3 ± 0.7 .

Figure 3C summarizes data for the change in fluorescence to 5 μ M U46619 treatment in cells that were pretreated with either 10 μ M gentamicin or 10 μ M 2-APB. As observed, both of these treatments inhibited the intracellular increase in calcium induced by U46619 ($p < 0.05$). These treatments did not affect the excitability of the cell because the F/F_0 change to KCl was 2.17 ± 1.04 and 2.19 ± 0.86 for gentamicin and 2-APB, respectively. For this series of experiments, the av-

Fig. 3. The IP₃ pathway mediates U46619-induced increase in intracellular calcium in vitro. A, fluorescent measurements (in relative fluorescent units, R.F.U.) and fluorescent images (inset) of a cardiac myocyte that had spontaneous calcium oscillations. After the addition of 5 μ M U46619, there was an increase in calcium oscillations and ultimately calcium overload. For the fluorescent images, warmer colors (yellows/reds) indicate increased fluorescence (increase in intracellular calcium). B, changes in fluorescence mediated by 0.5, 5, 50, and 50 μ M U46619 plus pretreatment with the TXA₂R antagonist SQ29548 (10 μ M). Measurements are indicated as a change in fluorescence after drug treatment divided by the initial fluorescence (F/F_0). C, comparison of the fluorescent response to U46619 (5 μ M) with no pretreatment and pretreatment with 10 μ M gentamicin or 2-APB. For all experiments combined, the average number of hearts tested per treatment group was 5.8 ± 1.3 , and the average number of cells tested per heart was 4.3 ± 0.5 *, significant difference between 50- and 0.5- μ g treatments (B) and significant difference between no pretreatment and gentamicin and 2-APB (C). †, significant difference between 50 μ g of U46619 and 50 μ g of U46619 + 10 μ M SQ29548.

erage number of hearts used per treatment was 7.7 ± 2.2 , and the average number of cells tested per heart was 3.3 ± 0.6 .

In Vivo Arrhythmia Inhibition. After observing the inhibition of the calcium responses in cells, we determined in vivo whether pretreatment of animals with gentamicin or 2-APB could also inhibit U46619-induced arrhythmias. Similar to what we have previously documented (Wacker et al., 2006), only ventricular arrhythmias were observed after U46619 treatment (Fig. 4). U46619 induced ventricular premature beats, bigeminy, salvos, and ventricular tachycardia, but did not induce ventricular fibrillation in our studies. The percentage of animals that developed each type of ventricular arrhythmia is summarized in Table 1. There were no arrhythmias after treatment with the ethanol vehicle control ($n = 8$). Figure 5 illustrates the average number of arrhythmias for each dose of U46619 for a given pretreatment dose. The probability of having an arrhythmia (inset) declined with increasing doses of both gentamicin and 2-APB. The estimated logistic regression slope for gentamicin was -1.83 ± 0.66 ($p < 0.001$; Fig. 5A), and for 2-APB it was -0.55 ± 0.25 ($p < 0.05$; Fig. 5B). Figure 6 displays the average Arrhythmia Severity Score for each dose of U46619 for a given pretreatment dose. The inset displays the arrhythmia score averaged for all three U46619 doses for a given pretreatment dose. Both treatments resulted in a significant decrease in the Arrhythmia Severity Score. The linear regression equation for gentamicin treatment was: Average arrhythmia score = $0.807 - 0.251 \times \text{gentamicin dose}$ ($p < 0.01$; Fig. 5C). The linear regression equation for 2-APB was: Average arrhythmia score = $0.858 - 0.201 \times \text{2-APB dose}$ ($p < 0.05$; Fig. 5D).

There were no significant changes in HR or mean arterial blood pressure (MABP) after the 0.225-, 2.5-, or 22.5-mg doses of gentamicin. With the 225-mg dose of gentamicin, HR was transiently reduced from 208 ± 16 to 196 ± 20 beats/min and MABP was reduced from 67 ± 9 to 62 ± 6 mm Hg. There was no change in HR after injection of any of the doses of 2-APB. There was a transient increase in MABP after injections of 2-APB; 67 ± 11 to 79 ± 12 mm Hg, 71 ± 14 to 82 ± 15 mm Hg, and 70 ± 9 to 87 ± 10 mm Hg at the 0.05-, 0.5-, and 5-mg doses, respectively. In two animals pretreated with 2-APB, abnormal T waves were observed, and these animals were removed from the study.

Discussion

The first step in testing the hypothesis that TXA₂ could directly induce arrhythmias in our rabbit model was to verify the expression of cardiac TXA₂Rs. We found the presence of TXA₂R mRNA and protein in both the atria and ventricle. These results are consistent with results observed by other

TABLE 1

Percentage of animals with ventricular arrhythmias induced by U46619

Pretreatment	U46619 μg	n	None %	VPB %	Bigem. %	Salvos %	VT %
No pretreatment	10	28	71	29	7	4	4
	20	28	64	36	14	7	7
	30	28	32	68	36	25	18
2-APB, 0.05 mg	10	9	100	0	0	0	0
	20	9	67	33	22	11	11
	30	9	56	44	44	33	33
2-APB, 0.5 mg	10	9	67	33	0	0	0
	20	9	89	11	11	11	0
	30	9	89	11	11	11	0
2-APB, 5 mg	10	9	89	11	0	0	0
	20	9	89	11	0	0	0
	30	9	67	33	22	11	11
Gentamicin, 0.225 mg	10	4	100	0	0	0	0
	20	4	100	0	0	0	0
	30	4	50	50	25	25	0
Gentamicin 2.25 mg	10	6	100	0	0	0	0
	20	6	100	0	0	0	0
	30	6	100	0	0	0	0
Gentamicin, 22.5 mg	10	4	100	0	0	0	0
	20	4	100	0	0	0	0
	30	4	100	0	0	0	0
Gentamicin, 225 mg	10	6	100	0	0	0	0
	20	6	100	0	0	0	0
	30	6	100	0	0	0	0

VPB, ventricular premature beat; Bigem., bigeminy; VT, ventricular tachycardia.

groups that have found binding sites for TXA₂R in the hearts of other species (Lasserre et al., 1992; Bowling et al., 1994). It is interesting to note that, in this study and our previous study (Wacker et al., 2006), we have observed only ventricular arrhythmias (ventricular premature beats, bigeminy, salvos, and ventricular tachycardia). This is surprising given that TXA₂Rs are in the atria, rabbit atria has a higher concentration of IP₃Rs than the ventricle (Domeier et al., 2008), and TXA₂ plays a role in inflammatory-mediated tachycardia in the mouse (Takayama et al., 2005). Our laboratory has documented that left atrial injections of U46619 activate cardiac vagal reflexes and induce bradycardia (Wacker et al., 2002); therefore, it is possible that the actions of these vagal reflexes may inhibit atrial arrhythmias in our model. Alternatively, the surgical placement of the left atrial catheter or the delivery of the drug into the left atrium may have also influenced the origin of the observed arrhythmias. Nonetheless, the differences in atrial and ventricular responses to U46619 should elicit further exploration.

Because the heart contains other cell types (such as smooth muscle cells and fibroblasts), we also tested isolated rabbit cardiac myocytes in single-cell RT-PCR experiments. We observed expression of TXA₂R mRNA from the cardiac myocytes, but not in the media samples, indicating that TXA₂

Fig. 4. The TXA₂R agonist, U46619, induces ventricular arrhythmias in vivo. Sample ECG trace of an anesthetized rabbit with ventricular arrhythmias 90 s after left atrial injection of 30 μg of U46619. Arrows indicate widened and abnormally shaped QRS complexes associated with ventricular premature beats and bigeminy.

Fig. 5. Gentamicin and 2-APB significantly reduce the probability of U46619-induced arrhythmias in vivo. Animals were given 10, 20, and 30 μ g of U46619, and the number of ventricular arrhythmias were recorded. The number of arrhythmias were decreased with increasing doses of gentamicin (A, 0.225, 2.25, 22.5, and 225 mg; $n = 4, 6, 4$, and 6, respectively) as well as 2-APB (B, 0.05, 0.5, and 5 mg; $n = 9, 9$, and 9, respectively) compared with animals that received no pretreatment ($n = 28$). Insets in A and B represent the probability of an arrhythmia occurring as a function of dosage of gentamicin or 2-APB as estimated by logistic regression (A, $p < 0.001$; B, $p < 0.05$).

mRNA was located in the cardiac myocytes, and did not result from possible contamination of mRNA in the media from other cell types. To our knowledge, this is the first study to specifically detect TXA₂R mRNA/protein in cardiac muscle tissue and single, isolated cardiac myocytes.

Next, we wanted to determine whether direct application of U46619 to isolated, adult rabbit cardiac myocytes induces changes in intracellular calcium. This is in agreement with other studies that have also found that TXA₂ mimetics can induce increases in intracellular calcium in neonatal rat cardiac myocytes (Hoffmann et al., 1993; Dogan et al., 1997). We found that the U46619-induced increases in cytosolic calcium were inhibited by the specific TXA₂R antagonist SQ29548, which supports our previous work (Wacker et al., 2006), demonstrating that SQ29548 also inhibited the U46619-induced arrhythmias. Thus, the U46619 actions in vivo and in vitro were TXA₂R-specific. In addition, U46619 increased the frequency of spontaneous calcium oscillations or induced calcium waves in myocytes that previously had none. This is in agreement with another study that demonstrated that U46619 increased the beating rate and induced tachyarrhythmias and chaotic activity in spontaneously beating neonatal rat cardiac myocytes (Li et al., 1997). These data provide support for the hypothesis that TXA₂ can induce arrhythmias in vivo via a direct receptor-mediated action on cardiac myocytes.

To further test this hypothesis and link in vitro calcium responses to in vivo ventricular arrhythmias, we wanted to determine whether blocking the IP₃ pathway could inhibit the U46619-induced responses. We focused on IP₃ as a mechanism for two reasons. First, TXA₂R activates the Gq protein

and can stimulate phospholipase C and subsequent IP₃ formation in numerous cell types, including cardiac myocytes (Nakamura et al., 1996). Second, it is becoming apparent that IP₃Rs play a significantly more prominent role in cardiac muscle physiology than previously thought. IP₃Rs are hypothesized to be involved in compartmentalized signaling, alter the kinetics of calcium release and reuptake, and mediate specific cellular responses to stresses (Kockskämper et al., 2008). Of specific importance to this study, IP₃Rs play a role in inducing arrhythmias during ischemia-reperfusion (Du et al., 1995; Woodcock et al., 2000) and arrhythmias mediated by endocrine agents (Jacobsen et al., 1996; Mackenzie et al., 2002; Zima and Blatter, 2004; Chu et al., 2006; Proven et al., 2006). Therefore, it is important to investigate the role that IP₃ plays in TXA₂-mediated arrhythmias.

In this study, we used pharmacological inhibitors that have been widely used to inhibit the formation of IP₃ (gentamicin) or block IP₃Rs (2-APB). Gentamicin is an aminoglycoside antibiotic that inhibits IP₃ release by binding to and sequestering its precursor, phosphatidylinositol 4,5-bisphosphate. Gentamicin has specifically been shown to inhibit IP₃ generation both in vitro and ex vivo by other known stimulators of the Gq/phospholipase C pathway—thrombin, norepinephrine, angiotensin II, phenylephrine, and bradykinin (Ramsammy et al., 1988; Jacobsen et al., 1996). Furthermore, gentamicin has been used to inhibit arrhythmias induced by ischemia-reperfusion in vivo (Du et al., 1995) and by thrombin and norepinephrine in isolated, perfused hearts (Jacobsen et al., 1996). Similar to these studies, in our experiments we found that gentamicin inhibited U46619-in-

Fig. 6. Gentamicin and 2-APB significantly reduce the Arrhythmia Severity Score. Arrhythmias that occurred with each dose of U46619 were also documented by a scoring system. Increasing doses of gentamicin and 2-APB decreased the Arrhythmia Severity Score at each dose of U46619. Insets in A and B represent the average arrhythmia score for each animal as a function of dosage of gentamicin or 2-APB as calculated by linear regression analysis (A, $p < 0.01$; B, $p < 0.05$).

duced calcium responses in vitro and also inhibited the U46619-induced arrhythmias in vivo.

To further validate the role of IP₃ in the U46619 responses, 2-APB was used in this study to block IP₃Rs. 2-APB is a membrane-permeable inhibitor of IP₃Rs that has been used to block IP₃-induced responses by endothelin, insulin-like growth factor, adenophostin, and IP₃ esters themselves in cardiac myocytes (Mackenzie et al., 2002; Ibarra et al., 2004; Zima and Blatter, 2004; Chu et al., 2006; Proven et al., 2006). In addition, 2-APB was specifically shown to inhibit the calcium release induced by another TXA₂ mimetic, STA₂, in platelet preparations (Maruyama et al., 1997). These authors demonstrated that 2-APB did not affect the amount of IP₃ released by STA₂ or affect other signal transduction pathways induced by other agonists. Here, we found that 2-APB also inhibited the TXA₂R-mediated increases in calcium in cardiac myocytes as well as arrhythmias in vivo.

The results from the gentamicin and 2-APB experiments complement each other because they differentially inhibit the IP₃ pathway. We recognize that pharmacological agents can have nonspecific actions. Nevertheless, the utilization of a pharmacological agent that inhibits the formation of IP₃ and the other that inhibits the receptor activated by IP₃ to differentially inhibit the IP₃ pathway provides strong evidence against nonspecific actions. Furthermore, the fact that gentamicin and 2-APB inhibited the calcium response in vitro and also inhibited the arrhythmias in vivo support the hypothesis that direct calcium-inducing actions of TXA₂ on cardiac myocytes play a role in the TXA₂-mediated arrhythmias in vivo. Therefore, this study is the first to link in

vitro signal transduction mechanism of TXA₂-induced calcium changes in cardiac myocytes to in vivo occurrence of arrhythmias.

Significance. TXA₂ is released during myocardial ischemia and the level is proportional to the number of arrhythmias that occur (Coker et al., 1981; Hirsh et al., 1981). In addition, TXA₂ is released by inflammatory cytokines (Takayama et al., 2005; Kaehler et al., 2008). It is possible that endogenous TXA₂ that is released during tissue damage or inflammation induces *direct* effects on the heart in addition to the *indirect* actions caused by platelet aggregation and vasoconstriction. This hypothesis is supported by a recent in vivo study with use of TXA₂R knockout mice (Takayama et al., 2005). This study demonstrated that inflammation-associated tachycardia was in part mediated by TXA₂ release and subsequent direct stimulation of cardiac TXA₂Rs.

In this study, we found that acute application of U46619 induced elevated calcium in vitro and arrhythmias in vivo. Therefore, elevated levels of TXA₂ may induce arrhythmias via a mechanism that has not been well researched. It may also be important to consider how chronically elevated levels of TXA₂ affect cardiac muscle. For example, it has been shown that the circulating levels of TXA₂ are elevated in the obese (Davi et al., 2002). Because we have shown that TXA₂ can induce transient calcium changes acutely, it is possible that chronically altered calcium levels may lead to cellular changes such as cardiac hypertrophy, remodeling, and apoptosis. It has been found that rat cardiac myocytes treated for 24 h with 100 nM TXA₂R agonist, IBOP ([1S[1 α ,2 α (Z),

3 β (1E,3S*),4 α)]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo-[2.2.1]hept-2-yl]5-heptanoic acid), had a much larger incidence of apoptosis than vehicle (Shizukuda and Buttrick, 2002). Therefore, additional investigation into the direct effects of TXA₂ is warranted.

Aspirin treatment has been used traditionally in the prevention of cardiovascular disease by reducing the levels of TXA₂; however, there are still problems with this treatment regimen. For example, there is an increased risk of gastrointestinal bleeding with taking nonsteroidal anti-inflammatory drugs like aspirin (Bhatt et al., 2008). In addition, it has been estimated that approximately 28% of the population is aspirin-resistant (Krasopoulos et al., 2008) with TXA₂ synthesis and or TXA₂R activation being detected despite aspirin treatment (Eikelboom et al., 2002). Therefore, the exploration of alternative counter measures against the harmful actions of TXA₂R stimulation may be beneficial. Because the direct arrhythmic actions of TXA₂ in our study were sensitive to drugs inhibiting the IP₃ pathway, it may be useful to further investigate this pathway as an alternative target to prevent TXA₂-mediated cardiac dysfunction.

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