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Evidence that 2-aminoethoxydiphenyl borate provokes fibrillation in perfused rat hearts via voltage-independent calcium channels

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ABSTRACT

We tested whether 2-aminoethoxydiphenyl borate (2-APB) induces arrhythmia in perfused rat hearts and whether this arrhythmia might result from the activation of voltage-independent calcium channels. Rat hearts were Langendorff perfused and beat under sinus rhythm. An isovolumic balloon inserted into the left ventricle was used to record mechanical function while bipolar electrograms were recorded from electrodes sutured to the base and the apex of hearts. Western and immunofluorescence analyses were performed on rat left ventricular protein extracts and left ventricular frozen sections, respectively. Rat ventricular myocytes express Orai 1 and Orai 3, and ventricle also contains the Orai regulator Stim1. Rat hearts ($n=5$) perfused with Krebs–Henseleit (KH) alone maintained sinus rhythm at 4.8 ± 0.1 Hz and stable mechanical function. By contrast, perfusing hearts ($n=5$) with (KH + 22 μM 2-APB) provoked a period of tachycardic ectopy at rates of up to 10.8 ± 0.2 Hz. As perfusion with (KH + 22 μM 2-APB) continued, the rate of spontaneous ventricular depolarization increased to 21.8 ± 1.2 Hz and became disorganized. Heart mechanical function collapsed as developed pressure decreased from 87 ± 8.8 to 3.5 ± 1.9 mm Hg. Flow rate did not change between normal (16.6 ± 0.9 ml/min) and fibrillating (17.4 ± 0.8 ml/min) hearts. The addition of 20 μM 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy]ethyl-1H-imidazole (SKF-96365) to (KH + 22 μM 2-APB) perfusates ($n=4$) restored sinus rhythm and heart mechanical output. These data indicate that activating myocardial voltage-independent calcium channels, possibly the Orais, may be a novel cause of ventricular arrhythmia.

1. Introduction

Clinical data acquired in the settings of idiopathic ventricular fibrillation and atrial fibrillation support the concept that cardiac arrhythmia can arise in small regions of the heart (Haïssaguerre et al., 2002; Jais et al., 1997). These observations echo an earlier hypothesis which stated that if cardiac cells which are not normally automatic were to somehow depolarize spontaneously, persistently, and at high rates, they might act as focal sources for arrhythmia (Rothberger, 1922; Scherf et al., 1950). Nonetheless, abnormal impulse propagation remains the principal explanation for arrhythmia, including focal clinical arrhythmias (Panfilov and Pertsov, 2001), despite evidence that non-reentrant activity can sustain ventricular fibrillation (Robichaux et al., 2010).

Impediments to the development of a robust focal source hypothesis for arrhythmia include the fact that only few molecular mechanisms readily disrupt cardiac electrical stability, and these usually act upon voltage-dependent ion channels which alters the action potential and/or impulse propagation (Chen et al., 2003; Panfilov and Pertsov, 2001). We have reported that exposing superfused rat left atria and left ventricular papillary muscles to 10 to 20 μM 2-aminoethoxydiphenyl borate (2-APB) causes electromechanical activity in the absence of an external stimulus which initiates from normal resting potentials, produces normal action potentials, and occurs at rates of 10 Hz in calcium-loaded left atria superfused at 37 °C (Wolkowicz et al., 2007a, 2007b, 2011). The EC_{50} values for this automaticity are similar to the concentrations of 2-APB that activate the voltage-independent Orai channels (Peinelt et al., 2008). Our initial assessments of whether these channels might be sources for this automaticity show that atrial myocytes express the Orais and two voltage-independent calcium channels inhibitors suppress this automaticity (Wolkowicz et al., 2011). In addition, Huo and co-workers report that 2-APB induces calcium entry into quiescent myocytes (Huo et al., 2010), just as it does in non-excitable cells via Orai 1 and Orai 3 (Braun et al., 2003; Goto et al., 2010; Peinelt et al., 2008;

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Usmani et al., 2010). Thus calcium entry through voltage-independent channels and signaling downstream of these channels may be unexpected causes for high-frequency automaticity in superfused heart muscle.

It is the purpose of this paper, then, to investigate whether 2-APB initiates arrhythmia in the intact heart and to pin-point a potential mechanism for this arrhythmia. We report that adding 2-APB to the buffer of Langendorff-perfused rat hearts provokes high-frequency electrical activity and heart mechanical collapse. The voltage-independent calcium channel inhibitor 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl) propoxy] ethyl-1H-imidazole (SKF-96365) reverses this novel type of fibrillation. These data suggest myocardial voltage-independent calcium channels, possibly the Orais, as novel sources for focal types of ventricular arrhythmia.

2. Materials and methods

2.1. Materials

2-APB and SKF-96365 were from Tocris-Cookson (Ellisville, MO, USA). KH reagents were from Fisher Scientific (Pittsburgh, PA, USA). Chemiluminescence reagent was from GE Healthsciences (Piscataway, NJ, USA). Other reagents were at least laboratory grade.

2.2. Analyses of ventricular Orai and Stim1 expression

2.2.1. Western analyses

Triton X-100-soluble left ventricular protein extracts ($n=3$) were Western analyzed for Orai 1 and Orai 3 using the ProSci 4281 and 4117 antibodies, respectively (ProSci, Poway, CA, USA). Left atrial and ventricular extracts ($n=3$ per) were Western analyzed for Stim1 using the Cell Signaling Technology 4916 antibody (Cell Signaling Technology, Danvers, MA, USA) (Wolkowicz et al., 2011). *Immunofluorescence analyses*: Left ventricle frozen sections were fixed and analyzed for Orai 1, Orai 3, and alpha-actinin immunoreactive material. Staining in the absence of primary antibody or with rabbit anti-chloramphenicol acetyltransferase served as negative controls (Wolkowicz et al., 2011).

2.3. Langendorff perfusion

These investigations conform to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague–Dawley rats (325–400 g) were used throughout, and they were anesthetized with inhaled isoflurane prior to heart extirpation.

Isolated rat hearts were perfused in the Langendorff mode with oxygenated Krebs–Henseleit (KH) maintained at 37 °C (Nishizawa et al., 2005; Urthaler et al., 1997). All hearts were in normal sinus rhythm and were stabilized with a 30-min perfusion prior to the start of any protocol. An isovolumic balloon was inserted into the left ventricle and used to record mechanical function with DasyLab (8.0) software (National Instruments, Austin TX, USA). Electrodes were sutured to the heart base and apex, and connected to an AD Instruments BioAmp amplifier and PowerLab 2/20 data system to acquire bipolar electrograms. We used AD Instruments Chart v4.2.4 software (Colorado Springs, CO, USA) to analyze these data.

2.4. Perfusion protocols

(1) Five control hearts were perfused with KH for up to 40 min. (2) Five hearts were perfused for up to 20 min with KH containing 5 μM 2-APB. This concentration was chosen as it (i) activates Orai 1-linked calcium entry (DeHaven et al., 2008) and (ii) blocks voltage-independent calcium channels like the transient receptor potential proteins (TRPs) or the inositol 1,4,5-trisphosphate receptor (Birnbaumer, 2009). (3) Five hearts were perfused for up to 15 min with KH containing 22 μM 2-APB. This concentration near maximally activates papillary muscle automaticity and non-excitable cell calcium transport through Orai 1 and 3 (DeHaven et al., 2008; Peinelt et al., 2008; Wolkowicz et al., 2011). (4) Four hearts were perfused with KH containing 22 μM 2-APB. Once fibrillation occurred, 20 μM SKF-96365 was added to the perfusate and perfusion was continued up to 15 min longer. This concentration of SKF-96365 (i) suppresses 2-APB-induced automaticity without affecting muscle function and (ii) blocks voltage-independent calcium entry (Birnbaumer, 2009; Ju et al., 2007; Touchberry et al., 2011; Usmani et al., 2010; Wolkowicz et al., 2011). Bipolar electrograms and mechanical function were recorded from all hearts throughout perfusion. 2-APB was added directly to the perfusate from a 250 mM DMSO stock; SKF-96365 was added to the perfusate from a 100 mM aqueous stock.

2.5. Statistics

Data are the mean ± S.E.M. Fisher's least protected significance difference test compared two means. Two-way repeated measure analysis of variance compared means between different groups. Significance was assigned at $P<0.05$.

3. Results

3.1. Rat ventricular expression of Orai 1, Orai 3, and Stim1

To begin to test the hypothesis that the activation of voltage-independent calcium channels disrupts left ventricular electrical stability, we assessed whether left ventricular myocytes express Orai 1 and Orai 3. Western analyses show that rat left ventricular protein extracts contain both Orais (Fig. 1A). Immunofluorescence analyses of rat left ventricle frozen sections demonstrate that cells which express Orai 1 and Orai 3 (Fig. 1B and C: Upper panels, respectively) also contain muscle alpha-actinin (Fig. 1B and C: Middle panels). Thus rat left ventricular myocytes contain Orai 1 and 3 immunoreactive material. These Orais distribute both diffusely and discretely in myocytes (Fig. 1B and C: Lower panels, arrows); the cause for these two patterns requires further analyses. A non-specific rabbit primary antibody produces only weak fluorescence in cells that contain alpha-actinin (Fig. 1D). Rat left ventricular and atrial extracts also contain Stim1 (Fig. 1A: Stim1), the cell regulator of Orai 1 and Orai 3 (Shuttleworth et al., 2004; Stiber et al., 2008).

3.2. 2-APB induces a type of ventricular fibrillation

We next tested whether 2-APB, a modulator of voltage-independent calcium channels, affects the electromechanical stability of intact, perfused rat hearts.

Hearts perfused with KH alone maintain a sinus rate of 287 ± 7 contractions/min, possess stable mechanical function, and produce typical bipolar electrograms (Table 1: sinus rhythm) (Beinfield and Lehr, 1968; Nishizawa et al., 2005). Hearts then were perfused with (KH + 5 μM 2-APB), a concentration of 2-APB that activates Orai 1 and blocks several other voltage-independent calcium channels (Birnbaumer, 2009; Boyden et al., 2004; DeHaven et al., 2008; Mackenzie et al., 2004). These preparations largely maintain normal function (Table 1: Low 2-APB) but produce 4.8 ± 2.1 ectopic depolarizations/min. Each ectopic depolarization provokes a ventricular contraction (*cf.* Fig. 2: lower panel * with upper panel ‡). These spontaneous depolarizations have varying electrical morphologies (Fig. 2A: lower panel *), wider than normal QRS complexes (28.2 ± 1.4 vs. 17.4 ± 0.65 ms, respectively), and long RR intervals (282 ± 8.8 vs. 215 ± 3.4 ms, [ectopic-normal] RR vs. normal RR, respectively).