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Ventricular rate adaptation: A novel, rapid, cellular-based in-vitro assay to identify proarrhythmic and torsadogenic compounds

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ABSTRACT

Introduction: Delayed cardiac repolarization is an established risk factor for proarrhythmia and Torsades-de-Pointes (TdeP) that is typically measured in vitro during slow, regular stimulation. We have developed an alternative, novel, and rapid cellular-based approach for predicting drug-induced proarrhythmia that detects changes in electrical refractoriness based on mechanical responses (measured optically) during increasingly rapid trains of stimulation interspersed with pauses (mimicking the clinically observed short–long–short (SLS) stimulation sequence associated with the TdeP initiation).

Methods: Acutely isolated rabbit ventricular myocytes were superfused and electrically stimulated using an accelerating pacing protocol (APP) consisting of 12 consecutive pacing segments (10 beats per segment) with incrementally faster cycle lengths; trains were separated by pauses to identify loss of stimulus capture as well as to mimic clinically observed SLS sequences. Drug effects were evaluated based on a myocyte's ability to contract during progressively faster pacing segments (rate-adaptation); the earliest rate during which the myocyte fails to respond (longest cycle length with incomplete capture (CLIC)) was used to quantify electrophysiologic effects.

Results: Torsadogenic drugs known to delay repolarization during slow stimulation prolonged CLIC and dramatically limited the ability to respond to progressively rapid stimulation. The recognized proarrhythmic compounds E-4031, cisapride, grepafloxacin, and haloperidol rapidly prolonged CLIC at and above therapeutic concentrations in a concentration-dependent manner, while negative controls (captopril, indomethacin, and loratidine) do not affect rate-adaptation.

Discussion: Ventricular rate adaptation represents a novel approach for rapidly detecting drugs with torsadogenic risk using rapid rhythms that are typically not employed when evaluating proarrhythmic risk. This method is well suited for detecting and avoiding potential cardiac liabilities early in drug discovery ("frontloading") prior to final selection of candidate drugs.

1. Introduction

Delayed cardiac repolarization is routinely employed as a surrogate marker for Torsades-de-Pointes (TdeP), an arrhythmia that may elude researchers during clinical trials due to its low incidence, especially with non-cardiac drugs (Yao et al., 2008). Typical *in-vitro* assays used to evaluate repolarization delays involve measuring changes in the action potential duration from tissues (such as cardiac Purkinje fibers or papillary muscles) using technically difficult electrophysiologic recording techniques (Gintant, Limberis, McDermott, Wegner, & Cox, 2001; Kii et al., 2005). In addition, delayed repolarization is often evaluated during slow, regular stimulation that enhances prolongation of the cardiac action potential (reverse use-dependent block, Hondeghem & Snyders, 1990) that does not reflect the short–long–short rhythm associated with initiation of Torsades (Kirchhof, Franz, Bardai, & Wilde, 2009; Locati, Maison-Blanche, Dejode, Cauchemez, & Coumel, 1995; Roden, 2004).

This study describes a novel, alternative, cardiomyocyte-based, *in-vitro* assay that evaluates the electrophysiologic effects of drugs based on mechanical contractile responses (twitches) measured optically in response to a rapidly accelerating pacing protocol (APP). The APP consists of a stimulus train with 12 distinct sets of beats, where each stimulus set is progressively faster than the previous, and where each accelerated set is separated by a pause. Pauses interspersed between periods of rapid pacing create the characteristic short–long–short (SLS) rhythm associated with the initiation of drug-induced TdeP. In contrast to repolarization studies emphasizing slow stimulation and reverse use-dependent block, the APP identifies compounds that affect repolarization during rapid stimulation by detecting the failure

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of myocytes to respond to stimuli at progressively faster stimulation rates (rate adaptation). Using this approach, three negative controls and six positive controls (associated with QT prolongation and TdeP at therapeutic or supratherapeutic concentrations) were evaluated (using the APP paradigm), in an effort to measure, rank order, and validate the ability to identify potential "QT offenders".

2. Methods

2.1. Regulatory guidelines

All animal procedures in this study were approved by the Abbott Institutional Animal Care and Use Committee (IACUC) according to current, governing IACUC protocols, as well as conformed to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). Abbott Laboratories is accredited by the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Preparation of isolated rabbit ventricular myocytes

Myocytes were isolated using techniques similar to those described previously (Su et al., 2010). Briefly, adult, female, New Zealand White rabbits (2–3.5 kg) were anesthetized with Pentobarbital Sodium (65 mg/kg) and Heparin Sodium (~1000 units). Following a medial thoracotomy and cardiectomy, the heart was transferred to an ice-cold dish of modified Krebs–Ringer bicarbonate buffer solution, containing (mM): NaCl 166.7, KCL 5, $NaHCO_3$ 19, $MgSO_4$ 1.2, NaH_2PO_4 1.2, Glucose 15, and Taurine 20 (pH 7.4, gassed with 95/5% O_2/CO_2). The aorta was then mounted on a Langerdorff perfusion apparatus and continuously perfused (60 mm Hg retrograde perfusion pressure, 37 °C) using the modified Krebs–Ringer bicarbonate buffer solution. During the initial rinse period, the left ventricle was punctured to allow residual blood to exit the chamber and to aid perfusion. Immediately following, a digestion solution containing the modified Krebs–Ringer bicarbonate buffer solution, 0.2 mg/ml Protease (Type XIV, Sigma Aldrich, Saint Louis, MO), 1.5 mg/ml Collagenase (Type II, Worthington Biochemical, Freehold, NJ), and 50 μM $CaCl_2$ was perfused through the tissue. After sufficient digestion, the heart was rinsed for an additional 5 min with a buffer solution containing the modified Krebs–Ringer bicarbonate buffer solution and 50 μM $CaCl_2$. Following the rinse, the left ventricle was separated from the heart and minced in the Krebs–Ringer, 50 μM $CaCl_2$ solution. The minced tissue solution was then transferred to a vial and agitated with 95/5% O_2/CO_2 for 5 min to further disperse the cells. After the dispersion, an aliquot of the cell solution was added to an equal portion of solution containing: Calcium-free HEPES-buffered Tyrode's solution (NaCl 140 mM, KCl 5 mM, $MgCl_2$ 1 mM, Glucose 5 mM, HEPES 20 mM (Sigma Aldrich, Saint Louis, MO) (pH = 7.4)), 2% Bovine Serum Albumin (Sigma Aldrich, Saint Louis, MO), and 0.2 mM $CaCl_2$. The solution, agitated and left to settle, was followed by a subsequent addition of the same BSA/buffer solution 5 min later. After an additional 5 min of settling and acclimation, the supernatant solution was siphoned off from the remaining cell pellet, cells rinsed and placed in a 0.2 mM $CaCl_2$, HEPES-buffered Tyrode's solution.

Myocyte cell suspensions (calcium tolerant and quiescent) were then plated onto laminin (Sigma Aldrich, Saint Louis, MO) coated glass coverslips (8 mm Round, BellCo Glass, Vineland, NJ). Cells were left undisturbed at room temperature for a minimum of 1 h to allow for attachment before beginning experiments. All experiments were conducted within 10 h of myocyte isolation and 8 h of plating, while each compound was run on multiple days across multiple isolation preparations. All buffer chemicals, unless otherwise noted, were obtained from Fisher Scientific (Fairlawn, NJ).

For experiments, coverslips with plated myocytes were placed in a heated bath (35 °C) atop an inverted microscope and superfused with a 2 mM $CaCl_2$, HEPES-buffered Tyrode's solution (~1 ml/min). During acclimation, myocytes were field stimulated (0.5 Hz) with platinum electrodes using bipolar pulses (3 ms duration, twice threshold, not exceeding 10 V). Single myocytes were visualized (at 40×) and contractions monitored as changes in sarcomere length using the IonOptix SarcLen system (IonOptix, Milton, MA), see Fig. 1A and B.

2.3. Accelerated pacing protocol design and analysis

The electrophysiologic effects of drugs were evaluated using the APP (Fig. 1C and D). This protocol incorporates incrementally rapid stimulus trains to interrogate drug-induced changes in rate-adaptation, as well as to provide for the SLS rhythm associated with drug induced TdeP. The APP protocol consists of a "warming" phase followed by a "test" phase consisting of 12 pacing segments. During the warming phase, a gradual increase in stimulation rate is applied (from a base rate of 2 s basic cycle length) with the BCL increasing in the following manner (BCL in milliseconds): 1000(×2), 800(×2), 600 (×2), 400(×2), 600. This "warming" period is followed by a "test" phase comprised of 12 stimulus trains (each with 10 pulses) decrementing in cycle length from (in milliseconds): 350, 300, 290, 280, 270 …. 220, 210, 200. Each segment of 10 pulses was followed by a single "recovery pause" 200 ms longer than the prior stimulus interval, providing for the SLS rhythm sequences. After the final set of 10 pulses at 200 ms and recovery pause of 400 ms, the protocol ends

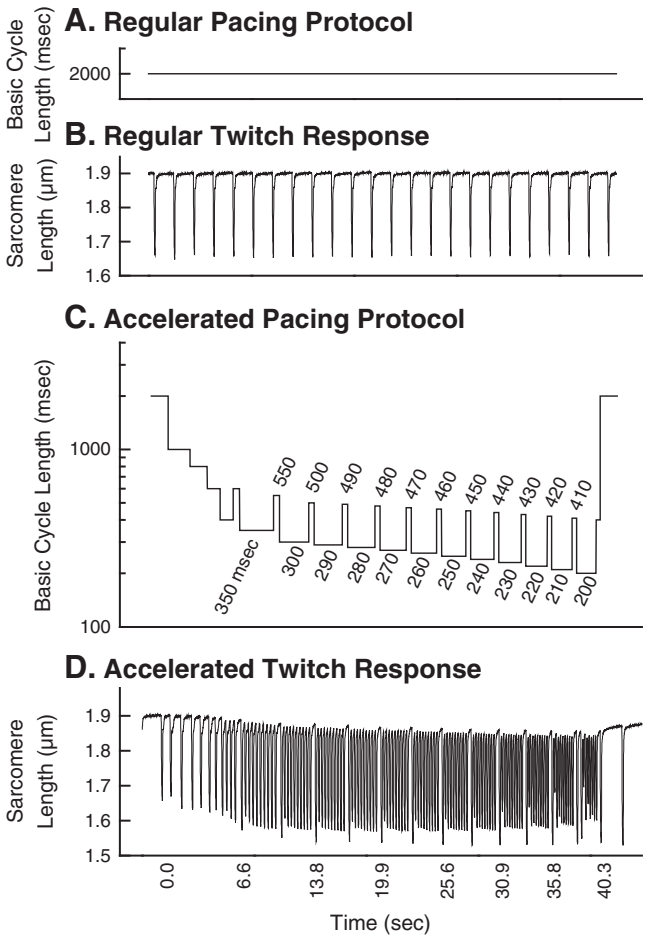

Fig. 1. An illustration of pacing protocols and myocyte twitch responses. Regular pacing protocol (2000 ms basic cycle length, Panel A) and representative twitches (Panel B). Panel C details the accelerated pacing protocol (APP). The basic cycle length of each of the 12 pacing segments (10 beats per segment) is listed below the step diagram, while the pause duration between each of the 12 pacing segments is listed above the step diagram. Panel D illustrates representative contractile responses recorded.