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Original article

Validation of a voltage-sensitive dye (di-4-ANEPPS)-based method for assessing drug-induced delayed repolarisation in Beagle dog left ventricular midmyocardial myocytes

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ABSTRACT

Introduction: Evaluation of drug candidates in in-vitro assays of action potential duration (APD) is one component of preclinical safety assessment. Current assays are limited by technically-demanding, time-consuming electrophysiological methods. This study aimed to assess whether a voltage-sensitive dye-based assay could be used instead. **Methods:** Optical APs were recorded using di-4-ANEPPS in electrically field stimulated Beagle left ventricular midmyocardial myocytes (LVMMs). Pharmacological properties of di-4-ANEPPS on the main cardiac ion channels that shape the ventricular AP were investigated using IonWorks™ and conventional electrophysiology. Effects of 9 reference drugs (dofetilide, E4031, D-sotalol, ATXII, cisapride, terfenadine, alfuzosin, diltiazem and pinacidil) with known APD-modulating effects were assessed on optically measured APD at 1 Hz. **Results:** Under optimum conditions, 0.1 μM di-4-ANEPPS could be used to monitor APs paced at 1 Hz during nine, 5 s exposures without altering APD. di-4-ANEPPS had no effect on either hI_{ERG}, hI_{Na}, hI_{Ks} and hI_{to} currents in transfected CHO cells (up to 10 μM) or $I_{Ca,L}$ current in LVMMs (at 16 μM). di-4-ANEPPS had no effect on APs recorded with microelectrodes at 1 or 0.5 Hz over a period of 30 min di-4-ANEPPS displayed the sensitivity to record changes in optically measured APD in response to altered pacing frequencies and sequential vehicle additions did not affect the optically measured APD. APD data obtained with 9 reference drugs were as expected except (i) D-sotalol-induced increases in duration were smaller than those caused by other I_{Kr} blockers and (ii) increases in APD were not detected using low concentrations of terfenadine. **Discussion:** Early in drug discovery, the di-4-ANEPPS-based method can reliably be used to assess drug effects on APD as part of a cardiac risk assessment strategy.

1. Introduction

Drugs that can cause Torsades de Pointes (TdP) polymorphic ventricular tachycardia have been a major focus of regulatory authorities and the pharmaceutical industry since the mid-1990s (Fenichel et al., 2004; Bass et al., 2007). This concern has led to the adoption of ICH S7B and ICH E14 guidance documents (Anon, 2005a,b), both of which rely on QT interval prolongation as a biomarker to predict the risk that a drug may produce TdP. Therefore, evaluation of the effects of new drugs on cardiac action potential duration (APD) is regarded as an essential activity in the pharmaceutical industry (Lindgren et al., 2008).

Current methods involve action potential (AP) recordings from canine Purkinje fibres (Gintant et al., 2001; Abi-Gerges et al., 2004; Champeroux et al., 2005) or papillary muscles (Biliczki et al., 2002; Lu et al., 2007) and isolated hearts from rabbits (Hondeghem and Hoffmann, 2003; Hondeghem et al., 2003; Lawrence et al., 2006; Milberg et al., 2004) and guinea-pigs (Hamlin et al., 2004; Kågström et al., 2007). These models of APD involve a high degree of technical ability as they use conventional electrophysiological techniques, have very low throughput and involve significant animal usage. Additionally, guinea pig ventricular myocytes (Davie et al., 2004; Terrar et al., 2007) and beagle dog left ventricular midmyocardial myocytes (LVMMs; Abi-Gerges et al., 2008) have been shown to provide suitable preclinical models to assess the effects of new drugs on APD. Compared to the PFs, papillary muscles and isolated hearts, the throughput of the single myocyte assays is increased and animal demand is reduced. However, the technical difficulty of making microelectrode recordings in these cells limits the provision of myocyte APD data and therefore the number of compounds that can be tested. Hence, pharmaceutical companies have sought to molecularise the AP by implementing medium throughput electrophysiological techniques such as IonWorks™ which allow for rapid detection of effects on heterologously-expressed ion channels (Schroeder et al., 2003;

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Bridgland-Taylor et al., 2006; Harmer et al., 2008). However, no matter how comprehensive the panel of cardiac ion channels becomes, there may always be the ultimate need to test compounds in an integrated system (e.g. a myocyte). Therefore, a method that uses potentiometric dyes to record APs in ventricular myocytes could be a way to generate such data more easily. The non-invasive nature of dyes requires less technical skill and the use of single myocytes means that fewer animals would be required, providing a higher daily experimental APD assay throughput so that potentially APD-modulating drugs could be discarded at a relatively early stage of drug discovery. Recently, although Hardy, Lawrence, Standen, and Rodrigo (2006) has demonstrated the feasibility of using a fast voltage-sensitive dye (di-8-ANEPPS) for recording drug-induced changes in AP morphology of guinea pig ventricular myocytes, prolongation of basal APD suggests that this dye modulates cardiac ion channels and may influence, therefore, the effects of drugs on APD. Thus, a structurally-related dye with no effects on the cardiac action potential is essential.

Since (i) the distribution of ion channel proteins and ionic currents that determine the AP shape and duration are similar in canine and human ventricles (Szabo et al., 2005), (ii) beagle dog is a commonly used preclinical species to test the effects of new drugs on cardiac repolarisation (Gralinski, 2003) and (iii) repolarisation of the midmyocardial ventricular myocytes usually determines the end of the T-wave (Antzelevitch, 2007) so data from these myocytes may better relate to QT measurements, we wanted to determine in this investigation if di-4-ANEPPS (a structurally-related dye to di-8-ANEPPS) can be used to record drug-induced changes in APD of beagle dog LVMMs. In particular, we set out to: (i) test di-4-ANEPPS against the main ion channels that shape the ventricular AP, (ii) determine a trade-off between excitation light intensity and dye concentration and (iii) assess the effects of 9 reference drugs on APD.

2. Methods

2.1. Isolations of LVMMs

Alderley Park female beagle dogs were used (weight 10.38–15.03 kg; age 12–33 months). They were maintained in accordance with the Guide for The UK Home Office Code and Practice for the Housing and Care of Animals used in Scientific Procedures. The procedures were authorized under a project licence granted under the Animals (Scientific Procedures) Act 1986.

Midmyocardial myocytes were isolated enzymatically from the left ventricular midmyocardium of the heart as previously described (Volders et al., 1998). Briefly, hearts were excised from anesthetized dogs (45 mg/kg pentobarbitone) and washed in an O_2-gassed Ca^{2+}-free standard myocyte Tyrode solution at approximately 4 °C. A cannula was quickly inserted into and sutured to the left anterior descending artery under continuous perfusion with the same Tyrode solution. Subsequent perfusion was done at 37 °C with O_2-gassed Ca^{2+}-free standard myocyte Tyrode solution for 5–10 min and then for 18–22 min with the same solution plus collagenase. Collagenase was then washed out with O_2-gassed, 0.2 mM Ca^{2+}-standard myocyte Tyrode solution for 5–8 min. Finally, LVMMs were isolated by careful harvesting of the middle third of the transmural wall of the perfused wedge and were stored at room temperature in standard myocyte Tyrode solution.

2.2. Cell culture and preparation

2.2.1. CHO K1 cells expressing hERG (hI_{ERG})

CHO K1 cells expressing hERG channels described by Persson, Carlsson, Duker, and Jocobson (2004) were grown to semi-confluence at 37 °C in a humidified environment (5% CO_2) in the following medium: Ham's F-12 nutrient mixture and L-glutamine (Sigma) supplemented with 10% foetal calf serum (FCS; Invitrogen) and 600 μg/ml Hygromycin (Invitrogen). Cells that were split for use in the IonWorks™ were incubated at 37 °C for 24 h and then incubated at 28 °C for 48–72 h.

2.2.2. CHO K1 cells expressing hNav1.5 (hI_{Na})

CHO K1 cells expressing hNav1.5 channels described by Persson et al. (2004) were grown to semi-confluence at 37 °C in a humidified environment (5% CO_2) in the following medium: Ham's F-12 nutrient mixture and Glutamax™ (Invitrogen) supplemented with 10% FCS and 1000 μg/ml Geneticin (Invitrogen). Cells that were split for use in the IonWorks™ were incubated at 37 °C for 24 h and then incubated at 28 °C for 24 h.

2.2.3. CHO K1 cells expressing hKv4.3/hKChIP2.2 (hI_{to})

CHO K1 cells expressing hKv4.3/hKChIP2.2 channels described by Persson, Carlsson, Duker, and Jocobson (2005) were grown to semi-confluence at 37 °C in a humidified environment (5% CO_2) in the following medium: Ham's F-12 nutrient mixture and Glutamax™ (Invitrogen) supplemented with 10% FCS and 1100 μg/ml Geneticin and 600 μg/ml Hygromycin. Cells that were split for use in the IonWorks™ were incubated at 37 °C for 24 h, then at 28 °C for 48 h.

2.2.4. CHO K1 cells expressing hKvLQT1/hminK (hI_{Ks})

CHO K1 cells expressing hKvLQT1/hminK (Millipore) were grown to semi-confluence at 37 °C in a humidified environment (5% CO_2) in the following medium: Iscoves nutrient mixture and Glutamine (Invitrogen) supplemented with 10% FCS and 400 μg/ml Geneticin, 100 μg/ml Hygromycin, 2% HT supplement (50×, Invitrogen) and 1% non-essential amino acids (100×, Invitrogen). Cells that were split for use in the IonWorks™ were incubated at 37 °C for 48 h.

2.2.5. Preparation of CHO-hI_{ERG} and CHO-hI_{Na} cells for IonWorks™

Prior to use in IonWorks™, the monolayer of cells were washed with ~3 ml of Versene (1:5000, Invitrogen) pre-warmed to 37 °C. This solution was then aspirated out and another 3 ml of Versene was dispensed into the flask, and the cells were incubated at 37 °C for 6 min. Cells were then detached from the bottom of the flask by tapping the flask gently. 10 ml of PBS (Dulbeco's phosphate containing Ca^{2+}/Mg^{2+}) was dispensed down the inside face of the flask to wash off any remaining cells and the cells in the flask were then dispensed into a 15 ml centrifuge tube and centrifuged at 50 *g* for 4 min. The supernatant was discarded and the remaining pellet of cells was resuspended in 3 ml of PBS. An automated reader (Cedex; Innovatis) was used to determine cell number and therefore the volume of PBS to be added to the cell suspension to give the desired final cell concentration for use in IonWorks™. For IonWorks™ HT (hI_{ERG}) and Quattro (hI_{Na}) mode, cell concentrations of 0.25×10^6 cells/ml and 1×10^6 cells/ml were used respectively.

2.2.6. Preparation of CHO-hI_{Ks} and CHO-hI_{to} cells for IonWorks™

The method used was the same as that prescribed for the preparation of CHO-hI_{ERG} and CHO-hI_{Na} cells, except for the following changes: cells were washed with 3 ml pre-warmed PBS (no Ca^{2+}/Mg^{2+}, Sigma) instead of Versene, and cells were incubated with 3 ml pre-warmed 0.05% Trypsin/EDTA solution (Invitrogen) for 3 min, instead of 3 ml of Versene for 6 min. Both cell lines were adjusted to 1×10^6 cells/ml for use on IonWorks (both run in Quattro mode).

When performing the CHO-hI_{Na}/CHO-hI_{to} duplex assay, the cell counts were determined and the cell concentration for both cell suspensions was adjusted to 1×10^6 cells/ml. The cells were mixed together in order to attain a 60:40 ratio CHO-hI_{Na}:CHO-hI_{to}.

2.3. Electrophysioloy

2.3.1. For IonWorks™

There is no capability to warm solutions in the IonWorks™ device; hence it was operated at room temperature (~21 °C).

2.3.1.1. Measuring hI_{ERG}. A single voltage pulse was applied to evoke the pre- and post-compound hI_{ERG} currents (Bridgland-Taylor et al., 2006). A holding potential of −70 mV was applied for 20 s, followed by a