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Original article

The effects of plasma proteins on delayed repolarization *in vitro* with cisapride, risperidone, and D, L-sotalol

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Abstract

Introduction: Drug-induced long QT syndrome (LQTS) has been linked to arrhythmias (including Torsades de Pointes and sudden cardiac death), and has led to an increased awareness of the potential risk of delayed repolarization *in vitro* and *in vivo*. However, *in vitro* assessments of delayed repolarization have not been fully predictive of *in vivo* effects. **Methods:** To define the extent to which plasma protein binding (ppb) contributes to such disparities in repolarization studies, we compared drug-induced prolongation of the canine Purkinje fiber action potential duration (APD_{90}) *in vitro* during superfusion with 100% Tyrode's solution (Tyrodes), canine plasma [50% plasma/50% Tyrodes] and a 5% solution of recombinant human serum albumin in Tyrodes (HSA). Drugs evaluated included cisapride (>98% ppb), risperidone (90% ppb), and D, L-sotalol (negligible ppb). Effects on APD were monitored using standard microelectrode techniques under physiologic conditions and temperature ($[K^+]$=4 mM, 37 °C) during slow stimulation (2 s basic cycle length). **Results:** The effects of cisapride and risperidone on Purkinje fiber APD_{90} were significantly attenuated in the presence of plasma proteins. However, with cisapride, the extent of reduction with plasma proteins was significantly less than predicted based on calculated free drug levels. **Discussion:** We conclude that while plasma protein binding does reduce APD prolongation seen with bound drugs, this effect is not well correlated with the calculated plasma protein binding or expected clinical free fraction. Because of the complex drug interactions that occur in plasma, the electrophysiological effects seen with bound drugs are not well correlated with the calculated free fraction and thus caution should be exercised when assigning a predictive safety window. Thus, the canine Purkinje fiber assay is useful for defining the modulation of delayed repolarization due to plasma protein binding of novel therapeutic agents.

Keywords: Action potential; Canine Purkinje fiber; Cardiac repolarization; Methods; Plasma protein binding; Q–T prolongation

1. Introduction

Clinical experiences highlight the need for a reliable repolarization assay for the preclinical evaluation of potential new drugs. The International Conference on Harmonization (ICH) S7B document suggests both *in vivo* and *in vitro* non-clinical assays be employed to determine potential risk of delayed repolarization associated with new pharmaceutical agents. The *in vitro* studies typically include assessment of a particular cardiac repolarizing potassium current (IKr), which is encoded by hERG (human ether a go-go related gene). Another assay evaluates changes in the cardiac action potential configuration or terminal repolarization. Here we focus on the latter, the *in vitro* repolarization assay, in which drugs are evaluated on the basis of the extent of prolongation of the action potential duration (APD). This assay typically involves microelectrode recordings of acutely isolated syncytial preparations (such as Purkinje fibers or papillary muscles) superfused with physiologic salt solutions. Changes in the APD values with escalating drug concentrations *in vitro* are recorded, and results compared to *in vivo* studies (EKG recordings and QT interval measurements) as part of an overall integrated risk assessment.

By their very nature, *in vitro* experiments allow for more precise control of experimental conditions by isolating tissues from their physiologic environment. For example, *in vitro*

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experiments typically utilize physiologic salt solutions to superfuse tissues with drug concentrations equivalent to plasma levels measured clinically. However, it is well known that many drugs bind to plasma proteins in the circulation, and that plasma protein binding can reduce the amount of free drug available. By using protein free superfusates, we postulated that the electrophysiological effects of drugs tested in *in vitro* assays could, due to lack of protein binding, overestimate the effects of drugs *in vivo* with identical drug concentrations.

The aim of the present study was to compare the effects of plasma protein binding on delayed repolarization *in vitro* to calculated predictions of free drug concentrations. Drugs were selected based upon their ability to delay cardiac repolarization and differences in plasma protein binding, including cisapride (98% in humans; McCallum, Prakash, Campoli-Richards, & Goa, 1998: 95% in canine; Veereman-Wauter et al., 1990), risperidone (90% in humans; 91.7% in canine; Mannens, Meuldermans, Snoeck, & Heykants, 1994), and D, L-sotalol (negligible protein binding; Campbell and Williams 1998). Results demonstrate that plasma protein binding can significantly reduce the electrophysiological effects of compounds evaluated *in vitro* but the extent of effect may be significantly less than that predicted from plasma protein binding values. Thus, estimates of free drug concentration based on plasma protein binding may lead to overestimates of the potential risk for delayed repolarization and overly optimistic estimates of a "therapeutic window" for novel drug candidates.

2. Methods

2.1. Solutions

Tyrode's solution contained in mM: NaCl, 131; $NaHCO_3$, 18; NaH_2PO_4, 1.8; $MgCl_2$, 0.5; dextrose, 5.5; KCl, 4; $CaCl_2$, 2 aerated with 95% O_2/5% CO_2. Salt chemicals were purchased from Fisher Scientific (Fair Lawn, NJ).

To evaluate the effects of plasma protein binding, we compared the effects of drugs during superfusion with a) Tyrode's solution, b) plasma solution, consisting of 50% canine plasma/50% Tyrode's solution, and c) human serum albumin (HSA), Tyrode's solution with 5% Albumin (Human). Plasma (obtained from fasted female beagles, Marshall BioResources, North Rose, NY) was flash-frozen, stored at −80 °C and thawed immediately prior to use. This 50% canine plasma/50% Tyrode's solution contained approximately 160 μg/ml of alpha 1-acid glycoprotein (AAG) based on reported measurements of AAG from female beagle serum of 320 μg/ml (Kuribayashi et al., 2003). Human serum albumin (Albuminar-25, Aventis Behring L.L.C., Kankakee, IL) solutions were made by diluting a 25% albumin solution (25 g in 100 mL solution, osmotically equivalent to 500 mL of plasma) into 100% Tyrodes for a final concentration of 5% albumin. All solutions were pre-aerated and then gently bubbled with 95% O_2, 5% CO_2, no anti-foaming agent was used. Dilutions of both plasma and albumin were used to a), minimize plasma/albumin requirements, b), minimize possible ionic variations between plasma/albumin samples, and c), prevent enhanced normal automaticity (observed in preliminary experiments during superfusion with 100% plasma). Electrolytes of the plasma or HSA solutions were measured (AEROSET clinical chemistry system, Abbott Diagnostics, Abbott Park, IL, see Table 1). After equilibration in the bath, solutions were re-circulated to minimize the amount of plasma and/or HSA used.

Table 1
A table comparing the calculated salt concentrations from 100% Tyrode's solution with measured salt concentrations (sodium, chloride, glucose, potassium, calcium, and albumin) from the 50% canine plasma/50% Tyrode's solution and 5% human serum albumin solution (n=3)

	100% Tyrode's [a]	50% Plasma/ 50% Tyrodes	5% Human serum albumin
Sodium	150 mM	144.3±2.02 mM	153±0.7 mM
Chloride	136 mM	123.9±2.43 mM	128±0.3 mM
Glucose	5.5 mM	4.86±0.19 mM	4.92±0.18 mM
Potassium	4 mM	3.89±0.08 mM	3.68±0.03 mM
Calcium	2 mM	2.32±0.01 mM	1.68±0.02 mM
Albumin	–	4.77 g/dL	4.6 g/dL

[a] Indicates calculated concentration.

Risperidone and sotalol were purchased from Sigma Chemical (St. Louis, MO) and cisapride was purchased from Sequoia Research Products, (Oxford U.K.). Drugs were made as stock solutions in dimethyl sulfoxide (DMSO). Maximum DMSO concentrations did not exceed 0.1% by volume. When drug stock solutions were added to their respective superfusates (100% Tyrodes, 50% canine plasma/50% Tyrodes, or HSA), they were allowed to mix for approximately 1 h.

2.2. Electrophysiology

The Abbott Institutional Animal Care and Use Committee approved the use of animals for these studies. Free-running canine cardiac Purkinje fibers were removed and placed in warmed (37 °C) superfusion chamber (8–10 ml/min) and stimulated (2× threshold, biphasic waveform, typically 1–2 ms in duration) using platinum electrodes located in the chamber floor (see Gintant, Limberis, McDermott, Wegner, & Cox, 2001). Fibers were impaled with 3 M KCl-filled microelectrodes (resistance 10–30 M ohm), and electrical activity monitored using high input impedance electrometers (model IE-210, Warner Instruments, Hamden CT.). Studies were initiated after a minimum of 30 min *in vitro* equilibration with stimulation. Fibers are considered suitable for study if (during stimulation at 2 s basic cycle length) the following criteria are satisfied: a), the membrane potential just prior to action potential upstroke is more negative than −80 mV, b), the action potential duration is between 300–500 ms (spanning approx. 1.2 standard deviations from the mean value of 405 ms), and c), the normal automatic rate does not exceed the stimulation cycle length (2 s BCL).

Fibers were exposed to three ascending drug concentrations, with electrophysiological effects at each concentration evaluated during stimulation at 30, 75, and 150 bpm (2 s, 800 ms, and 400 ms basic cycle lengths [BCL], respectively) chosen to simulate bradycardia, normal, and tachycardia respectively. The