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Original article

# Optimization of $Ca_v1.2$ screening with an automated planar patch clamp platform

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ABSTRACT

**Introduction:** $Ca_v1.2$ channels play an important role in shaping the cardiac action potential. Screening pharmaceutical compounds for $Ca_v1.2$ block is very important in developing drugs without cardiac liability. $Ca_v1.2$ screening has been traditionally done using fluorescence assays, but these assays have some limitations. Patch clamping is considered the gold standard for ion channel studies, but is very labor intensive. The purpose of this study was to develop a robust medium throughput $Ca_v1.2$ screening assay in PatchXpress® 7000A by optimizing cell isolation conditions, recording solutions and experimental parameters. Under the conditions established, structurally different standard $Ca_v1.2$ antagonists and an agonist were tested. **Methods:** HEK-293 cells stably transfected with $hCa_v1.2$ L-type Ca channel were used. For experiments, cells were isolated using 0.05% Trypsin. Currents were recorded in the presence of 30 mM extracellular $Ba^{2+}$ and low magnesium intracellular recording solution to minimize rundown. $Ca_v1.2$ currents were elicited from a holding potential of −60 mV at 0.05 Hz to increase pharmacological sensitivity and minimize rundown. Test compounds were applied at increasing concentrations for 5 min followed by a brief washout. **Results:** Averaged peak $Ca_v1.2$ current amplitudes were increased from 10 pA/pF to 15 pA/pF by shortening cell incubation and trypsin exposure time from 2.5 min at 37 °C to 1 min at room temperature and adding 0.2 mM cAMP to the intracellular solution. Rundown was minimized from 2%/min to 0.5%/min by reducing the intracellular free $Mg^{2+}$ from 2.7 mM to 0.2 mM and adding 100 nM $Ca^{2+}$. Under the established conditions, we tested 8 structurally different antagonists and an agonist. The $IC_{50}$ values obtained ranked well against published values and results obtained using traditional clamp experiments performed in parallel using the expressed cell line and native myocytes. **Discussion:** This assay can be used as a reliable pharmacological screening tool for $Ca_v1.2$ block to assess compounds for cardiac liability during lead optimization.



## 1. Introduction

Voltage Gated Calcium Channels are a superfamily of membrane proteins that provide the major influx pathway for calcium ($Ca^{2+}$) in many excitable and non-excitable cell types (Hosey, Chien, & Puri, 1996). Depending on their threshold of activation, $Ca^{2+}$ channels are classified into low or high voltage-activated channels. The $Ca_v1.2$ channel is a member of the high voltage activated class of $Ca^{2+}$ channels known traditionally as the L-type $Ca^{2+}$ channel and is activated at relatively depolarized membrane potentials (>−20 mV). $Ca_v1.2$ channels are present predominantly in cardiac muscle, endocrine cells and neurons, where they are responsible for excitation-contraction coupling, hormone secretion and gene regulation (Catterall, 2000).

$Ca_v1.2$ channels are the most abundant members of the voltage-gated $Ca^{2+}$ channel family present in cardiac myocytes (Katz, 1997). In conjunction with the $K^+$ channels, $I_{Kr}$ (hERG) and $I_{Ks}$ (KCNQ1 + KCNE1), and the cardiac $Na^+$ (Nav1.5) channel, as well as other ion channels, $Ca_v1.2$ plays a key role in shaping the cardiac action potential (Alseikhan, DeMaria, Colecraft, & Yue, 2002). $Ca_v1.2$ channels contribute to the maintenance of the action potential plateau, accelerate pacemaker activity in the sinoatrial node, and support conduction through the atrioventricular node (Katz, 1996). In addition, $Ca_v1.2$ channels play a major role in peripheral vasoconstriction and are the targets of $Ca^{2+}$ channel blockers used to treat hypertension (Godfraind, 1994). Because of their importance in normal cardiovascular function, screening of new drug candidates for activity on $Ca_v1.2$ channels is considered an important safety measure in developing new pharmaceuticals devoid of undesirable cardiovascular side effects.

Higher throughput screening of the $Ca_v1.2$ channel has been traditionally achieved using $Ca^{2+}$ fluorescence assays, which are amenable to a multi-well plate-based screening format, such as the

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FLIPR® Assay (Xia, Imredy, Koblan, Bennett, & Connolly, 2004). Although fluorescence techniques provide a robust screening method for $Ca_v1.2$ block, fluorescent and other compounds can interfere with the assay readout yielding confounding results. In addition, if more than one $Ca^{2+}$ entry pathway exists in the host cell, $Ca^{2+}$ fluorescence cannot distinguish among $Ca^{2+}$ entry pathways.

Voltage clamping of ion channels provides the most direct and highest quality functional information regarding interactions of potential drugs with ion channels. Patch-clamping of cardiac myocytes has been the traditional method of measuring functional L-type $Ca^{2+}$ channel block. Recent development of the planar patch electrode to substitute for the traditional glass pipette electrode has allowed automation of whole-cell patch clamp. So far, most higher throughput automated planar patch-clamp methods require cells with a spherical rather than cylindrical shape of the heart cell, and thus automated patch clamp systems rely on the availability of cell lines stably expressing the ion channel of interest. Using stable expression of ion channels in host cell lines, a number of high throughput patch-clamp assays have been successfully implemented for routine cardiac ion channel screening of $K^+$ and $Na^+$ channels (Dubin et al., 2005; Guo & Guthrie, 2005; Kiss et al., 2003; Trepakova et al., 2007; Zheng, Spencer, & Kiss, 2004). While cell lines stably expressing hERG, $I_{Ks}$, and $I_{Na}$ may routinely express currents with amplitudes $\geq$nA/cell, it has been much more difficult to produce highly expressing cell lines with $Ca_v1.2$ currents owing to the need for stable coexpression of multiple ion channel subunits to reconstitute physiologically representative currents (Lory, Varadi, Slish, Varadi, & Schwartz, 1993; Nishimura, Takeshima, Hofmann, Flockerzi, & Imoto, 1993). Another confounding factor for developing patch-clamp assays for the $Ca_v1.2$ current is the current 'rundown' observed with dialysis of the intracellular constituents during whole-cell recording, making it difficult to distinguish from onset of pharmacological blockade (Bean, 1984).

Using a HEK-293 cell line stably expressing the human L-type cardiac $Ca^{2+}$ channel subunits α1C, α2δ, and β2a (Xia et al., 2004), we are able to record $Ca_v1.2$ currents using the PatchXpress® 7000A automated patch-clamp platform. This platform routinely allows the achievement of gigaohm seals owing to the glass substrate of the planar patch electrode. In this aspect the PatchXpress closely approximates conventional clamp and allows recording of relatively small current amplitudes. The automation of the labor intensive aspects of patching, sealing, and recording from $Ca_v1.2$ expressing cells that the PatchXpress may afford can greatly increase the efficiency by which quality patch clamp data is obtained. We have optimized methods to maximize peak current amplitude and attenuate rundown using this automated patch clamp platform. We develop assay conditions that have allowed the evaluation and characterization of the effect of structurally different $Ca^{2+}$ channel standard antagonists and agonists. We thus help validate and evaluate an important pharmacological screening tool for $I_{Ca}$.

## 2. Materials and methods

### 2.1. Cells

#### 2.1.1. HEK-293 $Ca_v1.2$ cells

HEK-293 cells stably expressing the human $Ca_v1.2$ L-type Ca channel (Soldatov, 1992) along with the inward rectifier potassium channel Kir2.3 (Perier, Radeke, & Vandenberg, 1994) were grown and passaged in T75 tissue culture flasks (Corning, NY). The line (designated HEK-$Ca_v1.2$-E74) is derived from a C1-6-37-3 parent line previously described (Xia et al., 2004) except that the β2a subunit was recloned into the line under hygromycin B selection. Human L-type $Ca^{2+}$ channel subunits α1C (Harpold et al., 1998), α2δ (Ellis, Williams, Harpold, Schwartz, & Brenner, 1997), and β2a subunits (Harpold et al., 1999) were commercially acquired (SIBIA Neurosciences, La Jolla, CA) and are stably expressed using vectors pcDNA3.1, pRcCMV (both Invitrogen, CA), and pHygCMV (Clontech, CA), respectively. The inward rectifier Kir2.3 (obtained as a gift from Carol Vandenberg) was stably co-expressed using vector pZeoCMV (Stauderman et al., 1998). Expression of the $Ca_v1.2$ subunits was verified using Western blots. Kir2.3 expression was monitored by electrophysiological recording (see Xia et al., 2004). Growth media contained DMEM Glutamax culture media supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, as well as selection antibiotics Geneticin G418 (100–800 μg/ml) for α1C, Zeocin (40 μg/ml) for Kir2.3, and Hygromycin B (100–250 μg/ml) for β2a. HEK-$Ca_v1.2$ cells were incubated at 37 °C in filtered ambient air supplemented with 5% $CO_2$. Cells were grown to approximately 70–80% confluency and passaged/plated twice a week. All tissue culture reagents were obtained from Invitrogen Corp.

For PatchXpress experiments, prior to electrophysiological recording, cells were rinsed with phosphate-buffered saline (PBS), dissociated by incubation in a 0.05% Trypsin solution for 2 min at room temperature, harvested into bath solution (see below for composition), centrifuged twice at 700 rpm, and resuspended in 170 μl of bath solution.

For conventional patch clamp, cells were rinsed with PBS, dissociated with 0.05% Trypsin solution, re-suspended in culture medium, and stored at room temperature (20–25 °C) with gentle rocking for up to 8 h. Prior to recording, cells were added drop-wise into a test chamber and were superfused with external bath solution at a rate of 2 ml/min.

#### 2.1.2. Cardiac myocytes

Guinea pig ventricular myocytes were isolated by enzymatic digestion of the heart using a Langendorff perfusion method (Fermini et al., 1995).

Briefly, male guinea pigs were injected intraperitoneally with 0.4 ml/kg heparin (Baxter, 1000 U/ml). After 20 min, the guinea pigs were euthanized by guillotine. The heart was rapidly removed and the aorta cannulated for retrograde perfusion of the coronary arteries (Langendorff). Oxygenated $Ca^{2+}$-free HBS (in mM: 132 NaCl, 4 KCl, 1.2 $MgCl_2$, 10 HEPES, 11.1 glucose, pH = 7.35) at a temperature of 35 °C was perfused through the heart for 5 min at a rate of 4–7 ml/min. Next, $Ca^{2+}$-free HBS with 0.2 mg/ml of collagenase (Type II, Worthington, 262 units/mg) and 0.07 mg/ml protease (Sigma P-5417, 4.3 units/mg) were perfused for an additional 15 min. The digested left and right ventricles were cut free into warmed, oxygenated KB solution (in mM: 70 Glutamic acid, 30 KCl, 10 $KH_2PO_4$, 5 $MgCl_2$, 15 Taurine, 10 Glucose, 10 HEPES, 0.5 EGTA, pH adjusted to 6.9 with KOH) and minced into small sections which were dissociated further with trituration. The solution containing the myocytes was filtered through a mesh and myocytes were allowed to settle to the bottom of the solution at room temperature. After aspiration of the solution, the cells were re-suspended at room temperature in 15 ml conical tubes filled with KB with 0.5 mM $CaCl_2$. The capped conical tubes were gently rotated at room temperature to keep the ventricular myocytes in suspension until use.

### 2.2. Solutions

#### 2.2.1. HEK-293 $Ca_v1.2$

Bath recording solution for PatchXpress and conventional patch-clamp experiments contained (in mM): 102 NaCl, 4 KCl, 30 $BaCl_2$, 1.2 $MgCl_2$, 11.1 Glucose, 10 HEPES, pH 7.4 adjusted with NaOH. All $Ca_v1.2$ currents were recorded with 30 mM $Ba^{2+}$ as the external charge carrier unless otherwise noted. The base internal solution contained (in mM): 80 CsCl, 10 EGTA, 4 $MgCl_2$, 5 TrisATP, 1 $Na_2GTP$, 20 Phosphocreatine Tris, 20 TEACl, and 10 HEPES, adjusted to pH 7.2 with CsOH. To change the free $Mg^{2+}$ concentration, $MgCl_2$ was added to the base solution. For testing of $Ca^{2+}$ standards, 0.2 mM cAMP and