British Journal of Pharmacology (2009), 156, 143–152
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0007-1188/08
www.brjpharmacol.org

RESEARCH PAPER

Characterization of BK channel splice variants using membrane potential dyes

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Background and purpose: Large conductance calcium- and voltage-activated potassium (BK) channels are encoded by a single gene that displays extensive pre-mRNA splicing. Here we exploited a membrane potential assay to investigate the sensitivity of different BK splice variants to elevations in intracellular free calcium and their inhibition by the BK channel blocker paxilline.
Experimental approach: Murine BK channel splice variants were expressed in human embryonic kidney 293 cells and their properties analysed in response to ionomycin-induced calcium influx in both fluorescent membrane potential (fluorescent-imaging plate reader) and patch clamp electrophysiological assays. The dose-dependent inhibition of distinct splice variants by the BK channel-specific blocker paxilline was also investigated.
Key results: Ionomycin-induced calcium influx induced a robust hyperpolarization of human embryonic kidney 293 cells expressing distinct BK channel splice variants: stress regulated exon (STREX), e22 and ZERO. Splice variant expression resulted in membrane hyperpolarization that displayed a rank order of potency in response to calcium influx of STREX > e22 > ZERO. The BK channel inhibitor paxilline exhibited very similar potency on all three splice variants with IC_{50}s in membrane potential assays of 0.35 ± 0.04, 0.37 ± 0.03 and 0.70 ± 0.02 μmol·L^{-1} for STREX, ZERO and e22 respectively.
Conclusions and implications: BK channel splice variants can be rapidly discriminated using membrane potential based assays, based on their sensitivity to calcium. BK channel splice variants are inhibited by the specific blocker paxilline with similar IC_{50}s. Thus, paxilline may be used in functional assays to inhibit BK channel function, irrespective of the variant expressed.
British Journal of Pharmacology (2009) **156**, 143–152; doi:10.1111/j.1476-5381.2008.00011.x; published online 5 December 2008

Keywords: BK channel; paxilline; FLIPR; KCNMA1

Abbreviations: BK, large conductance calcium- and voltage- activated potassium channel; FLIPR, fluorescent-imaging plate reader; FMP, FLIPR membrane potential dye; HEK293, human embryonic kidney 293 cell; mRNA, messenger RNA; STREX, stress regulated exon

Introduction

Large conductance calcium- and voltage-activated potassium (BK) channels are ubiquitously expressed and control an eclectic array of physiological processes ranging from regulation of blood pressure (Brenner *et al.*, 2000b; Sausbier *et al.*, 2005), neurotransmitter release (Raffaelli *et al.*, 2004), micturition (Meredith *et al.*, 2004), epithelial transport (Bailey *et al.*, 2006; Sausbier *et al.*, 2006) and sexual function (Werner *et al.*, 2005). Indeed, mutations or deletion of the BK channel pore-forming α-subunits, or accessory β-subunits, may lead to major disorders including hypertension (Brenner *et al.*, 2000b; Sausbier *et al.*, 2005), epilepsy (Brenner *et al.*, 2005; Du *et al.*, 2005), ataxia (Sausbier *et al.*, 2004) and incontinence (Meredith *et al.*, 2004). This diversity in physiological function of BK channels is also reflected in their heterogeneity of channel properties in different tissues and cell types, which can be modified for example during development (MacDonald *et al.*, 2006) or in response to changes in physiological homeostasis (Xie and McCobb, 1998; Benkusky *et al.*, 2000). Unusually for potassium channels, the pore-forming α-subunit of BK channels is encoded by a single gene (Butler *et al.*, 1993), thus the diverse properties of BK channels result from a number of distinct post-transcriptional regulatory mechanisms including assembly with a family of four distinct regulatory β-subunits (β1–β4) (Orio *et al.*, 2002), extensive pre-mRNA splicing of the pore-forming α-subunit (Shipston, 2001) and regulation/assembly with a diverse array of intracellular signalling cascades.

Alternative pre-mRNA splicing is a major post-transcriptional mechanism to generate physiological diversity from a single gene. Alternative splicing of BK channels is under hormonal control and leads to channels with distinct properties including differences in calcium/voltage sensitivity (Saito *et al.*, 1997; Chen *et al.*, 2005), subcellular targeting (Zarei

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Received 10 July 2008; revised 31 July 2008; accepted 1 September 2008

et al., 2004) and regulation by cellular signalling pathways (Tian *et al.*, 2001; Erxleben *et al.*, 2002; McCartney *et al.*, 2005). Several sites of alternative pre-mRNA splicing within the BK channel α-subunit have been described, the majority of which are located within the intracellular C-terminal domain of the channel (Shipston, 2001). Analysis of alternatively spliced variants of the intracellular c2 site of splicing of BK channels has demonstrated how splicing at a single site can dramatically modify channel properties (Chen *et al.*, 2005). For example, at this site the cysteine-rich, 59-amino-acid, stress-regulated exon (STREX) variant has a left-shifted half maximal voltage for channel activation compared with the 29-amino-acid insert e22, or the insertless null variant known as ZERO (Saito *et al.*, 1997; Chen *et al.*, 2005). Furthermore, inclusion of STREX modifies the regulation of BK channels by diverse cellular signalling pathways (Tian *et al.*, 2001; Erxleben *et al.*, 2002; Chen *et al.*, 2005; McCartney *et al.*, 2005).

To date, functional analysis of BK channel splice variant properties has been analysed using the low-throughput 'gold-standard' method of patch clamp electrophysiology. Furthermore, the majority of the analysis of BK channel properties is performed at supra physiological calcium and voltage conditions, to optimize signal to noise ratios, which are often different from the conditions experienced by BK channels in native tissues and it has been assumed, but not tested, that the widely used BK channel-specific inhibitor, paxilline (Sanchez and McManus, 1996), inhibits all BK channel variants with similar potency.

We thus sought to develop a higher-throughput assay that would allow us to simultaneously: (i) rapidly characterize the functional properties of splice variants of the BK channel and discriminate between splice variants; (ii) analyse splice variant properties under conditions of near physiological voltages and elevations of intracellular free calcium; and (iii) determine the potency of the BK channel blocker paxilline on different splice variants under these conditions. Membrane potential dyes have been extensively exploited to examine voltage-gated potassium channels in fluorescence-based assays, although most suffer from slow response times, widespread drug interference effects and the majority of assays exploit a high-potassium stimulus to drive channel activation by robust depolarization of the cell membrane. In this study, we exploited the fluorescent-imaging plate reader (FLIPR) membrane potential dye from Molecular Devices that displays good temporal responses (Whiteaker *et al.*, 2001; Wolff *et al.*, 2003) and employed the calcium ionophore, ionomycin (Kauffman *et al.*, 1980) to drive BK channel activation predominantly through calcium influx. We found that BK channel splice variants STREX, e22 and ZERO, resulting from alternative splicing at the intracellular site C2 of alternative splicing in murine BK channels, display differential sensitivity to calcium influx and that paxilline inhibits each variant with similar potency.

Methods

Cell culture

Human embryonic kidney 293 (HEK293) cells were used over a passage range of 10–40. Cells were passaged every 3–5 days at approximately 80% confluency using trypsin-EDTA and maintained in 25 cm^2 Greiner flasks with Dulbecco's modified Eagle's medium containing 10% foetal calf serum in a 37°C incubator with 95% (v/v) air and 5% CO_2.

For electrophysiological experiments, HEK293 cells were cultured on glass coverslips (12 mm diameter Chance Glass Ltd, UK) in a sterile six-well tissue culture plate until approximately 40% confluent. Cells were transiently transfected with 1.2 μg of the appropriate DNA and 5 μl of Lipofectamine 2000 and transfection efficiencies of ~70% were routinely obtained. The murine BK channel variant constructs in pcDNA3.1 used in this work (ZERO, e22 and STREX) have been described previously and are expressed at similar levels at the plasma membrane in HEK293 cells as determined by Western blot and immunocytochemistry (Chen *et al.*, 2005). In addition, the single channel conductance of all three variants is similar (Chen *et al.*, 2005). Cells were subsequently incubated for 24 h at 37°C in 95% (v/v) air, 5% (v/v) CO_2 before 1 ml of HEK293 media was added to dilute the transfection reaction. They were then used for electrophysiological recordings 3–5 days after transfection.

Cells for fluorescent membrane potential (FMP) assays were maintained and transfected as above. In addition, stable cells maintained under selection with geneticin (0.8 mg ml^{-1}) were also used in some assays with similar results. Cells were replated into poly-D-lysine-coated black 96-well plates (Greiner) and incubated for a further 48 h before being used for the FMP assay.

FMP assay

Forty-eight hours after plating in black-walled, clear-bottom plates, the culture medium was aspirated and the cells incubated in FLIPR® Membrane Potential Red or Blue dye (Molecular Devices, Sunnyvale, CA) for 30 min at 37°C to allow dye loading. The dye was prepared as recommended by the manufacturer with 2 mmol·L^{-1} extracellular free calcium unless otherwise stated. Assays were performed at 22°C using a FlexStation® II system (Molecular Devices). To activate BK channels expressed in HEK293 cells the calcium ionophore ionomycin was applied at a final concentration of 1 μmol·L^{-1} using the automated liquid-handling function of the FlexStation® II programmed to administer 50 μl of ionomycin in extracellular solution to a total well volume of 200 μl, 16 s after the experiment began. The fluorescence changes were read at high sensitivity at 180 s at intervals of 1.52 s with excitation/emission wavelengths of 530/565 nm respectively. Ionomycin was stored in DMSO at –20°C and working stocks made fresh prior to the assay. The final DMSO vehicle concentration was 0.01%, which had no significant effect in the assay.

For each independent experiment, splice variant analysis was performed in parallel on the same plate with HEK293 cells expressing each variant and mock-transfected controls with a minimum of quadruplicate replicates. In all plates, a DMSO control and a paxilline pre-treated control for each variant were included. Data are presented as means ± SEM where n = the number of independent experiments.

The stability and well-to-well variation for each plate were determined using the experimental Z' parameter (Zhang *et al.*, 1999) where:

$$Z' = 1 - [3 \times (\text{standard deviation upper} - \text{standard deviation lower})]/[3 \times (\text{mean upper} - \text{mean lower})]$$

The upper and lower values define the numerically high and low relative fluorescent units (RFU) respectively in each assay plate used for the Z' calculation. For the FMP assay, as the highest RFU values are obtained upon depolarization, the upper values were taken as the response to ionomycin in non-transfected HEK293 cells (or ionomycin in the presence of maximally effective concentrations of paxilline in transfected cells). The lower RFU values are at hyperpolarized potentials, thus the lower RFU values were taken from the response to ionomycin in STREX-transfected cells. Plates with Z' values between 0.3 and 1 were considered acceptable for analysis.

To assay for elevation in intracellular free calcium cells were incubated with FLIPR® Calcium 3 dye (Molecular Devices) essentially as described above.

To determine the effect of paxilline cells were pre-treated for 10 min with the respective concentration of drug before assaying as above. The ionomycin-induced fluorescent response of BK-transfected cells in the presence of 10 $\mu mol \cdot L^{-1}$ paxilline was essentially indistinguishable from that determined in mock-transfected cells in the presence or absence of paxilline, demonstrating that 10 $\mu mol \cdot L^{-1}$ paxilline fully blocked each BK channel variant. Paxilline (10 $\mu mol \cdot L^{-1}$) alone had no effect on mock-transfected HEK293 cells.

Data were analysed with SoftMax Pro and data exported to Igor Pro, Microsoft Excel and/or Prism for further analysis. To compare between variants or analyse the dose–response to paxilline, the RFUs were determined 70 s into the assay. Raw data from each experiment was normalized to control baseline levels. In dose–response assays, maximal inhibition (100%) was defined as the fluorescent response equivalent to that observed in mock-transfected HEK293 cells in response to ionomycin. In each individual assay (minimum four replicates), the fluorescent signal for each variant in response to ionomycin, in the absence of paxilline, was defined as the maximal response (i.e. 0% inhibition). The fluorescent response in the presence of each respective paxilline concentration was then expressed as a percentage of maximal inhibition. Percentage inhibitions at each paxilline concentration from a minimum of four independent experiments for each BK channel splice variant were then combined and dose–response curves fitted using a Boltzmann function.

Electrophysiology

Whole-cell and cell-attached patch clamp recordings of BK channels were made at room temperature (20–24°C) from transfected HEK293 plated on glass coverslips. Channel activity was recorded using an Axopatch-200 patch clamp amplifier (Axon Instruments, USA). Borosilicate glass patch pipettes (Harvard Apparatus, Kent) with resistances of 5–10 MΩ for cell-attached recordings and 4–7 MΩ for whole-cell recordings.

All recordings were made with HEK cells under physiological potassium gradients. The patch pipette solution contained (in $mmol \cdot L^{-1}$): 140 KCl; 5 NaCl; 1 $MgCl_2$; 10 glucose; 10 HEPES; 1 BAPTA; and $CaCl_2$ to give a free calcium concentration of 0.1 $\mu mol \cdot L^{-1}$ for whole-cell recordings and 0.33 $\mu mol \cdot L^{-1}$ for cell-attached recordings. The bath solution contained (in $mmol \cdot L^{-1}$): 135 NaCl; 2.5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 glucose; 10 HEPES. Whole-cell recordings were made using current clamp to monitor changes in the resting membrane potential of HEK cells. After breaking into the cell, the membrane potential was allowed to stabilize, over approximately 5 min, before ionomycin (1 $\mu mol \cdot L^{-1}$) and paxilline (10 $\mu mol \cdot L^{-1}$) were added to the bath and voltage responses recorded. Single-channel BK activity was monitored using cell-attached recording with 0 mV applied to the membrane. The channel activity observed was therefore driven by the resting membrane potential of the HEK cell and channel opening recorded as downward deflections representing inward movement of potassium, as there would be approximately equimolar concentrations of potassium in the HEK cells and the patch pipette. Whole-cell data were analysed using pClamp 9 (Molecular Devices) and single-channel data analysed using Win EDR (J. Dempster, University of Strathclyde).

Statistical analysis

All data are presented as means ± SEM, with n = number of independent experiments unless otherwise indicated. Statistical analysis between groups was determined by ANOVA with Tukey's *post hoc* test. Significance was defined at $P < 0.05$.

Results

Assay of BK channel splice variants using a fluorescent membrane potential assay

In an attempt to discriminate between BK channel splice variants using the membrane potential assay, we utilized the calcium ionophore ionomycin to stimulate BK channels expressed in HEK293 cells via calcium influx (Fig. 1a). In HEK293 cells expressing the STREX splice variant, application of 1 $\mu mol \cdot L^{-1}$ ionomycin resulted in a robust and rapid decrease in fluorescence indicating cellular hyperpolarization as would be expected for activation of a potassium-selective channel. In contrast, ionomycin stimulated an increase in fluorescence indicating membrane depolarization in mock-transfected HEK293 cells. The nature of this calcium-dependent depolarizing response to ionomycin in native HEK293 cells has not been fully characterized, although previous studies (e.g. Fliegert *et al*., 2007) have suggested that it involves capacitative calcium entry and other store-operated cation influx pathways. The ionomycin-induced depolarization might be expected to facilitate BK channel activation in transfected cells, but it also sets a limit on the sensitivity of the assay as the net electrophysiological response to ionomycin is thus a product of the ionomycin-induced hyperpolarization, due to calcium-dependent activation of BK channels, and the depolarizing component, due to endogenous, more slowly activating mechanisms such as store-operated cation currents. Importantly, even though we have not blocked the ionomycin-induced depolarizing component, the calcium-dependent activation of the BK current is the faster response and so we can clearly discriminate the contribution of BK channels with a signal to noise ratio that allowed robust

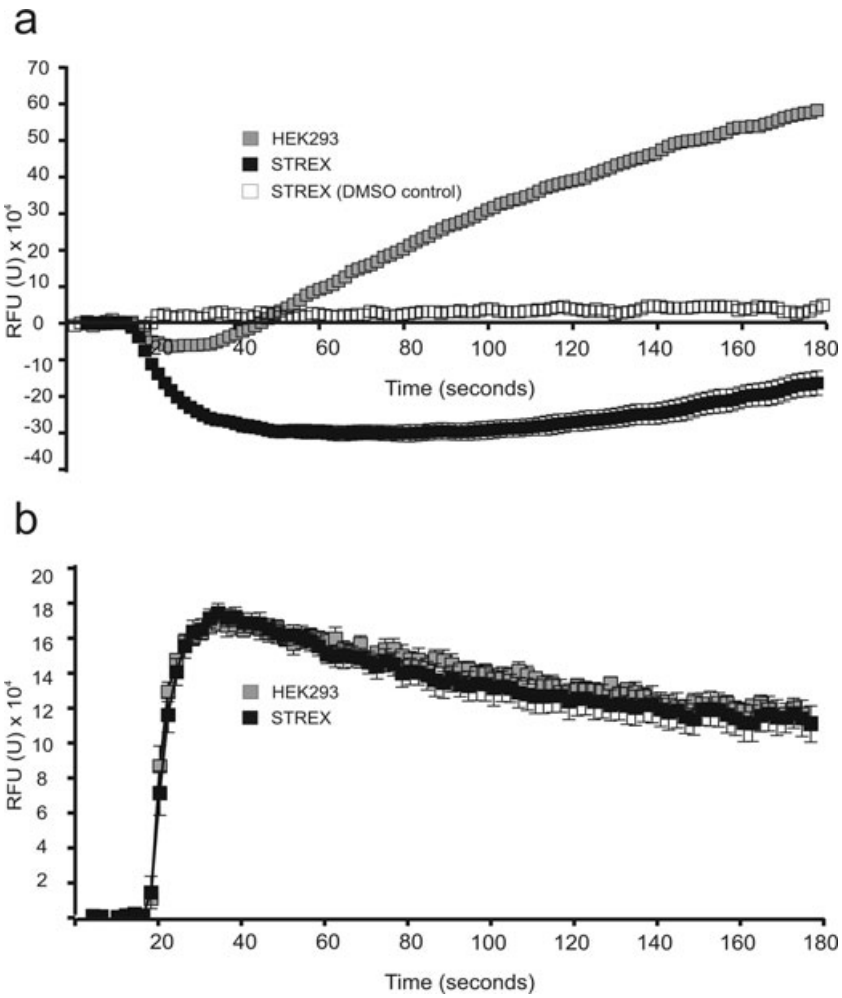

Figure 1 Ionomycin induces calcium influx and membrane hyperpolarization in HEK293 cells expressing the STREX BK channel splice variant. a. Time course of change in relative fluorescent units (RFU) in the membrane potential assay. Cells were stimulated with 1 μmol·L^{-1} ionomycin or DMSO (vehicle control) at $t = 16$ s in mock-transfected HEK293 cells or cells expressing the STREX BK channel splice variant. A negative RFU indicates membrane hyperpolarization. Data are means ± SEM ($n = 8$). b. Time course of change in RFUs in the intracellular free calcium reporter assay. Mock-transfected HEK293 cells or cells expressing the STREX variant were stimulated with 1 μmol·L^{-1} ionomycin at $t = 16$ s. Data are means ± SEM ($n = 3$). BK, large conductance calcium- and voltage-activated potassium channel; HEK293, human embryonic kidney 293 cell; STREX, stress-regulated exon.

analysis of BK channel properties with this assay. The vehicle control (0.01% DMSO) had no significant effect on the baseline fluorescent signal in HEK293 cells expressing the STREX variant (Fig. 1a) or mock-transfected HEK293 cells (not shown). The FLIPR® Membrane Potential dye used in these studies is available in two formats (red and blue) to allow potential dye interactions to be eliminated when screening compound libraries. There was no significant difference in the amplitude or time course of the ionomycin-induced hyperpolarization between the red or blue dye (not shown). Temperature modulated the amplitude of the responses, as conducting the assays at 37°C reduced the maximum hyperpolarization to 60.0 ± 3.7% ($n = 8$) of the response seen at 22°C. Thus, to improve the signal to noise ratio and allow direct comparison of responses with conventional patch clamp electrophysiological analysis, all subsequent assays were performed at room temperature (22°C).

To verify that the ionomycin-induced hyperpolarization was predominantly a result of calcium entry, we first assayed the elevation of intracellular free calcium using the Calcium 3 dye (Molecular Devices, Sunnyvale, CA). Ionomycin elicited a rapid and sustained increase in fluorescence (Fig. 1b), representing an increase in intracellular free calcium concentration. Removal of extracellular calcium significantly attenuated the ionomycin-induced rise in intracellular free calcium to 7.6 ± 3.6% ($n = 4$) of the maximal response in the presence of 2 mmol·L^{-1} extracellular calcium. Thus, the predominant effect of ionomycin is to induce calcium influx, with the residual rise in intracellular free calcium in the absence of extracellular calcium most likely to result from release of calcium from intracellular stores in this system (Fliegert *et al.*, 2007). There were no significant differences in calcium influx between the transfected and non-transfected HEK293 cells (Fig. 1b), verifying that lipofectamine transfection of HEK293 cells with BK channels does not alter the ionomycin-induced calcium entry into the cell.

To further validate the calcium dependency of the ionomycin-induced hyperpolarization response in the FMP

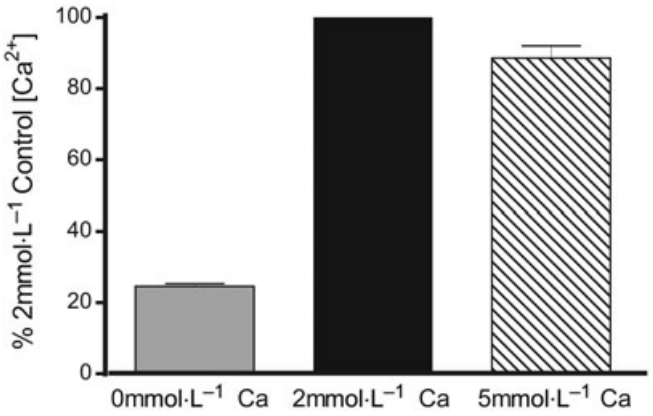

Figure 2 Depletion of extracellular calcium attenuates the membrane hyperpolarization induced by ionomycin. Membrane potential assays were performed as in Figure 1a using the STREX variant with different extracellular calcium concentrations. Data are normalized to the peak hyperpolarization determined at 70 s in the presence of 2 mmol·L^{-1} extracellular calcium. Data are means ± SEM ($n = 8$). STREX, stress-regulated exon.

assay, STREX channels expressed in HEK293 cells were investigated in the absence of extracellular calcium. Buffering extracellular free calcium to <10 nmol·L^{-1} with the calcium chelator BAPTA resulted in a significantly attenuated ionomycin-induced hyperpolarization in HEK293 cells expressing the STREX variant ($P < 0.001$ ANOVA with Tukey's test). The peak hyperpolarization, determined at 70 s, was reduced by >75% compared with that observed with 2 mmol·L^{-1} extracellular calcium (Fig. 2). The residual activation is most likely to represent release of calcium from intracellular stores in this system (Fliegert *et al.*, 2007). Increasing extracellular calcium concentration to 5 mmol·L^{-1} had no significant effect on the ionomycin-induced hyperpolarization compared with 2 mmol·L^{-1}. Taken together, these data strongly suggest that the ionomycin-induced hyperpolarization in HEK293 cells expressing the STREX channel variant is predominantly a result of BK channel activation by influx of calcium.

Ionomycin-induced calcium influx differentially activates BK channel splice variants

To directly demonstrate that the ionomycin-induced reduction in fluorescence results from membrane hyperpolarization, we performed conventional whole-cell current clamp recordings. Ionomycin induced a robust hyperpolarization (by 51 ± 3 mV) in HEK293 cells expressing the STREX channel variant (Fig. 3a,b). This was correlated with a mean 35 ± 3% change in dye emission (% change in RFU) in the FMP assay. Assuming a linear response, this suggests that the sensitivity of the assay is approximately a 1.5 mV change in membrane potential for a 1% change in RFU.

In contrast, ionomycin elicited no significant hyperpolarization in mock-transfected HEK293 cells and resulted in an ~9 mV depolarization (Fig. 3b). There was no significant difference in resting membrane potential between mock-transfected HEK293 cells and cells expressing the STREX or ZERO variants (Fig. 3b: HEK293 –30 ± 4 mV, ZERO –29 ± 2 mV and STREX –34 ± 6 mV; $n = 3$). However, ionomycin induced a significantly greater hyperpolarization in HEK293 cells expressing the STREX variant compared with those expressing the ZERO BK variant (Fig. 3b) with the peak hyperpolarization being –82 ± 3 and –48 ± 5 mV for STREX and ZERO channels respectively (***$P < 0.001$ ANOVA with Tukey's test; $n = 3$).

As the ionomycin-dependent calcium influx allowed clear discrimination of the ZERO and STREX variant channels in electrophysiological assays, we asked whether the membrane potential assay could be used to discriminate between distinct variants. Previous electrophysiological analysis has demonstrated clear differences in the apparent calcium sensitivities of the BK channel variants ZERO, e22 and STREX (Chen *et al.*, 2005). These variants are the result of alternative pre-mRNA splicing at a single intracellular site of splicing, c2, in the BK channel C-terminus. Importantly, these variants display similar single-channel conductance and are expressed at equivalent levels at the cell surface in HEK293 cells as determined by Western blotting and immunocytochemistry (Chen *et al.*, 2005 and data not shown). Thus, any observed differences in HEK293 cell membrane hyperpolarization in response to ionomycin-induced calcium influx must result from differences in the apparent calcium/voltage sensitivities or kinetics of the BK channel variants. Cells expressing STREX channels showed the greatest hyperpolarization (Fig. 4a) and the e22 and ZERO BK variants produced significantly smaller responses. Expressed as a percentage of the STREX response, the other variants displayed a rank potency to activation by the ionomycin-induced calcium influx STREX > e22 > ZERO (Fig. 4a,b). The hyperpolarization of e22- and ZERO-expressing cells was 64 ± 17% and 45 ± 13% of the STREX response respectively ($P < 0.001$ ANOVA with Tukey's test; $n = 8$).

Paxilline inhibits BK channel variants with similar potency

The ability to discriminate between splice variants expressed in HEK293 cells, using the membrane potential assay, allowed us to address whether the specific BK channel blocker paxilline was equally potent on the three splice variant α-subunits. The concentration dependence of paxilline block of the ionomycin-induced hyperpolarization was investigated for each variant. Paxilline dose dependently inhibited the ionomycin-induced hyperpolarization (Fig. 5) with IC_{50} values of 0.35 ± 0.04, 0.37 ± 0.03 and 0.70 ± 0.02 μmol·L^{-1} (Fig. 5b) calculated for ZERO, STREX and e22 variants respectively. The IC_{50} for the e22 variant was significantly greater than those for the ZERO and STREX variants ($n = 4$, $P < 0.001$, F test). Paxilline had no effect on non-transfected HEK293 cells (not shown) and, at maximally effective concentrations between 1–10 μmol·L^{-1}, the effect of ionomycin on the fluorescent response was indistinguishable from that in HEK293 cells alone, demonstrating complete inhibition of BK channels. To further confirm that paxilline mediated its effect solely through inhibition of BK channels, we analysed the ability of paxilline to prevent membrane hyperpolarization in the whole-cell current clamp assays (Fig. 6) and activation of unitary BK currents in cell-attached patches (Fig. 7). First, treatment of HEK293 cells expressing STREX channels with maximally effective

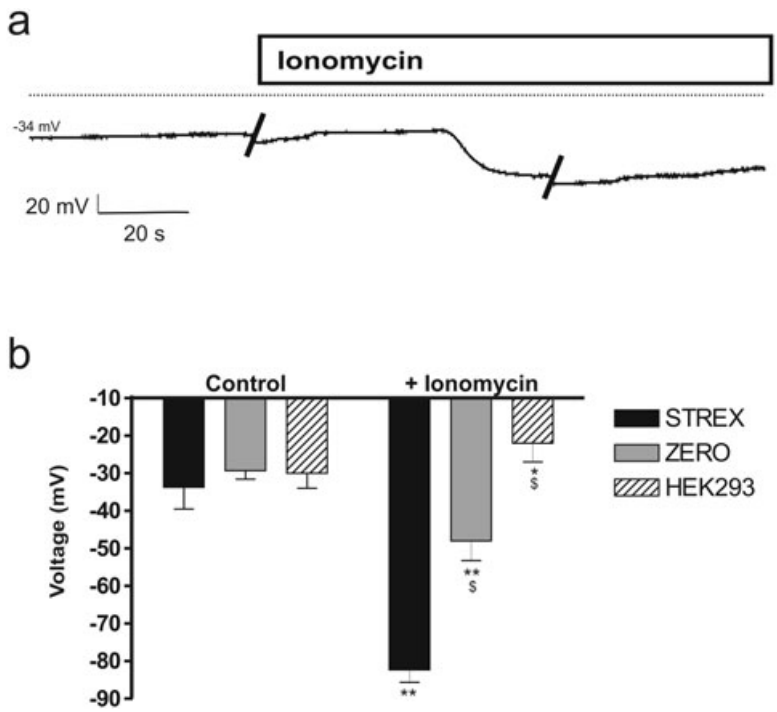

Figure 3 Ionomycin-induced membrane hyperpolarization in whole-cell current clamp recordings of transfected HEK293 cells. a. Representative trace of membrane potential from a HEK293 cell expressing the STREX BK channel splice variant. Ionomycin induces a robust membrane hyperpolarization from a resting membrane potential of −34 mV. b. Summary bar chart of mean resting membrane potential of mock-transfected HEK293 cells and cells transfected with either the STREX or ZERO variant before (Control) or after (+ ionomycin) application. Data are means ± SEM (n = 3–7/group). *P < 0.05, **P < 0.01 ANOVA with Tukey's *post hoc* test, compared with respective pre-treatment control. $^{\$}P$ < 0.01 compared with ionomycin STREX group. BK, large conductance calcium- and voltage-activated potassium channel; HEK293, human embryonic kidney 293 cell; STREX, stress-regulated exon.

concentrations of paxilline resulted in a small but significant decrease in resting membrane potential (Fig. 6) as a result of inhibition of STREX channel activity under resting conditions. The ability of STREX channels to make a small but significant contribution to the membrane potential is also supported by the clear STREX channel activity observed in cell-attached recordings under resting conditions (see Fig. 7). In HEK293 cells expressing the ZERO variant, channel blockade caused a reduction in membrane potential that was not statistically significant, in accordance with the low activity of these channels in the absence of calcium influx at rest. Importantly, subsequent stimulation with ionomycin failed to induce membrane hyperpolarization in cells expressing STREX channels (Fig. 6), or ZERO variant (data not shown). Finally, in cell-attached patch recordings, application of ionomycin resulted in a robust (6.75 ± 1.96 fold, n = 3) stimulation of STREX single-channel activity that was abolished upon subsequent application of paxilline (Fig. 7).

Discussion

The major findings of this study are that: (i) BK channel splice variants can be characterized in both high-throughput membrane potential dye-based assays, as well as by conventional patch clamp assays, based on their different sensitivity to activation by ionomycin-induced calcium influx; and (ii) BK channel splice variants were inhibited by the widely used, and selective, BK channel inhibitor paxilline with similar IC_{50}s, under conditions of near physiological calcium and voltage conditions. Thus, paxilline may be used in functional assays to inhibit BK channel function, irrespective of the variant expressed.

The magnitude of the ionomycin-induced hyperpolarization of HEK293 cells expressing different splice variants will be dependent upon several factors including: (i) the number of functional channels at the plasma membrane; (ii) single-channel conductance; (iii) proportion of cells transfected in the population; and (iv) the apparent calcium/voltage sensitivity and kinetics of the channel variants. The three variants assayed here all display similar cell-surface expression levels and single-channel conductance in HEK293 cells (Chen *et al.*, 2005). Thus, by using a combination of ionomycin to induce a calcium influx to stimulate BK activity, with the FLIPR membrane potential dye as a reporter of transmembrane potential, we were able to discriminate between these BK channel splice variants based on their sensitivity to calcium influx. Under our assay conditions, intracellular free calcium most likely rises to <1 $\mu mol \cdot L^{-1}$ (Fliegert *et al.*, 2007) and at these calcium levels, the three BK channel splice variants examined here display dramatically different half maximal voltages of activation (Chen *et al.*, 2005). The STREX variant has higher levels of activity at hyperpolarized membrane potentials for a given calcium concentration compared with either e22 or ZERO and in the membrane potential assays, the

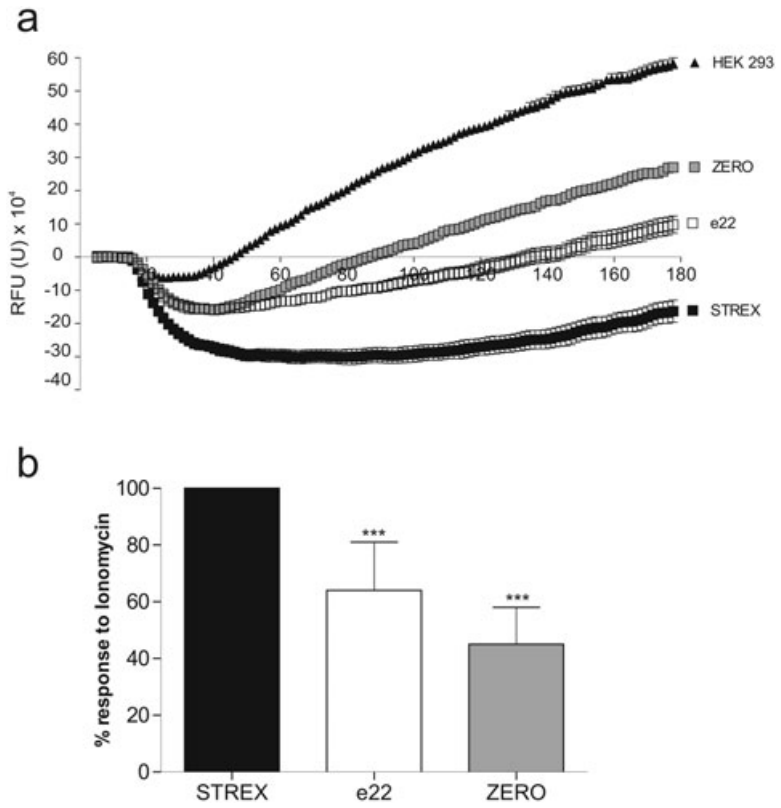

Figure 4 Fluorescent membrane potential assay discriminates BK channel splice variants. a. Time course plots of mean change in membrane potential assay RFU in response to ionomycin for mock-transfected HEK293 cells or cells expressing the STREX, e22 or ZERO splice variants respectively. Data are means ± SEM, $n = 8$. b. Summary bar chart of membrane hyperpolarization (determined at 70 s) in response to ionomycin for each variant normalized to the response of HEK293 cells expressing the STREX variant (100%). Data are means ± SEM, $n = 8$. ***$P < 0.001$ ANOVA with Tukey's *post hoc* test, compared with STREX. BK, large conductance calcium- and voltage-activated potassium channel; HEK293, human embryonic kidney 293 cell; RFU, relative fluorescent unit; STREX, stress-regulated exon.

rank order of ionomycin-induced hyperpolarization was STREX > e22 > ZERO, in accordance with the sensitivity of these variants in conventional electrophysiological assays (Chen *et al.*, 2005). Importantly, the ionomycin-induced hyperpolarization determined in the fluorescent membrane potential assays was also evident using conventional whole-cell current clamp recordings under identical conditions. Furthermore, the fluorescent membrane potential assay utilized here allows us to examine the properties of BK channel splice variants at near physiological calcium and voltage conditions in a medium-to high-throughput mode. This should allow examination of functional responses to pharmacological manipulation under conditions typically experienced by BK channels in native cells. Furthermore, as the magnitude of the hyperpolarization response is also dependent on the number of channels at the cell surface, as well as their single-channel conductance, this assay should allow us to screen for variants and/or channel mutants that display either cell-surface trafficking defects or dramatic alterations in single-channel conductance (see Zarei *et al.*, 2004; Chen *et al.*, 2005).

Alternative pre-mRNA splicing, as well as assembly with accessory β-subunits, can also dramatically modify calcium/voltage sensitivity of BK channels. This assay should allow screening of a wide variety of variants, mutations and accessory subunits. Important in this regard will be the selection of the membrane potential dye for investigation. For example, micromolar concentrations of the membrane potential dye, di-8-ANEPPS, increases the open probability of BK channels expressed in pituitary tumour cells (Wu *et al.*, 2008) and the oxonol dye $DiBAC_4(3)$ (Morimoto *et al.*, 2007) at 300 nmol·L^{-1} has been shown to activate BK channels coexpressed with the β1 and β4 subunits. Using the FLIPR membrane potential dye, we saw no effect of the dye in isolated patch clamp recordings when used at the same concentrations used for loading cells on BK channel α-subunits expressed in HEK293 cells (data not shown). Coupled with the improved temporal resolution of FLIPR membrane potential dye compared with di-8-ANEPPS or $DiBAC_4(3)$ (Whiteaker *et al.*, 2001; Yamada *et al.*, 2001; Wolff *et al.*, 2003), the assay conditions we exploited to analyse BK channel splice variant properties may provide useful insights into other modes of BK channel regulation.

One of the most potent and selective BK channel blockers is the tremorgenic alkaloid paxilline but the effect of paxilline on different splice variants was unknown. This issue is important as it is widely assumed that paxilline will inhibit BK channels, irrespective of their molecular composition, in contrast to the known modification of BK channel sensitivity to Iberiotoxin by β4 subunits (Behrens *et al.*, 2000; Brenner *et al.*, 2000a; 2005; Meera *et al.*, 2000). However, the effect of paxilline on different splice variants has not been systematically examined. Using the fluorescent membrane potential assay, we were able to show that paxilline dose-dependently inhib-

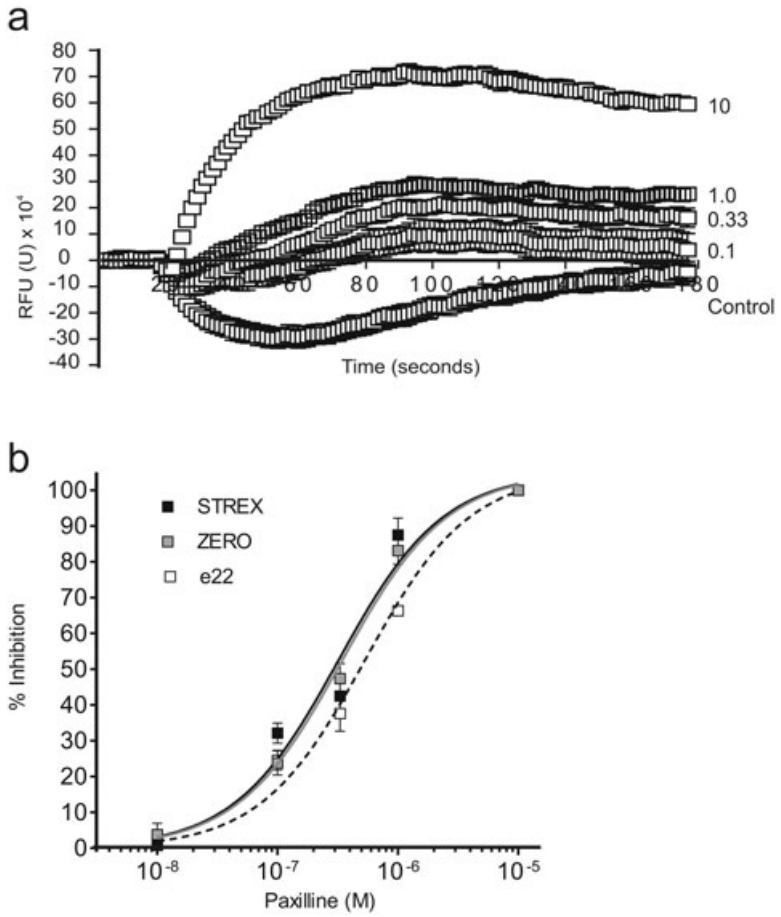

Figure 5 Paxilline inhibits BK channel splice variants with similar potency. a. Representative fluorescent membrane potential assay of HEK293 cells expressing the STREX variant pre-treated for 10 min with varying concentration of the BK channel inhibitor paxilline. Data are means ± SEM for four replicates per group from a representative assay. b. Summary dose–response curves for each variant expressed as the percentage inhibition of the ionomycin-induced response. Data are means ± SEM, $n = 4$ independent experiments. The IC_{50} values for the splice STREX, ZERO and e22 splice variants were 0.35 ± 0.04, 0.37 ± 0.03 and 0.70 ± 0.02 μmol·L^{-1} respectively. The IC_{50} for the e22 variant was statistically significantly different when compared with STREX or ZERO; $P < 0.001$; *F* test. BK, large conductance calcium- and voltage-activated potassium channel; HEK293, human embryonic kidney 293 cell; STREX, stress-regulated exon.

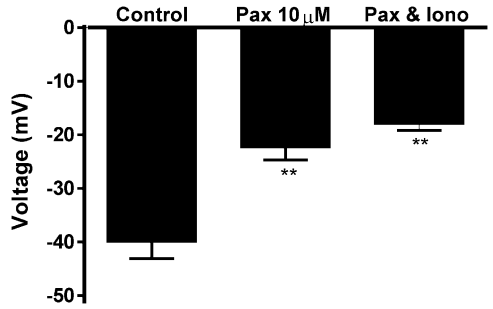

Figure 6 Paxilline blocked the ionomycin-induced hyperpolarization in whole-cell patch clamp assays. Summary data of the effect of paxilline (Pax 10 μmol·L^{-1}) on resting membrane potential of HEK293 cells expressing the STREX BK channel variant. Paxilline induced a significant membrane depolarization compared with control. However, in the presence of paxilline, ionomycin (Pax & Iono) failed to induce membrane hyperpolarization. Data shown are means ± SEM, $n = 3$ independent experiments. $^{**}P < 0.01$ ANOVA with Tukey's *post hoc* test, compared with pre-treatment control.

ited the ionomycin-induced hyperpolarization in HEK293 cells expressing the STREX, ZERO or e22 variants. Paxilline had a similar potency on all three splice variants, although there was a small (twofold), but significant, reduction in potency for the e22 variant compared with STREX or ZERO. The calculated IC_{50} values were 300 700 nmol·L^{-1}; these are approximately an order of magnitude greater than those derived from the effects of paxilline on inside-out patches in electrophysiological assays on single-ion channels from smooth muscle (Sanchez and McManus, 1996). Several factors may account for this difference, although dye interference can probably be excluded as it did not alter the effects of paxilline on mock-transfected HEK293 cells in our assays, nor did it modulate BK channel activity *per se*. The most straightforward explanation for the raised IC_{50} values is that the membrane potential and whole-cell assays utilized here reflect the functional impact of BK channels on membrane potential. Thus, when investigating the inhibition of such a large conductance channel, any small residual channel open

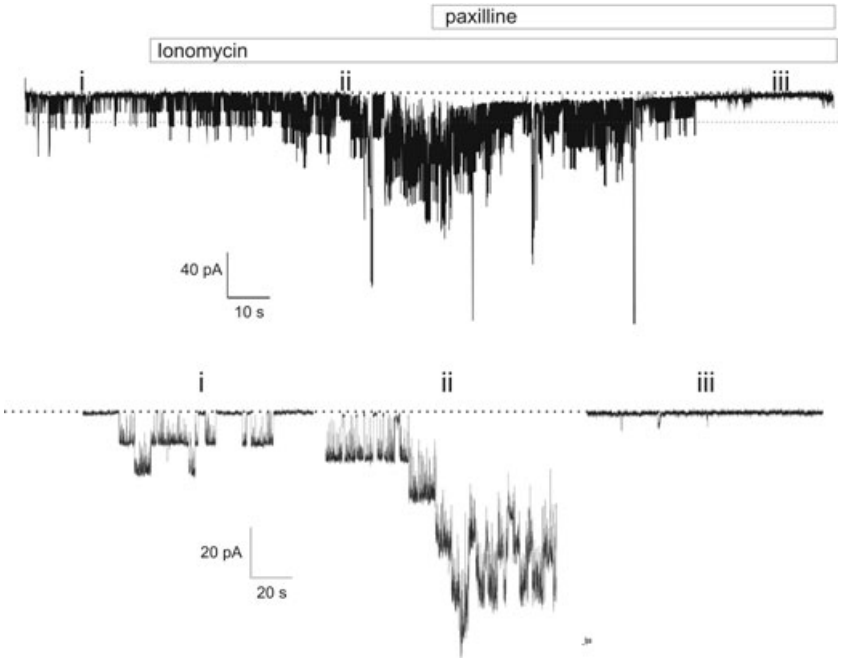

Figure 7 Ionomycin activates single BK channels in cell-attached patches. Representative continuous single-channel trace (upper trace) and expanded traces under different conditions (lower traces) in a cell-attached patch from a HEK293 cell expressing the STREX BK channel. Cells were bathed in physiological solution and the pipette potential was 0 mV. Application of ionomycin to the bath activates BK channel activity in the cell-attached patch. Note upon ionomycin application, BK single-channel amplitude increases as predicted as a result of cellular hyperpolarization upon BK channel activation in an unclamped cell. Subsequent application of paxilline fully inhibits single-channel activity. BK, large conductance calcium- and voltage-activated potassium channel; HEK293, human embryonic kidney 293 cell; STREX, stress-regulated exon.

probability, or single-channel conductance, will have a significant impact on the membrane potential. This is an important consideration when examining the potential functional impact of inhibiting BK channels in native cells.

Taken together, our data reveal that ionomycin-induced calcium influx can be exploited to discriminate between BK channel splice variants in both high-throughput fluorescent membrane potential assays and in patch clamp electrophysiological assays under conditions at physiological intracellular free calcium and voltage conditions in intact cells. Importantly, we have demonstrated that the BK channel inhibitor paxilline is practically equi-potent on these distinct splice variants and thus can be used to inhibit BK channels, irrespective of their splice isoform composition in native systems. This approach should also allow us to examine the functional consequence of BK channel association with distinct accessory subunits and/or mutations that affect calcium/voltage sensitivity.

Acknowledgements

FS was in receipt of an MRC PhD studentship. We are grateful to Jim McCulloch for use of the Flexstation. This work was supported by the Wellcome Trust.

Conflicts of interest

None.

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