Stable expression of the human large-conductance Ca^{2+}-activated K^+ channel α- and β-subunits in HEK293 cells

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Abstract We have generated HEK293 cell lines stably expressing high levels of either the human BK channel α-subunit alone or the BK channel α-subunit and β-subunit together. For co-expression a plasmid with three expression cassettes was constructed. Patch-clamp recordings on inside-out patches from the transfected cells resulted in macroscopic currents reflecting the expression of 200–800 BK channels per patch. No decrease in channel expression could be detected in cells grown for more than 50 passages. The α-subunit when expressed alone conducted currents which were sensitive to intracellular Ca^{2+} in the physiological range. In the presence of the β-subunit the steady-state activation curves were shifted by −20 to −30 mV and channel deactivation kinetics were slowed. The BK channel opener NS1608 (10 μM) shifted the steady-state activation curves for the α-subunit as well as for the αβ-subunits by −40 to −50 mV.

Key words: BK channel; Maxi-K channel; *hslo*; Beta-subunit; Stable expression

1. Introduction

Large-conductance Ca^{2+} activated K^+ channels (BK channels) represent a functional subtype of potassium channels with unique features. They display a high single-channel conductance and their gating mechanism is characterised by sensitivity to the concentration of intracellular Ca^{2+} and to membrane potential [1,2]. The α-subunit of the BK channel was first cloned from *Drosophila melanogaster* and later from murine and human tissues. The genes encoding these channels were designated *dslo*, *mslo* and *hslo*, respectively [2–6]. Several phenotypes of BK channels have been described in native cells [7,8], but only one functional BK channel gene has been identified hitherto. Thus, the molecular basis underlying the diversity may be attributed to alternative RNA splicing [5], post-translational protein modifications [9] or the association of accessory proteins with the pore forming α-subunit [10]. Recently the human BK-channel β-subunit homologue was cloned [11] and its function shown to be modulation of the BK channel. The BK channel is probably formed by tetramers of the α-subunit [12], and in some tissues the tetramer associates with the β-subunit [11]. Previous studies of the BK channel have primarily been performed in *Xenopus* oocytes and in mammalian cells transiently expressing the channel. While such systems may be sufficient for many applications they do suffer from being either heterologous and/or short-lived with variable expression levels. A continuous source of mammalian cells expressing high levels of channels would therefore greatly facilitate the investigation of gating properties and pharmacological modulation of the channels.

Using the recently cloned human BK channel α and β cDNAs we have generated stable HEK293 cell lines expressing high levels of either homomeric α or heteromeric αβ channels. These stable cell lines allowed the analysis of currents from homogeneous populations of BK channel molecules, and the high expression levels made it possible to characterise in detail the effects of Ca^{2+}, V_m, paxilline, and NS 1608 on the channel gating.

2. Materials and methods

2.1. Plasmid constructs

The BK channel α-subunit cDNA in its fully spliced form [5] was excised as a *Kpn*I, *Not*I fragment and cloned into the expression vector pcDNA3 (InVitrogen), pD3/BKα. Expression was under control of the CMV promoter and selection of stable clones was possible using G418 (Gibco BRL). The BK channel β-subunit cDNA was cloned into the expression vector pZeoSV (InVitrogen), pSV/BKβ as previously described [11]. Expression was under the control of the SV40 promoter and selection of stable clones was possible using Zeocin (InVitrogen). A third plasmid with expression cassettes for both genes was constructed as follows: a *Bam*HI fragment containing the entire expression cassette of the β gene (SV40 promoter, β cDNA and SV40 polyA sequence) was excised from the pSV/BKβ plasmid and cloned into the *Bgl*II site at position 12 of the pD3/BKα construct. A plasmid with the SV40 and CMV promoters pointing in the opposite directions pDS/BKαβ was used for transfection.

2.2. Cell culture and transfections

HEK293 (ATCC CRL-1573) cells were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum and 2 mM Glutamax (Gibco BRL). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were transfected with 5 μg of expression plasmid using lipofectamine (Gibco BRL) according to the manufacturer's protocol. Cells transfected with pcDNA3 constructs were selected in medium supplemented with 0.5 mg/ml G418 and cells transfected with pZeoSV constructs were selected in medium supplemented with 0.25 mg/ml Zeocin. Cells cotransfected with both types of plasmid constructs were selected with both G418 and Zeocin. Single clones were picked and propagated in selection media until sufficient cells for freezing were available, thereafter the cells were cultured in regular culture media without selection agent.

2.3. RT-PCR

RT-PCR was used to confirm the presence of α and β mRNA in clones. The Lysate mRNA Capture Kit for RT-PCR (Amersham) was used according to the manufacturer's protocol. The following primers were used: α sense (1643s): 5′-TGTCCTTCCCTACTGTTTGTGAGC-3′, α antisense (2081as): 5′-CAGTGAAACATCCCAGTAGAGTCG-3′, β sense (−45s): 5′-CCACAGGACGCCGGGAAGACTAA-3′, β antisense (678as): 5′-GAGGGCAGGTGGAGAAGGCATTGT-3′, GADPH: positive control primers supplied with the kit.

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Abbreviations: BK channel, big K^+ channel; CMV, cytomegalovirus; neo, neomycin; SV40, simian virus 40; NS1608, NeuroSearch compound 1608

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2.4. Patch-clamp electrophysiology

One to four days prior to patch-clamp experiments cells were seeded in 35 mm petri dishes containing glass coverslips (∅ 3.5 mm). Macroscopic BK currents were recorded in inside-out patches as previously described [13]. The currents were activated by command voltage steps from $V_h = -150$ mV to potentials between −150 and 150 mV (30–50 ms duration, 10 mV increments) followed by a jump back to −150 mV, at which potential the brief tail currents were measured. All experiments were conducted at room temperature. The data values are given as mean ± S.E.M. Two solutions (A and B) were used in the patch-clamp experiments. The solutions contained: 146 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, and either 1.3 mM EGTA (A) or 1.07 mM EGTA (B), to give calculated free Ca^{2+} concentration of 500 nM (A) and 1.8 μM (B) (Eqcal, Biosoft, Cambridge, UK). pH was adjusted to 7.2 with KOH. Paxilline was from Sigma, USA. NS 1608 was synthesised at NeuroSearch A/S [14].

3. Results

To generate a mammalian cell line expressing the BK channel α-subunit, pD3/BKα was transfected into HEK293 and stable clones were isolated using G418 selection. Several stable clones expressed high levels of functional BK channels as measured by patch-clamp electrophysiology. One clone named HEK/α-1.2 has been characterised in detail [13]. In order to generate cell lines expressing both the BK channel α-subunit and β-subunit three approaches were taken. The first was to transfect HEK/α-1.2 with pSV/BKβ and select stable clones with Zeocin, the second was to co-transfect HEK293 with both pcDNA3/BKα and pZeoSV/BKβ and select stable clones with G418 and Zeocin. Both these strategies yielded clones expressing αβ-subunits as described by us earlier [11]. However, the clones were not stable in long-term culture. The third approach was to make a plasmid encoding both the α and β genes together with a selection marker. To construct such a plasmid the entire expression cassette of the β gene (SV40 promoter, β cDNA and SV40 polyA signals) was excised from the pSV/BKβ construct and inserted into the pD3/BKα construct. The resulting construct was named pDS/BKαβ (Fig. 1) and isolated in two forms depending on the orientation of the β cassette. The plasmid with the promoters

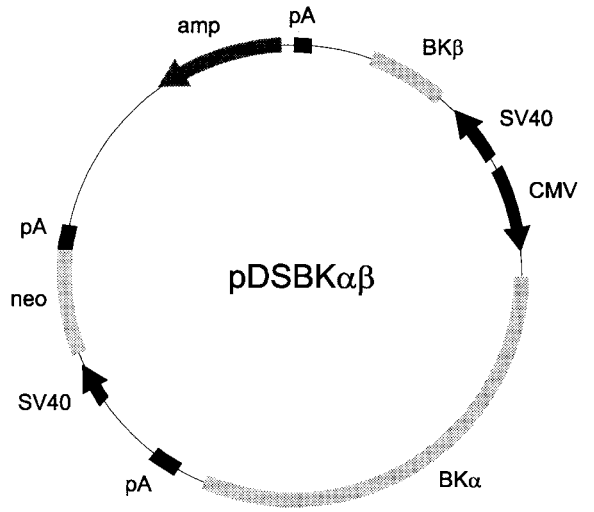

Fig. 1. The BK channel αβ expression vector pDS/BKαβ contains three eukaryotic expression cassettes. The promoters for these are shown as arrows, the expressed genes are shown light grey shaded areas and polyadenylation signals are shown as small boxes.

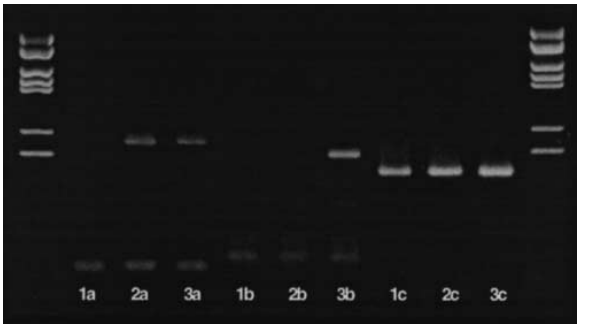

Fig. 2. RT-PCR of HEK (1a, 1b, 1c), HEK/α1-2 (2a, 2b, 2c) and HEK/αβ-B7 (3a, 3b, 3c). The PCR was performed with primers specific for the following genes: a: BKα, b: BKβ and c: GADPH.

SV40 and CMV pointing in the opposite direction was used to transfect HEK293 cells and stable clones were isolated using G418 selection. Twenty single clones were picked initially, cultured to passage level 5 and then tested for presence of mRNA by RT-PCR (Fig. 2). All showed expression of α and β although two clones seemed to have low levels of β expression. Eight clones were retested at passage 10, and four additional different clones were tested at passage 20. The presence of α and β mRNA could be detected by RT-PCR in all of these 12 clones. Some variation in band intensity among the β mRNA was noted (data not shown). A clone named HEK/αβ-B7 was chosen for detailed characterisation by electrophysiology studies.

Inside-out patches from HEK/αβ-B7 cells contained on average 500 channels (range 200–800) resulting in macroscopic K^+ currents. However, in a few patches it was possible to obtain single channel recordings from HEK/αβ-B7 cells and these revealed classical 230 pS BK channels in symmetrical K^+ (Fig. 3). The characteristics of these single channels were indistinguishable from BK channels in native cells with respect to unit conductance and sensitivity to charybdotoxin and TEA (data not shown).

The deactivation of α and αβ channels was characterised using the brief inward tail currents obtained following repolarisation to −150 mV in symmetric K^+ (Fig. 4a). The peak tail current, which is an index of channel open state probability, levelled off at high potentials indicating near maximal activation of the channels. Steady-state activation curves based on the normalised peak tail currents were constructed for the cells expressing α and αβ-subunits when exposed to Ca^{2+} at intracellular concentrations of 500 nM and 1.8 μM, and the data were fitted to Boltzmann functions (Fig. 4b). The activation curves were shifted towards more negative membrane potentials when the Ca^{2+} concentration was augmented, and the effect was quantified by the shift in the half-maximal activation potential, $V_{0.5}$ (Fig. 4c). The values in 500 nM free Ca^{2+} were: 95 ± 2 mV ($n = 6$) and 74 ± 2 mV ($n = 9$) for α and αβ, respectively, and the values in 1.8 μM free Ca^{2+} were: 18 ± 2 mV ($n = 6$) and −12 ± 5 mV ($n = 9$) for α and αβ, respectively. The β-subunit thus gave rise to a 21–30 mV shift depending on the concentration of free Ca^{2+}, and at both concentrations the shift was significant ($P < 0.01$). The BK channel deactivation kinetics after activation by a voltage step to 80 mV were also significantly influenced by the presence of the β-subunit, and the effect was quantified by fitting the decaying part of the tail current to a mono-exponential

function. (Fig. 4d). The values in 500 nM free Ca^{2+} were: 0.33 ± 0.04 ms ($n = 5$) and 1.49 ± 0.32 ms ($n = 9$) for α and αβ, respectively, and the values in 1.8 μM free Ca^{2+} were: 1.57 ± 0.26 ms ($n = 5$) and 5.09 ± 0.68 ms ($n = 3$) for α and αβ, respectively. Thus, the β-subunit slowed the deactivation by 3.1–4.5-fold ($P < 0.025$ and $P < 0.05$). The channel activation kinetics were not significantly influenced by the presence of the β-subunit. The effect of the BK channel opener NS1608 was not dependent on the presence of the β-subunit, since 10 μM NS1608 shifted the activation curves by an average of 52 mV and 48 mV for α and αβ, respectively. Furthermore, the BK channel blocker paxilline showed similar affinity for the homomeric and heteromeric channels (data not shown).

The transfected HEK cell lines expressing BK channels have proven to be stable. HEK/α-1.2 cultured for more than 40 cell culture passages has been used in patch-clamp electrophysiology studies without a decline in the currents measured (Fig. 5). HEK/αβ-B7 has also been found to be stable in passage numbers higher than 50 (data not shown).

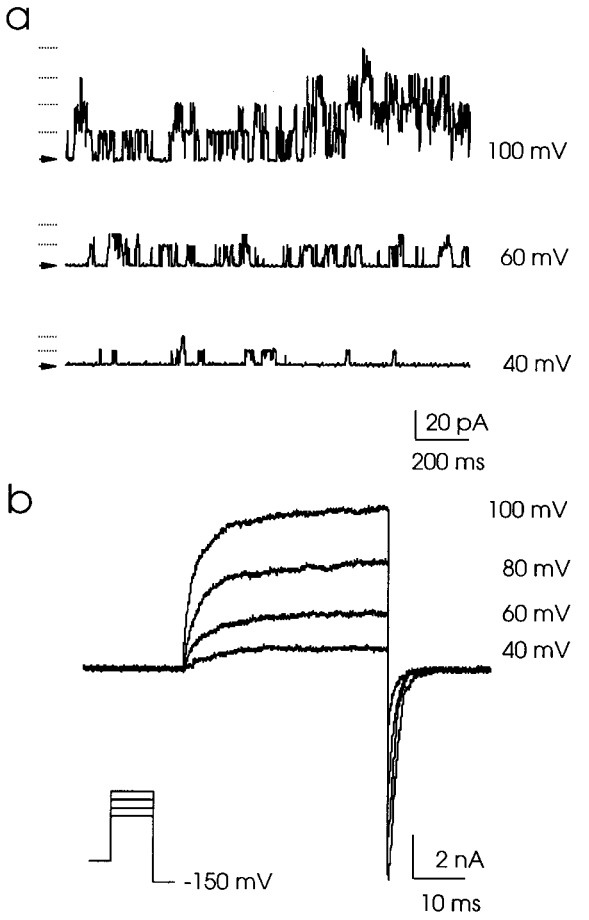

Fig. 3. Recordings of BK channel αβ-subunits in inside-out patch of HEK 293 cells. a: Patch containing four single channels. The channel open state probability increased with increasing positive potentials and the single channel conductance was calculated to be 230 pS. b: Patch containing at least 300 single channels. The potential was stepped from $V_h = -100$ mV to 40, 60 80, 100 mV where the steady-state activated macro currents corresponding to the single channel traces in (a) were recorded. The voltage was then stepped to −150 mV, at which potential the BK channel tail currents were determined. In both figures the pipette as well as the bath contained a high K^+ solutions with 300 nM free Ca^{2+} (150 mM KCl, 7.625 mM $CaCl_2$, 1.205 mM $MgCl_2$, 0.1 M EGTA and 10 mM HEPES at pH 7.2).

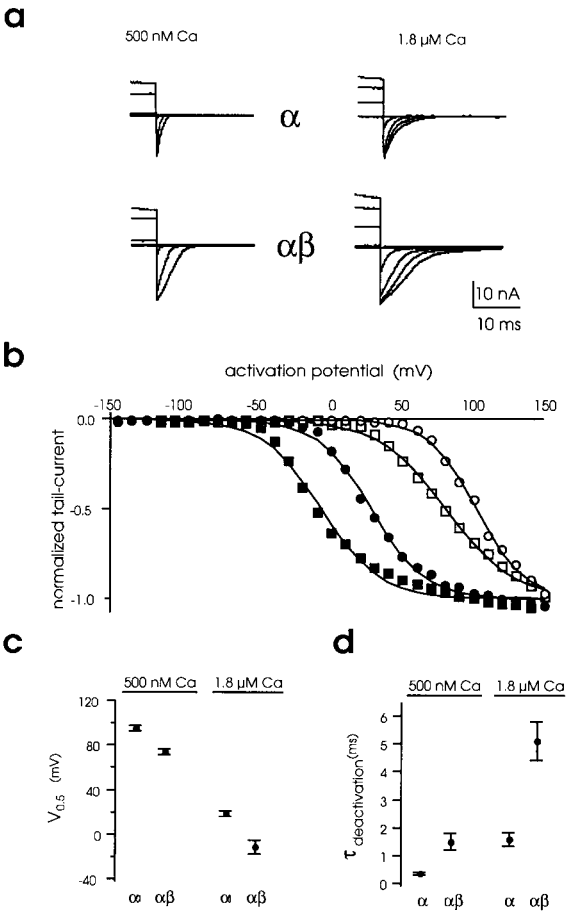

Fig. 4. Macro currents of BK channel α- and αβ-subunits in HEK293 cells. a: Currents activated in two inside-out patches containing the α-subunit (upper traces) or the αβ-subunits (lower traces). The traces depict the currents obtained by a command voltage step to −150 mV followed by a steady-state activation from −100 mV to 150 mV in 50 mV increments. The pipettes contained 500 nM free Ca^{2+} whereas the bath contained either 500 nM free Ca^{2+} in the experiments shown on the left or 1.8 μM free Ca^{2+} in the experiments shown on the right. b: Normalised peak tail currents obtained in 500 nM free Ca^{2+} (open symbols) and in 1.8 μM free Ca^{2+} (closed symbols) from patches containing the α-subunit (circles) or the αβ-subunits (squares). The lines show Boltzmann equations fitted to the data. c: Average half-maximal activation potential ($V_{0.5}$) ± S.E.M. obtained from Boltzmann fits similar to those shown in (b). d: Channel deactivation time constants found by fitting the decaying part of the tail current to a mono-exponential function.

4. Discussion

Several approaches were initially taken to generate stable clones expressing BK channel αβ-subunits. Transfection of β into a cell line already expressing α, or co-transfection of both subunits at once yielded clones which were stable for several passages. However, they were not stable in long-term cultures. Even though resistance to the given selection pressure was maintained, clones made by the first approach lost expression of the β-subunit, and clones made by the second approach lost expression of either one or both subunits. To circumvent the problems of having expression of one and not the other

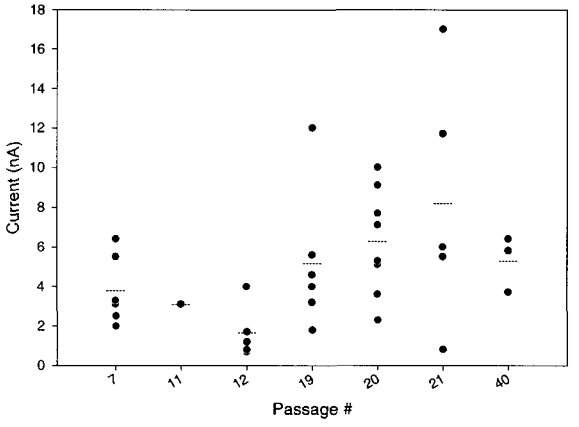

Fig. 5. Stability of HEK/α1-2. Outward currents were measured at 100 mV in solution A (500 nM free Ca^{2+}) in inside-out patches from HEK/α1-2 cells in different passages of cell culture.

subunit, we chose the strategy of linking the α and β genes on the same plasmid, and several long-term stable clones expressing both subunits were obtained. The HEK/α-1.2 clone has been used in patch-clamp experiments at passage numbers higher than 40 and the HEK/αβ-B7 at passage numbers higher than 50. Furthermore, the HEK/αβ-B7 clone was continuously followed by RT-PCR and no sign of instability has been noticed.

The β-subunit was found to increase the open state probability of the channel at all membrane potentials. This effect was seen as a leftward shift of the fitted activation curves by 20–30 mV and the shift was largest at the highest concentration of $[Ca^{2+}]_i$. The qualitative effects of the β-subunit on steady-state activation presented in this report are in concordance with the published data, although the exact size of the leftward shift varies from one report to the other [11,15–17].

The kinetics of activation and deactivation were studied by fitting the traces to mono-exponential functions. The co-expression of the β-subunit slowed the deactivation of the channel. We did not, however, find any significant differences between the activation time constants of α and αβ when measured at 80 mV. The slower deactivation kinetics may significantly influence the activity of the αβ heteromeric channel following transient depolarisations such as action potentials. The effect of the β-subunit on non-stationary kinetics appears similar to that of the BK channel opener NS1608. The effects of the β-subunit and NS 1608 are additive, which suggest the existence of several possible modulation sites on the α-subunit.

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