

Supporting Online Material for

BK_{Ca} -Cav Channel Complexes Mediate Rapid and Localized Ca^{2+} -activated K^+ Signaling

Henrike Berkefeld, Claudia A. Sailer, Wolfgang Bildl, Volker Rohde, Jörg-Oliver Thumfart, Silke Eble, Norbert Klugbauer, Ellen Reisinger, Josef Bischofberger, Dominik Oliver, Hans-Günther Knaus, Uwe Schulte,* Bernd Fakler*

*To whom correspondence should be addressed. E-mail: bernd.fakler@physiologie.uni-freiburg.de (B.F.); u.schulte@logopharm.com (U.S.)

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Supporting Online Material

Material and methods

Biochemistry

Plasma membrane-enriched protein fractions were prepared from freshly isolated adult rat brains according to (1) and solubilized at 4° C with ComplexioLyte48 (neutral detergent buffer, 1-1.25 mg/ml) with protease inhibitors added and cleared by ultracentrifugation (30 min at 150.000xg). For 2-dimensional gel electrophoresis, the solubilized material was supplied with Coomassie R250 (0.0125 % final concentration) and used for blue native gel electrophoresis (BN-PAGE) on a linear 1-11% PAGE gel (2, 3). After equilibration in 2x Laemmli buffer, excised BN-PAGE gel lanes were placed on a 10% SDS-PAGE gel (with a 4% stacking gel). For affinity purification, the solubilized material (6 mg of fractionated protein) was incubated for 2 hours at 4°C with 25 µg immobilized affinity purified rabbit anti-BK $\alpha_{(1118-1132)}$ ((4), termed anti-BK α), affinity-purified rabbit anti-BK $\alpha_{(1184-1132)}$ (termed *anti-BK* α^*), affinity-purified rabbit *anti-Cav1.2*₍₇₇₉₋₈₄₆₎ or various control IgG pools. After brief washing, bound proteins were eluted with Laemmli buffer (DTT added after elution). For MS-sequencing, eluates were shortly run on SDS-PAGE minigels, divided into three molecular-weight ranges and separately subjected to in-gel trypsin digestion and subsequent LC-MS/MS analysis (5).

Cultured tsA cells (1-2 days after transfection) and *Xenopus* oocytes (4-5 days after injection of cRNA) were homogenized in 500 µl PBS (with protease inhibitors) with glass potters and centrifuged for 3 min at 1000xg to remove yolk and/or nuclear material. Crude membrane preparations were obtained from the homogenates by ultracentrifugation (20 min at 100.000xg). Membranes were solubilized using ComplexioLyte48 (1 ml per 1.25 mg membrane protein) and subjected to analytical affinity purification as described before. After electroblotting of SDS-PAGE-resolved samples on PVDF membrane, the blot was split and subjected to Western analysis with rabbit *anti-Cav1.2*, rabbit *anti-Cav2.3*₍₈₉₂₋₉₀₇₎ (upper half >150 kDa), and rabbit *anti-BK* α ₍₉₁₃₋₉₂₆₎ (6) (lower half <150 kDa); antibody-stained bands were visualized by anti-rabbit IgG-HRP and ECL+.

Mass spectrometry

Trypsinized peptides were vacuum-dried and redissolved in 0.5% trifluoroacetic acid. Using an UltiMate 3000 HPLC system peptide samples were concentrated on a C18 PepMap100 precolumn (5 μ m) and separated on ReproSil-Pur 120 ODS-3 (C18; 3 μ m) manually packed into a PicoTipTM Emitter (75 μ m; tip: 8 ± 1 μ m). Peptides were eluted with an aqueous-organic gradient (solvent A:

0.5% acetic acid; solvent B: 80% acetonitrile / 0.5% acetic acid; gradient: 60 min from 3% B to 30% B, 15 min to 100% B; flow rate: 300 nl/min) and sprayed into a LTQ-FT mass spectrometer with a nano-electrospray source. Each scan cycle consisted of one FTMS full scan and up to five ITMS dependent MS/MS scans of the five most intense ions. The number of acquired MS/MS spectra per peptide ion was restricted by enabling dynamic exclusion (duration 30 sec, mass width 20 ppm) and monoisotopic precursor selection. Using the Mascot search engine extracted MS/MS spectra were searched against the NCBInr database (mammalia) accepting common variable modifications and one missed trypsin cleavage. Peptide tolerance was \pm 10 ppm and MS/MS tolerance was \pm 1.0 Da. Peptides with a Mascot score below 20 were skipped.

Two semiquantitative parameters were used to compare the amount of selected proteins:

(i) Relative exponentially modified sequence coverage (remSC) calculated as the ratio of exponentially modified sequence coverages (7) obtained for any given protein from eluates of the *anti-BK* α or the *anti-Cav1.2* antibody and control IgG pools; sequence coverage (SC) per se is the primary sequence actually identified in MS/MS spectra versus primary sequence theoretically identifiable in MS/MS analyses.

(ii) Relative peptide query score (rPQ-Score) is the ratio of peptide queries (PQ) obtained for any given protein from eluates of the *anti-BK* α or the *anti-Cav1.2* antibody and control IgG pools; the PQ value of a protein is defined as the sum of all Mascot queries (MS/MS spectra). For proteins not detected in the controls, a detection limit of 0.125 (queries) was used as a denominator in the rPQ score.

Specificity of co-purification was judged from quantitative comparison between the amounts of a given protein in eluates from *anti-BK* α / *anti-Cav1.2* and from control IgG pools (defining 'background binding activity'). For this purpose the rPQ scores of the 100 most abundant proteins of the IgG eluate (that were also present in the *anti-BK* α eluates) were determined and binned in 0.2 log intervals. The resulting distribution of the log rPQ scores was used to derive the specificity threshold at a log rPQ value of 0.6 (Fig. S1). Accordingly, proteins with values for the rPQ-Score > 4 were regarded specifically purified by the *anti-BK* α or the *anti-Cav1.2* antibodies.

Molecular biology

Preparation and injection of cRNA into *Xenopus* oocytes and site-directed mutagenesis were done as described (8), cell culturing and transfection of cDNAs were performed as detailed in (9). All cDNAs were verified by sequencing; genebank accessions of the clones used are (A48206 (BK α , gift of Dr. L. Salkoff),

NM_021452.1 (BKβ4), M67515.1 (Cav1.2), X57477.1 (Cav2.1(I1520H), gift of Dr. J. Yang), NM_017346.1 (Cavβ1b), NM_012828.1 (Cavβ3), AF286488.1, (α2δ)).

For profiling expression of Cav and BK_{Ca} channels in chromaffin cells by PCR (Fig. 6B), mRNA was isolated with the Trizol method, followed by reverse transcription (Superscript II) and ethanol precipitation of the cDNA as described in (*10*). PCR was performed with AmplitaqGold polymerase (Applied Biosystems) by applying 10min 95°C followed by 40 cycles with 95°C for 15s and 1min at 60°C. The following primers were used: Cav1.2 (fwd: catgggcgacagtgtcagaa, rev: catagcgtcttgcatccagtaca), Cav2.1 (fwd: cggccgcattcactataagg, rev: ggtaggtccatccgcaagag) and BK α (fwd: cccgtccacagcaaatcg, rev: gggataggcattatccgtcta); primers were designed to be intron-overspanning and optimized for an annealing temperature of 60°C using PrimerExpress 2.0 software.

Electrophysiology and data analysis

Electrophysiological recordings from giant inside-out patches excised from oocytes were performed at room temperature (22-24 °C) as described previously (8). Briefly, currents were recorded with an EPC9 amplifier, low-pass filtered at 1-3 kHz, and sampled at 5-10 kHz; capacitive transients were compensated with the automated circuit of the EPC9. Pipettes made from thick-walled borosilicate glass had resistances of ~0.3 MOhm when filled with (in mM) 115 NaMES, 5 KMES, 5 HEPES and 1.3 CaCl₂, pH adjusted to 7.2. Intracellular solution (K_{int}) applied via a gravity-driven multi-barrel pipette was composed as follows (mM): 120 KMES, 5 HEPES, 5 or 10 EGTA or BAPTA (pH 7.2). For determination of BK_{Ca} channel activation at defined [Ca²⁺]_i (Fig. 3D) the amount of CaCl₂ required to yield the free [Ca²⁺]_i indicated was calculated with WEBMAXC v2.22 and verified with a Ca²⁺ sensitive electrode.

Whole-cell patch-clamp recordings on Chinese hamster ovary (CHO) cells were done at room temperature (22-24 °C) as previously described (*11*). Briefly, currents were recorded with an Axopatch 200B amplifier, filtered at 2-10 kHz and sampled at 25 kHz. Recording pipettes made from quartz glass had resistances of 1-5 MOhm when filled with (in mM) 135 KCl, 3.5 MgCl2, 2 NaATP, 5 EGTA/BAPTA and 5 HEPES (pH 7.4). The bath solution contained (in mM) 144 NaCl, 5.8 KCl, 0.9 MgCl₂, 1.3 CaCl₂, 0.1 NaH₂PO₄, 5.6 D-glucose and 10 HEPES (pH 7.4).

Activation time course of BK_{Ca} currents was characterized by the time constant ($\tau_{activation}$) derived from a monoexponential fit to the rising phase of the current. Interval between the onsets of Cav and BK_{Ca} currents is defined as the period between the onset of the inward Cav current and its apparent maximum (deflection point) marking the onset of the outward BK_{Ca} current. Steady-state activation curves (Fig. 3D, Figs. S3, S4) were determined with a tail current

protocol as detailed in the legends. Data were normalized and fitted with a Boltzmann function: $I(norm) = 1 - 1 / (1 + exp((V - V_{1/2}) / k)); V_{1/2}$ is voltage required for halfmaximal activation, k is the slope factor. Curve fitting and further data analysis were done with Igor Pro 4.05A on a Macintosh G4. Data are given as mean ± SD throughout the manuscript.

Chromaffin cells were prepared as described by (*12*). Briefly, adrenal glands were removed from P2-4 rats; isolated chromaffin cells were obtained by papain treatment. Isolated cells were put on glass cover slips and incubated for at least 24h at 37°C and 9% CO₂ in DMEM supported with penicillin/streptomycin and 10ml/l insulin-transferrin-selenium-X. Solutions for whole-cell recordings were identical to those described above for experiments with CHO cells, except for addition of TTX (1 μ M) and apamin (200 nM, to block SK channels). Subtraction of currents recorded without the preconditioning depolarization step to 20 mV was used to isolate Cav-triggered BK_{Ca} outward currents.

Supplementary Figures

Fig. S1

Histogram illustrating the distribution of the log rPQ scores of the 100 most abundant proteins of the IgG eluate compared to BK_{Ca} and Cav channel subunits co-purified with the *anti-BK* α antibody. Bars in grey represent the number of IgG-bound proteins in the respective 0.2 log intervals, bars in red represent the indicated BK_{Ca} and Cav channel proteins (as listed in Table 1, Part I).

Dashed green line is specificity threshold. The bar filled in pink denotes a protein candidate that binds to the $BK_{Ca}\alpha$ subunit, and is presently investigated for its functional significance.

Fig. S2

A, Western probed full-length gels of co-purifications with *anti-BK* α (left) or *anti-Cav1.2* (right) antibodies of BK_{Ca} and Cav channels from culture cells expressing BK_{Ca} (BK α , BK β 4) and Cav1.2 (Cav1.2 α , Cav β 1b, α 2 δ) channels. Experimental conditions and Western blotting as in Fig. 2B.

B, Co-purification of BK_{Ca} and Cav channels from *Xenopus* oocytes expressing BK_{Ca} (BK α , BK β 4) and Cav2.1 (Cav2.1(I1520H), Cav β 1b or Cav β 3, α 2 δ) channels. Left panel, SDS-PAGE-separated eluate of an affinity purification with *anti-BK\alpha* Western-probed as indicated. Right panel, ion chromatogram as in Fig. 2A of one (out of nine) Cav2.1-specific peptides obtained, amino acid sequence of the peptide is indicated (m/z ratio of peptide was 713.37543; this m/z was not detected in affinity purifications with IgGs). MS/MS analysis was used instead of Western blotting since antibodies specific for the rabbit Cav2.1 subunit used are not available.

Fig. S3

A, Steady-state activation curve of Cav2.1(I1520H) channels recorded in i-o patches excised from oocytes expressing Cav2.1 α , Cav β 3 and α 2 δ under physiological ion conditions (1.3 mM extracellular Ca²⁺) with a tail-current protocol (inset). From a holding potential of -80 mV patches were depolarized to potentials between -80 and 50 mV (for between 5 and 10 ms); the following tail potential was -100 mV. Data points (normalized maximal current at tail potential) are mean ± SD of 7 patches. Continuous line is the fit of a Boltzmann function to the data with values for V_{1/2} and slope factor of -22.1 mV and 7.6 mV.

B, Functional coupling of Cav2.2 (Cav2.2 α , Cav β 3, α 2 δ) and BK_{Ca} (BK α , BK β 4) channels. Experimental conditions as in Fig. 3A, but with 10 mM EGTA at the cytoplasmic side of the i-o patches. Data points are mean ± SD of 3 experiments.

C, Experiments with Cav2.1 (Cav2.1(I1520H), Cav β 3, α 2 δ) and BK_{Ca} (BK α , BK β 4) channels as in Fig. 3A, but with 5 mM BAPTA at the cytoplasmic side of the i-o patches. Data points are mean ± SD of 5 experiments.

Fig. S4

A, **B**, Steady-state activation curve and representative current traces of Cav1.2 (A) and Cav2.3 (B) channels illustrating the functional characteristics of these channels when heterologously expressed in CHO cells (the respective Cav α subunit together with Cav β 1b and α 2 δ)

Steady-state activation curves were determined as in Fig. S2A; data points (normalized maximal current at tail potential) are mean \pm SD of 6 and 4 cells for Cav1.2 and Cav2.3, respectively. Continuous line is the fit of a Boltzmann function to the data with values for V_{1/2} and slope factor of -10.1 mV and 6.6 mV (Cav1.2) and 0.6 mV and 9.7 mV (Cav2.3).

C, Sensitivity of Cav1.2-BK_{Ca} coupling to Ca²⁺-buffers. Successive whole-cell recordings of BK_{Ca} currents activated by co-expressed Cav1.2 channels (25 ms after step depolarization to 30 mV) with the indicated Ca²⁺ buffers provided by the whole-cell pipette. Current responses at the time points indicated are shown in the lower panel. After measurements with one buffer solution, the pipette was gently withdrawn to allow re-sealing of the cell before repatching with the next buffer solution (see insets). Note the reversible abolishment of BK_{Ca} currents upon exchanging EGTA with BAPTA.

References

- 1. C. A. Sailer *et al.*, *J. Neurosci.* **22**, 9698 (2002).
- 2. H. Schagger, W. A. Cramer, G. von Jagow, *Anal. Biochem.* **217**, 220 (1994).
- 3. H. Schagger, G. von Jagow, Anal. Biochem. **199**, 223 (1991).
- 4. S. G. Wanner *et al.*, *Biochemistry* **38**, 5392 (1999).
- 5. U. Schulte *et al.*, *Neuron* **49**, 697 (2006).
- 6. H. G. Knaus *et al.*, *J. Neurosci.* **16**, 955 (1996).
- 7. Y. Ishihama *et al.*, *Mol. Cell. Proteomics* **4**, 1265 (2005).
- 8. B. Fakler *et al.*, *Cell* **80**, 149 (1995).
- 9. J. Ludwig et al., Proc. Natl. Acad. Sci. USA 98, 4178 (2001).
- 10. B. Liss, *Nucleic Acids Res.* **30**, 89 (2002).
- 11. W. Bildl *et al.*, *Neuron* **43**, 847 (2004).
- 12. J. B. Sorensen *et al.*, *Cell* **114**, 75 (2003).



Figure S1, Berkefeld et al.

culture cells: BK_{Ca} + Cav1.2



В

A





Figure S2, Berkefeld et al.





Figure S4, Berkefeld et al.