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BK_{Ca}-Cav Channel Complexes Mediate Rapid and Localized Ca²⁺-Activated K⁺ Signaling

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Large-conductance calcium- and voltage-activated potassium channels (BK_{Ca}) are dually activated by membrane depolarization and elevation of cytosolic calcium ions (Ca²⁺). Under normal cellular conditions, BK_{Ca} channel activation requires Ca²⁺ concentrations that typically occur in close proximity to Ca²⁺ sources. We show that BK_{Ca} channels affinity-purified from rat brain are assembled into macromolecular complexes with the voltage-gated calcium channels Cav1.2 (L-type), Cav2.1 (P/Q-type), and Cav2.2 (N-type). Heterologously expressed BK_{Ca}-Cav complexes reconstitute a functional "Ca²⁺ nanodomain" where Ca²⁺ influx through the Cav channel activates BK_{Ca} in the physiological voltage range with submillisecond kinetics. Complex formation with distinct Cav channels enables BK_{Ca}-mediated membrane hyperpolarization that controls neuronal firing pattern and release of hormones and transmitters in the central nervous system.

arge-conductance Ca^{2+} and voltageactivated K⁺ channels (BK_{Ca} or K_{Ca}1.1) are fundamental modulators of neuronal signaling (1, 2) by contributing to action potential repolarization (3, 4), mediating the fast phase of afterhyperpolarization (3, 5–8), controlling dendritic Ca²⁺ spikes (9), and establishing a feedback loop between membrane potential and cytosolic Ca²⁺ that regulates release of hormones and transmitters (10–13).

The physiological functions of BK_{Ca} channels arise from their unique allosteric activation by two distinct stimuli, membrane depolarization and cytosolic Ca^{2+} ions (14–16). Increasing Ca^{2+} concentrations ($[Ca^{2+}]_i$) shift the depolarization required for channel opening into the physiological voltage range. In fact, $[Ca^{2+}]_i$ of $\geq 10 \,\mu\text{M}$ are usually required for activating BK_{Ca} channels at membrane potentials around 0 mV (17). In central nervous system (CNS) neurons, such high levels of [Ca²⁺]_i are tightly restricted in time and space to local "Ca2+-signaling domains" centered around voltage-activated Ca²⁺ (Cav) channels (18, 19). In these domains, speed and magnitude of Ca²⁺ signals are inversely related to the distance from the Ca^{2+} source and are assessed experimentally by the distinct properties of the Ca²⁺ chelators EGTA and BAPTA. Thus, Ca^{2+} -sensitive processes affected by millimolar concentrations of BAPTA but not EGTA are assumed to be placed within ~20 nm from the Cav channels (nanodomain), while processes with an equal BAPTA/EGTA sensitivity are located between 20 and 200 nm (microdomain) or even further away from the Ca²⁺ source (*18*).

Functional characterization in various types of neurons provided two hallmarks for the activation of BK_{Ca} under normal conditions. First, BK_{Ca} channels reside in close spatial proximity to Cav channels, as they were robustly activated by Ca²⁺ influx through the Cav channels in the presence of EGTA, whereas BAPTA at millimolar concentrations largely attenuated or abolished the functional channel-channel coupling (3, 13, 20-22). Second, BK_{Ca} channels appear to be selectively activated by a subset of Cav channels with distinct functional properties and subcellular distribution. Thus, P/Q-, N- and L-type Cav channels activate BK_{Ca} either selectively or concertedly in nerve terminals, dendrites, or somata of various types of CNS neurons (3, 6, 13, 20, 23, 24).

Despite its fundamental importance for the physiology of BK_{Ca} channels, the mechanism underlying the intimate and selective association between BK_{Ca} and Cav channels is as yet unknown, and selective coupling between BK_{Ca} and Cav channels in heterologous expression systems has not been demonstrated.

Affinity Purification of BK_{Ca} Channel Complexes from Rat Brain

We used affinity purifications (AP) with two different $BK_{Ca}\alpha$ subunit-specific antibodies (anti- $BK\alpha$ and anti- $BK\alpha^*$ Abs) on solubilized plasma membrane–enriched protein fractions prepared from total rat brain (25). Separation by blue native

and subsequent denaturing gel electrophoresis showed that these protein fractions contained high-molecular-weight complexes of BKCa channels [Fig. 1A, (26)]. Total eluates obtained in APs with the two anti-BKa Abs and with several immunoglobulin G (IgG) pools (preimmunization IgGs and antibodies unrelated to BK α) serving as a control were subjected to analysis by nanoflow liquid chromatography tandem mass spectrometry (nano-LC MS/MS) (Fig. 1A). This approach identified the α subunit of BK_{Ca} channels (BK α) by retrieving \geq 66 different peptide fragments (for each anti-BKa Ab) covering ~75% of the BKa primary sequence (Fig. 1, B and C and Table 1, top). In addition, MS/MS spectra from the anti-BKa eluates unambiguously identified the two BK β subunits (BK β 2 and BK β 4) expressed in the CNS (17), as well as several Cav channel α and β subunits (Table 1, top). The Cava subunits specifically retained by both anti-BKa Abs were Cav1.2, Cav2.1 and Cav2.2 (Table 1 and fig. S1) (26) encoding the pore-forming subunits of the L-, P/Q- and N-type Cav channels, respectively (27). In fact, Cav2.1 was the protein most abundantly copurified with BKα; all together, MS/MS analyses detected 43 different peptides covering ~44% of the Cav2.1 amino acid sequence. Similar sequence coverage was obtained for the specifically copurified Cavß subunits Cav\beta1b, Cav\beta2, and Cav\beta3 (Table 1 and fig. S1). In contrast, Cav2.3, R-type Cav channels (27), and the Cav β 4 subunit were detected in the eluates from both anti-BKa Abs and control IgGs with similar abundance (Table 1 and fig. S1).

Coassembly with Cav1.2 channels was confirmed by subsequent reverse purification using an antibody specific for the Cav1.2 subunit (anti-Cav1.2) and suitable for AP from rat brain plasma membranes. As illustrated in Fig. 2A by the ion chromatogram (left) and the MS/MS spectrum (right) of one out of the eight unique peptides obtained, BK α was copurified by anti-Cav1.2 but not by the control IgG pools (Table 1, bottom).

Coassembly of Heterologously Expressed BK_{Ca} and Cav Channels

The copurification of BK_{Ca} with specific Cav channel subtypes from rat brain plasma membranes was reproduced by APs from culture cells that heterologously expressed BKCa channels and either Cav1.2 or Cav2.1 channels. For these experiments, the respective channel subunits BK α and BK β 4, as well as Cav1.2 or Cav2.1. CavB1b or CavB3, and $\alpha 2\delta$ (28), were transfected into culture cells or injected as cRNAs into Xenopus oocytes (26). Figure 2B and fig. S2A illustrate the results of coimmunoprecipitations using anti-BKa and anti-Cav1.2 on the BK_{Ca}-Cav1.2 coexpressions in culture cells. Thus, anti-BKa effectively and specifically retained the Cav1.2 subunit, and anti-Cav1.2 coprecipitated the BKa subunit with similar efficiency. An equivalent result was obtained from an AP using anti-BKa on Xenopus oocytes coexpressing BK_{Ca} and Cav2.1 channels. In this experiment, copurification of the Cav

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channel was verified by MS/MS analysis that retrieved 31 peptides for BKa and 9 and 11 peptides specific for the Cav2.1 and Cavβ3 subunits, respectively (fig. S2B).

Functional Characteristics of BK_{Ca}-Cav Complexes

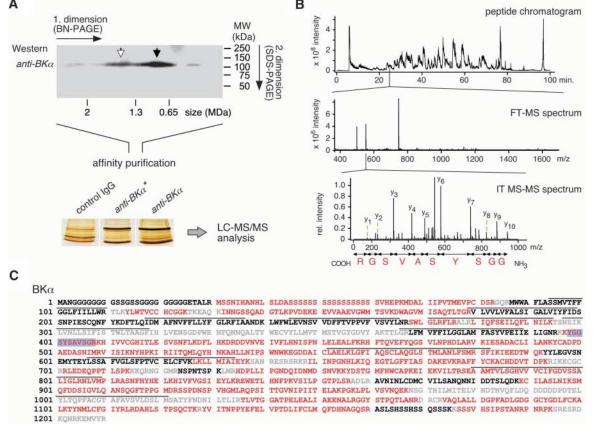
Next, the functional properties of BK_{Ca}-Cav2.1 channel complexes were investigated in giant insideout patches (26) excised from Xenopus oocytes coexpressing BK_{Ca} (BK α and BK β 4) and rundown deprived Cav2.1 channels [Cav2.1(I1520H), Cav β 3, and α 2 δ (29)]. Figure 3A shows typical current traces recorded in response to step depolarizations in the physiological voltage range. Thus, for voltage steps above the activation threshold of Cav2.1 channels, current responses were biphasic (in all 135 patches tested): an initial Ca²⁺ inward current that was followed by an outward K⁺ current, as would be expected for activation of BK_{Ca} channels by influx of Ca²⁺ ions through the Cav2.1 channels (Fig. 3A). The coupling between both channels was mandatory for activation of BK_{Ca} in the physiological voltage range as shown by control experiments lacking expression of the

Fig. 1. Affinity purification of BK_{Ca} channel complexes from CNS plasma membranes. (A) (Top) High-molecular-weight complexes of BK_{Ca} channels in solubilized plasma membrane-enriched protein fractions from rat brain visualized by twodimensional gel electrophoresis (26). Solubilized protein complexes were separated by blue native polyacrylamide gel electrophoresis (BN-PAGE, 1. dimension) and denaturing SDS-PAGE (2. dimension), and were subsequently Westernprobed with anti-BK α (42). BK_{Ca} channels $(4BK\alpha + 4BK\beta)$ indicated by filled arrowhead, main fraction of high-molecular weight complexes of BK_{Ca} channels denoted by open arrowhead. (Bottom) Eluates of APs with two different anti-BK α Abs (anti-BK α and anti-BK α^*) or a pool of preCav channels (Fig. 3B). The time course of the Cav2.1-BK_{Ca} coupling reflected by the interval between the onsets of Ca^{2+} and K^{+} currents (26) was voltage-dependent and markedly reduced by membrane depolarization. At potentials positive to 0 mV, this time interval was less than one millisecond (Fig. 3A, inset); mean values for the duration (n = 15 experiments) were 0.75 ± 0.24 ms (at 20 mV) and 0.95 ± 0.18 ms (at 10 mV). The amplitude of the Cav channel-activated BKCa current was strongly voltage-dependent and exhibited a bell-shaped steady-state current-voltage (I-V) relation with a peak amplitude at about 20 mV (Fig. 3B). Both the shape of the I-V relation and the time course of the Cav2.1-BK_{Ca} coupling are a reflection of several factors, including the voltagedependent gating properties of Cav2.1 (Fig. 3C and fig. S3A) and BK_{Ca} channels, the amplitude of the Ca^{2+} current (Fig. 3C), and the Ca^{2+} sensitivity of BK_{Ca} channels (Fig. 3D). In particular, increase in open probability and faster activation kinetics of the Cav channels promote accelerated coupling (Fig. 3A) and increase in the BK_{Ca} currents (Fig. 3B), whereas the decrease in Ca^{2+} current amplitude (due to a reduced driving force at voltages

approaching the Ca²⁺ reversal potential) leads to reduction and cessation of BK_{Ca} currents at voltages >20 mV (Fig. 3B). Similar results for coupling and I-V relation were obtained with Cav2.2 (Cav2.2 α , Cav β 3, and α 2 δ) channels (fig. S3B).

Comparison of BK_{Ca} currents evoked by Cav2.1 and those recorded in excised patches from oocytes expressing only BK_{Ca} with defined $[Ca^{2+}]_i$ (26) was used to estimate the Ca²⁺ concentration delivered to BK_{Ca} via the Cav channels. The robust BKCa activity observed at membrane potentials ≤0 mV (Fig. 3B) suggested that Cav2.1 channels might provide Ca²⁺ concentrations of $\geq 10 \ \mu M$ (Fig. 3D). This value was confirmed by the time constants obtained from monoexponential fits to the activation time course $(\tau_{activation})$ of BK_{Ca} currents. The values determined for $\tau_{activation}$ of Cav2.1-evoked BK_{Ca} currents were similar to the results obtained from BK_{Ca} currents at a $[Ca^{2+}]_i$ of 10 μ M across the entire voltage range tested (Fig. 4A).

The BK_{Ca}-Cav2.1 coupling was further probed for its sensitivity to Ca2+-buffers. Excised patches were successively perfused with intracellular solutions containing EGTA and BAPTA in 5 mM



B

immunization IgGs. Total eluates were shortly run into a SDS-gel, in-gel trypsinized, and analyzed by nano-LC MS/MS spectrometry. (B) (Top) Highperformance liquid chromatography (HPLC) chromatogram of peptides obtained by trypsinization of anti-BK α eluates in the mass range of 50 to 150 kD. (Middle and bottom) MS and MS/MS spectra of a peptide unique for BK α . The peptide had a mass/charge (*m*/*z*) ratio of 552.76154 and was eluted at the time point indicated. In the MS/MS spectrum, the complete y⁺-ion series

is indicated, and the amino acid sequence derived from the mass differences is given in carboxy-to-amino-terminal direction. (C) Coverage of the BK α amino acid sequence by the peptides identified with nano-LC MS/MS. Peptides identified by mass spectrometry are in red, those accessible to but not identified in MS/MS analyses are in black, and peptides not accessible to the MS/MS analyses used are in gray. Lines denote hydrophobic segments S0 to S10; the blue box highlights the BK α peptide shown in (B).

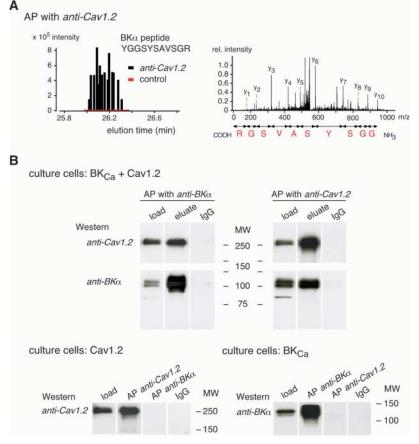
and 10 mM concentration. Cav channel-activated BK_{Ca} currents (at 20 mV) were unaffected by 10 mM EGTA (with respect to the standard 5 mM EGTA), whereas 5 mM and 10 mM BAPTA reduced their amplitude to $28 \pm 5\%$ (mean \pm SD of 9 patches) and $10 \pm 4\%$, respectively (Fig. 4B). This reduction resulted from a decreased $[Ca^{2+}]_i$ at the BK_{Ca} channels, as indicated by their markedly slowed activation time course (Fig. 4B, inset, and fig. S3C). The $\tau_{activation}$ values determined for a membrane potential of 20 mV were 13.4 ± 2.1 ms $(\text{mean} \pm \text{SD}, n = 9)$ and $19.9 \pm 2.1 \text{ ms} (n = 9)$ for 5 mM and 10 mM BAPTA, whereas in EGTA the respective value was $6.9 \pm 1.1 \text{ ms}$ (n = 12; all values were significantly different from each other, with P < 0.0005, pairwise Student's t test). Other properties of Cav2.1-activated BK_{Ca} currents, including the bell-shaped steady-state I-V, were similar in BAPTA- and EGTAbuffered intracellular solutions (fig. S3).

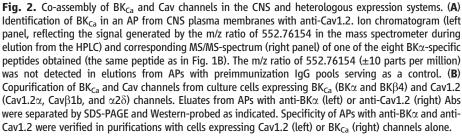
The distinct effects of EGTA and BAPTA on the Cav-activated BK_{Ca} currents place the chan-

nels within a "local nonequilibrium Ca²⁺ domain," a steep Ca²⁺ concentration gradient around a Cav channel that rapidly builds up after opening of the channel pore in the presence of mobile buffers (*18*, *19*). Figure 4C depicts such Ca²⁺ concentration profiles simulated for a Cav channel with a single-channel conductance of 1.7 pS [(*30*), for 1.3 mM external Ca²⁺] and EGTA or BAPTA at concentrations of 5 mM and 10 mM. Accordingly, the distance between BK_{Ca} and Cav2. 1 channels fitting the data on both amplitude and activation time course of BK_{Ca} currents may be estimated to ~10 to 15 nm.

Specificity of BK_{Ca}-Cav Channel Complex Formation

Cav1.2, Cav2.1, and Cav2.2 were specifically copurified with BK_{Ca} channels from rat brain, whereas Cav2.3 was not (Table 1 and fig. S1). We, therefore, investigated the specificity of BK_{Ca}-Cav coassembly using coexpression of either Cav1.2 or Cav2.3 (plus Cav β 1b and α 2 δ) with BK_{Ca} (BK α and





BKβ4) in Chinese hamster ovary (CHO) cells. For functional recordings, the patch-clamp technique was used in whole-cell configuration (26). Figure 5A shows representative current responses to depolarizing voltage steps recorded from coexpression of BK_{Ca} with either of the two Cav subtypes after equilibration of the intracellular milieu with the pipette solution. As indicated by the biphasic current response and the bell-shaped I-V relation, Cav1.2 channels effectively activated the coexpressed BK_{Ca} channels [Fig. 5, A (top) and B] (n of 48 cells), similar to the Cav2.1-BK_{Ca} coexpression in oocytes. Again, the Ca2+ provided through the Cav channels was mandatory for the BKCa currents as reflected by their complete decay paralleling the pronounced inactivation of the Cav1.2 channels (Fig. 5A and fig. S4A). The Cav1.2-BK_{Ca} coupling could not be disrupted with EGTA but was reversibly disrupted after replacing EGTA in the pipette solution with BAPTA (n = 13 cells) (fig. S4C).

In marked contrast to Cav1.2, Cav2.3 channels failed to promote activity of the coexpressed BK_{Ca} channels under standard conditions (n = 9cells) (Fig. 5, A and B). This failure was not due to an inefficient expression of BK_{Ca} but rather resulted from equilibration of the cytoplasm with EGTA as monitored in a series of experiments applying step-depolarizations every 30 to 45 s (Fig. 5C, D). Thus, immediately after establishing whole-cell configuration, Ca2+ influx through Cav2.3 channels elicited robust BK_{Ca} currents that vanished over the following 3-min period required for diffusion of EGTA into the CHO cell (Fig. 5D) (n = 8 cells). The Ca²⁺ currents through Cav2.3 channels were unaffected by EGTA (Fig. 5A and fig. S4B), as were both the Ca^{2+} and K^{+} currents in the Cav1.2-BK_{Ca} coexpressions used as a control (n = 16 cells) (Fig. 5A). In line with the distinct effect of EGTA, immunoprecipitation with the anti-BKa Ab failed to copurify the Cav2.3 protein from Cav2.3-BK_{Ca} coexpressing cells (Fig. 5D).

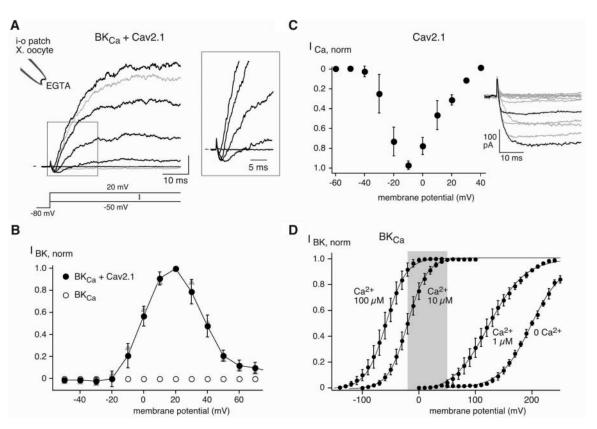
The molecular basis of this subtype specificity is encoded by the pore-forming α subunits. Thus, when cells coexpressing BK α and Cav1.2 α in the absence of the respective auxiliary subunits were used for coimmunoprecipitation, both the anti-BK α and the anti-Cav1.2 Abs effectively purified both channel α subunits (Fig. 5E), although to a somewhat lesser extent compared to the previous experiment with cells expressing all BK_{Ca} and Cav channel subunits (Fig. 2).

Properties of "Native" BK_{Ca}-Cav Channel Complexes

Finally, the functional properties obtained for heterologously reconstituted BK_{Ca} -Cav channel complexes were compared to those of their native counterparts. We used chromaffin cells as a model system because of their well-known coupling between Q- and L-type Cav and BK_{Ca} channels (*31*) and their suitability for electrophysiology and efficient intracellular dialysis.

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Fig. 3. Functional coupling of heterologously expressed BK_{Ca} and Cav2.1 channels. (A) Current response to the indicated voltage steps (-50 to 20 mV at 10 mV increments, holding -80 mV) recorded under physiological ion conditions (1.3 mM extracellular Ca²⁺) in a giant insideout (i-o) patch excised from an oocyte coexpressing BK_{Ca} (BK α and BK β 4) and Cav2.1 [Cav2.1(I1520H), Cav β 3, and α 2 δ] channels. Cytoplasmic solution was buffered with 5 mM EGTA. Current scale is 1 nA. (Inset) Current traces in black (-50, -20, -10, 0, and 20 mV) at expanded time scale. (B) Normalized (outward) currents through BK_{Ca} channels as a function of membrane potential recorded in excised i-o patches from oocytes expressing BK_{Ca} and Cav2.1 channels



(filled symbols) or BK_{Ca} channels alone (open symbols). Data points are mean \pm SD of 10 experiments [gray triangles are from the experiment in (A)]. (**C**) Ca²⁺ (inward) currents normalized to maximum and recorded under conditions as in (A) in excised i-o patches from oocytes expressing Cav2.1 channels as in (A). (Inset) Representative experiment, traces at -10 and 20 mV are in black. (**D**) Steady-state activation curves of BK_{Ca} channels recorded at the [Ca²⁺]_i

indicated in giant i-o patches from oocytes. Data points are mean \pm SD of 6 experiments. Gray bar denotes the voltage range of BK_{Ca} channel activation by coexpressed Cav2.1 channels from (A) and (B). Continuous lines are fits of Boltzmann functions to the data with values for V₁₂ and slope factor of 197.8 mV and 27.1 mV (0 [Ca²⁺]_i), 123.2 mV and 32.1 mV (1 μ M [Ca²⁺]_i), -19.9 mV and 17.1 mV (10 μ M [Ca²⁺]_i), and -58.1 mV and 17.5 mV (100 μ M [Ca²⁺]_i).

Figure 6A shows a typical sequence of Cavmediated inward and BK_{Ca}-mediated outward currents recorded in response to a stepdepolarization in the presence of 5 mM EGTA (n = 32 cells). Coupling of the tetraethylammonium (TEA)-sensitive BK_{Ca} currents to the Ca²⁺ influx is indicated by their deactivation after interruption of the Ca^{2+} influx by a voltage step to the Ca^{2+} reversal potential (Fig. 6A) (31). In addition, BK_{Ca} currents could be eliminated by application of nifedipine and ω-agatoxin IVA, specific blockers of Cav1.2 and Cav2.1 that are expressed in chromaffin cells (Fig. 6B). The spatiotemporal dynamics of the Cav-BK_{Ca} coupling was probed by replacing EGTA in the recording pipette with 5 mM BAPTA. The respective current transients exhibited similar overall properties as with EGTA, although the amplitude of the BK_{Ca} currents was decreased by roughly 80% (ratio of mean currents), and the deactivation at the Ca²⁺ reversal potential was markedly accelerated, as expected for a lower $[Ca^{2+}]_i$ at the BK_{Ca} channels (Fig. 6A).

Discussion

The central finding of this work is that two distinct classes of ion channels, BK-type Ca^{2+} -activated K⁺ channels and voltage-gated Ca^{2+}

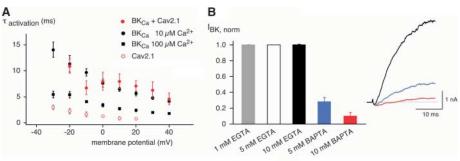
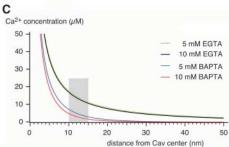


Fig. 4. Localization of BK_{Ca} and Cav2.1 channels within Ca²⁺ nanodomains. **(A)** Activation time constants ($\tau_{activation}$) of BK_{Ca} channels activated either by coexpressed Cav2.1 channels (red circles; experiments as in Fig. 3A) or by [Ca²⁺]₁ of 10 and 100 μ M (black circles and squares, respectively) together with $\tau_{activation}$ of Cav2.1 channels. Data points are mean ± SD of 12 (BK_{Ca}/Cav2.1), 6 (BK_{Ca}), and 5 (Cav2.1) experiments. **(B)** Normalized currents through BK_{Ca} channels recorded at 20 mV with the indicated buffers present on the cyto-

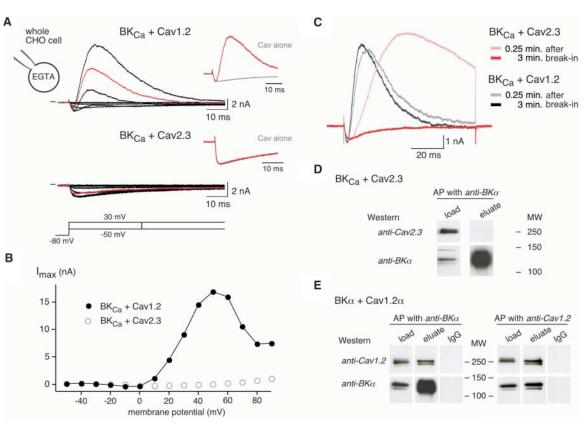


plasmic side of i-o patches from oocytes coexpressing BK_{Ca} and Cav2.1. Data points are mean \pm SD of 9 experiments. (Inset) Representative current traces with 10 mM EGTA (black), 5 mM BAPTA (blue), or 10 mM BAPTA (red). (**C**) Steady-state Ca²⁺ concentration profiles at the cytoplasmic opening of a single Cav channel. The profiles were determined with the CalC software v. 5.4.0 (*43*) (single channel conductance of 1.7 pS, driving force of 60 mV). The gray bar represents the range fitting the experimental data shown in Figs. 3 and 4.

channels, may be assembled into macromolecular channel-channel complexes in the CNS. Functionally, these complexes reconstitute Ca^{2+} nanodomains, where Ca^{2+} influx through the Cav channels provides the $[Ca^{2+}]_i$ required for rapid and robust activation of BK_{Ca} channels in the physiologically relevant voltage range.

Formation of BK_{Ca} -Cav channel complexes. For characterization of the molecular environment

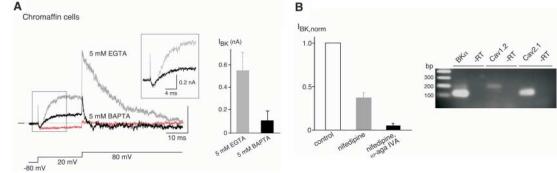
Fig. 5. BK_{Ca}-Cav coassembly is subtype-specific and determined by the α subunits. (A) Current response to the indicated voltage steps (-50 to 30 mV at 10 mV increments) recorded under physiological ion conditions in whole CHO cells coexpressing BK_{Ca} (BK α and BKβ4) and either Cav1.2 (top) or Cav2.3 (bottom) channels. Cav channels were assembled from Cav1.2 α /Cav2.3 α , Cav β 1b, and $\alpha 2\delta$. Traces recorded upon a voltage step to 20 mV are highlighted in red and superimposed in the inset with the response of the respective Cav subtype to the same voltage step. Time constants (mean \pm SD) for the decay of BK_{Ca} and Cav1.2 currents obtained from monoexponential fits were 28.4 \pm 9.9 ms and 27.6 ± 11.7 ms, respectively. (B) I-V relation of of BK_{Ca} channels, we started out from proteomic analysis combining APs of appropriately solubilized proteins with nano-LC MS/MS analysis of total eluates. When applied to plasma membrane preparations from total rat brain (25), this approach isolated BK_{Ca} channels with high efficiency and provided information on the BK α protein [sequence coverage of ~75%, splice variations] and on proteins associated with BK_{Ca} channels. Respective analyses by nano-LC MS/MS revealed two striking results with respect to the mechanism of native BK_{Ca} channel activation. First, the proteins most efficiently copurified with $BK\alpha$ were members of the Cav-channel family (Table 1), with particular abundance of Cav2.1. Second, proteins with a similar peptide yield and proposed scaffolding function were not identified, nor did mass spectrometry retrieve molecules



the experiments in (A); symbols as indicated. (C) Activation of BK_{Ca} by Cav2.3 channels is abolished by 5 mM EGTA washed into CHO cells after establishing whole-cell configuration. Current responses recorded upon voltage steps to 30 mV 0.25 min (light) and 3 min (dark) after whole-cell break-in into cells coexpressing BK_{Ca} channels with either Cav2.3 (red) or Cav1.2 (black) channels. (D) Failure of anti-BK α to purify the Cav2.3 α subunit from culture cells coexpressing BK_{Ca} (BK α

and BK β 4) and Cav2.3 channels (Cav2.3 α , Cav β 1b, and α 2 δ). Load and anti-BK α eluate were separated by SDS-PAGE and Western-probed by anti-BK α and an antibody against the Cav2.3 α subunit (anti-Cav2.3). (E) Copurification of BK_{Ca} and Cav channel α subunits from culture cells expressing BK α and Cav1.2 α . Eluates from APs with anti-BK α (left) and anti-Cav1.2 (right) Abs were separated by SDS-PAGE and Western-probed as indicated.

Fig. 6. Recombinant BK_{Ca} -Cav channel complexes match the characteristics of their native counterparts. (**A**) (Left) Current responses to the indicated voltage protocol [adapted from (22)] recorded in chromaffin cells with either 5 mM EGTA (gray) or 5 mM BAPTA (black) in the whole-cell pipette; the trace in red shows block of the BK_{Ca} current by 5 mM extracellular TEA (at 5 mM intracellular EGTA). Current scale is 0.5 nA. (Inset) Current traces at



expanded time scale. (Right) Mean \pm SD of currents through BK_{ca} channels (n = 5) from experiments as on the left. (**B**) Identification of L-type (Cav1.2) and P/Q-type (Cav2.1) channels as the Cav channels coupling to BK_{ca} channels in chromaffin cells. (Left) Mean \pm SD of BK_{ca}-mediated currents (n = 5) before and after addition

of the L-type Cav channel blocker nifedipine (5 μ M) and the P/Q-type channel blocker ω -agatoxin IVA (1 μ M). (Right) PCR amplification of transcripts coding for BK α and the Cav α subunits indicated from chromaffin cells; control reactions (without reverse transcription) are referred to as –RT.

suggested to link BK_{Ca} and Cav channels (32). For the Cav channel α subunits identified, quantitative comparisons (between eluates of the anti-BKa Abs and control IgGs) indicated specific copurification for Cav1.2, Cav2.1, and Cav2.2, whereas Cav2.3 was dubbed nonspecific by our specificity scores (Table 1 and fig. S1).

Analyses using biochemistry and electrophysiology on heterologously coexpressed Cav and BK_{Ca} channels confirmed the subtype-specific assembly suggested by the proteomic approach. Thus, Cav1.2/Cav2.1 and BK_{Ca} channels were effectively copurified from culture cells and Xenopus oocytes without a requirement for additional exogenous partners (Figs. 2 and 5, and fig. S2). The functional properties of the Cav-BK_{Ca} coupling fully matched the criteria of Ca²⁺ nanodomains, with an estimated distance between channels of ~ 10 nm (18) (Fig. 4 and fig. S4), a value very similar to the 9.5 nm recently determined for the diameter of the voltage-gated K^+ channel Kv1.2 in its crystallized form (33). Mechanistically, channel-channel assembly appears to be determined by the α subunits of BK_{Ca} and Cav channels, although a ubiquitously expressed partner protein that escaped our MS/MS analyses cannot be completely ruled out.

Relevance of BK_{Ca}-Cav channel complexes. Formation of stable macromolecular complexes with Cav channels affects the physiology of BK_{Ca} channels in several ways. First, complex formation provides a simple molecular solution to the issue of how BK_{Ca} channels may be supplied with micromolar [Ca²⁺]_i without affecting other Ca2+-dependent metabolic processes. Second, complex formation puts the activity of BK_{Ca} channels under tight control of their Cav partners. In the context of an excitable cell, this tight coupling ensures that activation of BK_{Ca} channels occurs fast enough to shape the action potential by contributing to its repolarization (4, 7, 8) and to generate the fast afterhyperpolarization following single Na⁺ or Ca²⁺ spikes in various types of CNS neurons (1, 3, 6, 7, 34).

BK_{Ca} signaling via coassembled Cav channels will be shaped by the distinct distribution of Cav channels to particular types of cells or subcellular compartments (Table 1) (35, 36). In fact, all Cav channel subtypes identified were found as partners of somatic BK_{Ca} channels in distinct types of CNS neurons (3, 6, 13, 20, 23, 24, 37). In their preferred subcellular localization (38, 39), the presynaptic compartment, however, BK_{Ca} channels appear to be fueled by P/Q- and N-type Cav channels (6, 40), in line with our efficient copurification of the Cav subunits Cav2.1 and Cav2.2. Functionally, presynaptic BK_{Ca} channels were shown to control transmitter release by narrowing the action potential and reducing Ca²⁻ influx into the presynaptic elements (6, 12, 40)and to operate as an "emergency brake" that prevents cell damage in the case of globally increased $[Ca^{2+}]_i$ (41). Both functions may be related to the molecular arrangement of BK_{Ca} channels: Control of transmitter release would well fit with the properties of BK_{Ca}-Cav complexes, whereas emergency braking may be attributed to uncomplexed BK_{Ca} channels.

The BK_{Ca}-Cav channel complexes represent a molecular unit providing effective and precisely timed hyperpolarization of the membrane potential in response to local Ca^{2+} influx.

Table 1. (Top) BK_{Ca} and Cav channel subunits affinity-purified with anti-BK α from CNS plasma membranes and identified by nano-LC MS/MS. (Bottom) BK_{Ca} and Cav channel subunits affinitypurified with anti-Cav1.2 from CNS plasma membranes. Procedures used for affinity-purification and mass spectrometry, as well as the criteria for protein identification, are detailed in (26). remSC is relative exponentially modified sequence coverage. rPQ-Score is relative protein query score. Values for remSC > 5 and rPQ-Scores > 4 indicate specific purification by anti-BK α or anti-Cav1.2 over control IgG pools. * indicates lower estimates of the rPQ score with no matching peptide fragments in the controls.

	Protein ID	remSC	rPQ-Score
BK _{Ca} subunits	BK α (K _{Ca} 1.1,KCNMA1)	48.1	322.3
	ΒΚβ2 (ΚСΝΜΒ2)	∞	16.0*
	ΒΚβ4 (ΚСΝΜΒ4)	∞	112.0*
Cav subunits	Cav2.1 (α1A)	∞	496.0*
	Cav1.2 (α1C)	∞	40.0*
	Cav2.2 (α1B)	5.5	4.9
	Cav2.3 (α1Ε)	3.8	2.4
	Cav β1b	∞	124.0*
	Cav β2	∞	100.0*
	Cav β3	6.3	4.1
	Cav β4	2.3	2.5
Cav subunits	Cav1.2 (α1C)	œ	216.0*
	Cav β1b	∞	128.0*
	Cav β2	∞	104.0*
	Cav β3	∞	104.0*
	Cav β4	6.3	5.0*
BK _{Ca} subunits	ВКа	∞	24.0*

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Supporting Online Material

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Materials and Methods Figs. S1 to S4

- References
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