Coassembly of Big Conductance Ca\(^{2+}\)-activated K\(^{+}\) Channels and L-type Voltage-gated Ca\(^{2+}\) Channels in Rat Brain*

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Morten Grunnet†§ and Walter A. Kaufmann¶

From the †Department of Medical Physiology, the Panum Institute, University of Copenhagen, Blegdamsvej 3, Copenhagen DK-2200, Denmark, §Neurosearch A/S, Pedersstrupvej 93, Ballerup 2750, Denmark, and the ¶Department of Physiology, Centre for Molecular Biology and Neuroscience, University of Oslo, Oslo N-0317, Norway

Based on electrophysiological studies, Ca\(^{2+}\)-activated K\(^{+}\) channels and voltage-gated Ca\(^{2+}\) channels appear to be located in close proximity in neurons. Such colocalization would ensure selective and rapid activation of K\(^{+}\) channels by local increases in the cytosolic calcium concentration. The nature of the apparent coupling is not known. In the present study we report a direct coassembly of big conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (BK) and L-type voltage-gated Ca\(^{2+}\) channels in rat brain. Saturation immunoprecipitation studies were performed on membranes labeled for BK channels and precipitated with antibodies against α, and α\(_{1D}\) L-type Ca\(^{2+}\) channels. To confirm the specificity of the interaction, precipitation experiments were carried out also in reverse order. Also, additive precipitation was performed because α\(_{1C}\) and α\(_{1D}\) L-type Ca\(^{2+}\) channels always refer to separate ion channel complexes. Finally, immunochemical studies showed a distinct but overlapping expression pattern of the two types of ion channels investigated. BK and L-type Ca\(^{2+}\) channels were colocalized in various compartments throughout the rat brain. Taken together, these results demonstrate a direct coassembly of BK channels and L-type Ca\(^{2+}\) channels in certain areas of the brain.

It is becoming evident that membrane proteins rarely function as independent units. Instead, these proteins are commonly coassembled in larger functional domains. Such domains may consist of various membrane proteins, a number of folding proteins, and different intracellular components with catalytic functions. As an example, in a series of studies various types of neurons express some or all of these channels in different proportions. BK channels are localized in the presynaptic membrane of hippocampal neurons facing the synaptic cleft in terminals of Schaffer collaterals (23). In neurons firing with high frequency, the membrane depolarization, together with presynaptic Ca\(^{2+}\) influx, is thought to be sufficient to activate BK channels. This BK activation will prevent neuronal hypereexcitability by initiating a repolarization of the membrane. BK channels thereby act as a kind of “emergency brake” (2, 24, 25). It has also been demonstrated that activation of BK channels can be accomplished in less than 0.5 ms by Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels during a single action potential in hippocampal pyramidal cells (2, 26–28). Keeping the enormous cellular Ca\(^{2+}\) buffering capacity in mind, this rapid activation requires an intimate association of both channel types. The nature of the apparent coupling is still not known. It is uncertain whether these channels are located in close proximity or actually linked together. Further, it is debated which types of voltage-gated Ca\(^{2+}\) channel are in-

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† Supported by a traveling scholarship from the University of Copenhagen. To whom correspondence should be addressed: Dept. of Medical Physiology, 12626, The Panum Institute, University of Copenhagen, Blegdamsvej 3, Copenhagen DK-2200, Denmark. Tel.: 45-3532-7565; Fax: 45-3532-7526; E-mail: mgrunnet@mmf.ku.dk.

‡ The abbreviations used are: BK channels, big conductance Ca\(^{2+}\) channels; BSA, bovine serum albumin; CAPS, 3-cyclohexylaminopropanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; IbTX, iberiotoxin; IK channels, intermediate conductance potassium channels; RIA, radioimmunoassay; SK channels, small conductance Ca\(^{2+}\)-activated potassium channels; TBS, Tris-buffered saline.
and H2O2 were from Sigma. All other reagents employed were of the peroxidase-conjugated goat anti-rabbit IgG was from Jackson & Jack-PerkinElmer Life Sciences. Affinity-purified alkaline phosphatase- or homogenization in ice the homogenate was subjected to 2 °C, the homogenate was subjected to 2 x low speed centrifugation at 823 × g for 3 min at 4 °C. The supernatants were then centrifuged at 13,000 × g for 10 min at 4 °C, and the pellet was resuspended in homogenization buffer and loaded on a 7.5/10% Ficoll gradient afterward. The gradient was subjected to high speed centrifugation at 150,000 × g for 30 min at 4 °C. The fraction between the synaptosome suspension and 7.5% Ficoll (“supernatant”) was centrifuged in homogenization buffer at 200,000 × g for 45 min at 4 °C, the pellet resuspended in 20 mM Tris/ HCl, pH 7.4, snap frozen in liquid nitrogen, and stored at −80 °C. The fraction between the 7.5 and 10% Ficoll (“interface”) was centrifuged in homogenization buffer at 14,280 × g for 30 min at 4 °C. The pellet was resuspended in 5 ml of homogenization buffer and transferred to 500 ml of 5 mM Tris/ HCl, pH 7.4, for 45 min at 4 °C on a stirring plate for cell lysis. The suspension was centrifuged at 200,000 × g for 45 min at 4 °C and the pellet processed like the supernatant fraction.

Antibody Production and Affinity Purification—All applied antibo-dies were purified from polyclonal sera raised in rabbits using immuno- genic peptides of the following sequences: BK channel α subunit, residue positions 913–926 (VND TNV QFL DQD DD) of mSlo (anti-αslo913-926), L-type α1c subunit, residue positions 818–835 (anti-α1c 818-835), and L-type α1d subunit, residue positions 2121–2137 (anti-α1d 2121-2137). Antibodies were raised, affinity purified from re-spective immune sera, and characterized by enzyme-linked immunosor-bent assay and Western blot analysis as described previously (24, 30–32). Specificity of immunolabeling for all antibodies was confirmed (see Fig. 2).

Immunoblotting—After running membranes on SDS-PAGE, proteins were transferred for 60 min under constant voltage (100 V) in a buffer consisting of 25 mM Tris, 190 mM glycin, 10% (v/v) methanol. After protein transfer, polyvinylidene difluoride membranes were incubated for 2 h at room temperature in blocking buffer consisting of 10% low fat milk powder, 0.5% (w/v) Triton X-100, 0.1% (w/v) Tween 20, dissolved in 150 mM NaCl, 20 mM Tris/ HCl, pH 7.4. Mem- branes were then incubated overnight on a rocking plate at 4 °C with primary antibodies diluted 1:1,000 in blocking buffer. Membranes were washed in washing buffer consisting of 0.5% (w/v) Triton X-100, 0.1% (w/v) Tween 20, dissolved in 150 mM NaCl, 20 mM Tris/ HCl, pH 7.4, and incubated with affinity-purified alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma) diluted 1:2,000 in blocking buffer for 90 min on a rocking plate at room temperature. After five washes in washing buffer, blots were developed in staining buffer consisting of 100 mM NaCl, 5 mM MgCl2, 100 mM Tris/ HCl, pH 9.5, 0.045% nitro blue tetrazolium, and 0.035% 5-bromo-4-chloro-3-indolyl phosphatase until the staining reaction was then allowed to take place for >4 h at gentle rotation at 4 °C. After washing three times with RIA buffer the suspension was trans-ferred to counting tubes and counted in a Cobra II auto γ counter or a Tri-Carb 2000TR liquid scintillation analyzer, both from Packard Bio-science. For β counting, 3 ml of scintillation fluid was used for each sample. When 125I-IbTX-D19Y/Y36F was used as ligand, paxilline was added to all buffers (final concentration, 375 mM).

Immunoprecipitation—For these immunoprecipita-tion studies BK antibodies were prebound to Sepharose 4B by washing 0.25 ml of Sepharose in 8.5 ml of dialyzed affinity-purified BK antibody for 60 min at 4 °C a gentle rotation. Dialyzing was performed in 0.1 M NaHCO3, Sepharose-antibody complexes were isolated by gentle centrifugation for 5 min at 2,000 × g. The supernatant was discarded and the pellet resuspended in 10 ml of 100 mM glycine, pH 7.4, to block unbound Sepharose sites. Blocking was allowed to proceed for 60 min at 4 °C under gentle rotation. The suspension was filtered and washed with 1 liter of ice-cold Tris-buffered saline (TBS) (containing 150 mM NaCl, 20 mM Tris/ HCl, pH 7.4, at a metal grid. The Sepharose-antibody complex was recovered from the grid with a spatula, resuspended in 6 M guanidinium hydrochloride, and dialyzed against buffer containing 0.4% Triton X-100 before being loaded at the column. This was obtained by washing 2-ml membranes in 20 mM Tris/ HCl, pH 7.4, 200 μl of buffer consisting of 10 mM NaCl, 20 mM Tris, 0.1% BSA followed by a 10-min centrifugation at 48,000 × g. The supernatant was discarded and the pellet solubilized in 4 ml of buffer consisting of 2% (w/v) digitonin, 150 mM NaCl, 20 mM Tris/ HCl, pH 7.4, and the supernatant containing solubilized membranes loaded at the column in a total volume of 7 ml of 2% (w/v) digitonin, 150 mM NaCl, 20 mM Tris/ HCl, pH 7.4 and incubated overnight at 4 °C under gentle rotation. The column was washed five times with 0.1% digitonin, 150 mM NaCl, 20 mM Tris/ HCl, pH 7.4, and then eluted with 10 ml of 0.1% digitonin, 150 mM NaCl, 0.1% C. The column was kept on ice and neutralized immediately to pH 7.4 with HCl. After dialyzing overnight in 1 liter of 0.05% SDS, 5 mM Tris/ HCl, pH 7.4, membranes were concentrated to 0.5 ml using a Centricon prep (Amicon, Inc., Beverly, MA). SDS sample buffer was added and the samples heated to 56 °C for 10 min before being analyzed by SDS-PAGE. Im-munoblotting was performed as described above with the exception that secondary antibody was peroxidase conjugated and diluted 1:100,000 in 3% BSA, developed with Supersignal (Pierce) according to the manu-facturer’s instructions, and exposed on Hyperfilm ECL (Ambersham Bioscience).

Binding Studies—Binding studies were performed as described pre-viously (29).

Immunohistochemistry—Adult Wistar rats were deeply anesthetized with pentobarbital (150 mg/kg, intraperitoneal). Brains were perfusion fixed through the ascending aorta with 50 ml of phosphate-buffered saline (50 mM, pH 7.4) plus heparin (3,000 IU/liter) followed by 250 ml of 4% paraformaldehyde and 0.1% glutaraldehyde in 50 mM phosphate- buffered saline. Brains were then removed immediately from the same fixative for 60 min at 4 °C, and transferred to 5, 10, and 20% sucrose in phosphate-buffered saline. They were rapidly frozen in iso- pentane (−45 °C) and stored at −70 °C. Serial coronal and sagittal sections (40 μm) were cut on a sliding cryostat (Microm, Walldorf, Germany) and kept in 0.1 M TBS (pH 7.2, containing 0.05% NaN3) at 4 °C.

Consecutive brain sections were processed for immunohistochemis-try using the peroxidase anti-peroxidase technique for free floating samples. Sections were rinsed in 0.1 M TBS-T (Tris-buffered saline containing 0.4% Triton X-100) over a period of 2 h at room temperature.
Endogenous peroxidase activity was blocked with 0.9% H$_2$O$_2$ and 20% methanol in TBS followed by rinsing sections in TBS-T. Nonspecific binding sites were then blocked by incubating sections for 90 min on a rocker plate in TBS-T containing 2% BSA, 2% normal goat serum, and 0.2% milk powder. Primary antibodies were applied in TBS-T containing 1% BSA overnight at room temperature on a rocker plate. Antibody dilutions were: affinity-purified anti-BK Alpha-18, 2.2 ng/$\mu$l (1:2,000); CNC1 crude serum, 1:1,000; affinity-purified CND8, 1.8 ng/$\mu$l (1:1,500). Tissue sections were rinsed 4 x 30 min in TBS-T and incubated with horseradish peroxidase-coupled goat anti-rabbit IgG (Dako, Glostrup, Denmark) diluted 1:400 in TBS-T buffer containing 1% BSA overnight at room temperature. The antigen-antibody complex was visualized by a reaction using 0.63 mM 3,3′-diaminobenzidine and 0.002% H$_2$O$_2$ in TBS, pH 7.6. The reaction was allowed to proceed for 10–12 min and terminated by washing sections in TBS. Tissue sections were mounted on chromalum gelatin-coated glass slides, air dried for 1 h, flushed with tap water followed by dH$_2$O, dehydrated in increasing concentrations of ethanol, cleared in n-butylacetate, and coverslipped with Eukitt.

Alternatively, sections were processed according to the avidin-biotinylated horseradish peroxidase complex technique. Primary antisera were applied as described before. Thereafter, sections were washed in TBS-T and goat anti-rabbit biotinylated secondary antibodies (1:200 in TBS-T containing 0.1% BSA; Vector Laboratories) were applied for 90 min at room temperature. Sections were rinsed in TBS-T again and treated with an avidin-biotinylated horseradish peroxidase complex (1:200 in TBS-T containing 0.1% BSA) for 90 min at room temperature. The sections were reacted with 0.05% 3,3′-diaminobenzidine and 0.003% H$_2$O$_2$ in TBS for 6–7 min, rinsed in TBS, mounted on glass slides, air dried, dehydrated, and covered with Eukitt.

**Fig. 1.** Optimizing immunoprecipitation conditions. An initial precipitation was conducted of $^{125}$I-IbTX-D19Y/Y36F-labeled membranes with antibodies raised against different kind of Ca$^{2+}$ channels. A, Alpha-18 directed against BK $\alpha$ subunits in rat, CNC1 against $\alpha_{1C}$ subunits in rat, CND8 against $\alpha_{1D}$ subunits in rat, CNA-C1 against $\alpha_{1A}$ subunits in rat, RCP15 against denaturated Ca$^{2+}$ channel $\beta$ subunits in rat, and CNCMCA-gly against $\alpha_{1C}$ subunits in rabbit. For optimization of solubilization, $^{125}$I-IbTX-D19Y/Y36F-labeled membranes were treated with different detergents and subsequently precipitated with 5 and 10 $\mu$l of CND8 antibodies as depicted in B (n = 4–11). The specificity of the immunoreaction was controlled by precipitation of $^{125}$I-IbTX-D19Y/Y36F-labeled membranes with, respectively, Alpha-18, CNC1, or CND8 antibodies compared with the corresponding preimmune serum or an early bleed (in the case of CNC1), demonstrated in C (n = 4).
Coassembly of K⁺ Channels and Ca²⁺ Channels

The specificity of the immunostaining was controlled by applying the respective preimmune serum or the immune serum preadsorbed with the synthetic immunogenic peptides (concentration, 10 μM).

Statistics—The results from saturation immunoprecipitation experiments and saturation binding experiments were subjected to Michaelis-Menten analysis in which the equilibrium dissociation constant \( K_d \) and maximal receptor concentration \( B_{max} \) were determined using Equation 1

\[
y = \frac{(B_{max} \times x)}{(K_d + x)}
\]

where \( y \) is the receptor concentration and \( x \) is the radioligand or antibody concentration. Resulting values are given as the mean ± S.E.

Protein Determination—The protein concentrations of the membrane preparations were determined according to Bradford using BSA as a standard.

RESULTS

Optimization of Immunoprecipitation Studies—Functional interaction of voltage-gated Ca²⁺ channels and Ca²⁺-activated K⁺ channels has been suggested from a number of different electrophysiological experiments, but demonstration of direct coupling is still missing. Different kinds of immunoprecipitation were performed to investigate direct coupling of potassium channels of the BK type and L-type Ca²⁺ channels. Radiolabeled ligands exist for both BK and L-type Ca²⁺ channels. This raises the possibility of performing semiquantitative immunoprecipitation experiments. Membranes are labeled with a radioligand recognizing specifically either BK or L-type channels. Subsequently, immunoprecipitation is performed with antibodies recognizing the protein thought to interact with either of the two channels. Finally, the presence of BK or L-type channels in the precipitate is detected by counting the amount of radioactivity.

In an initial experiment shown in Fig. 1A, rat brain membranes were labeled with the modified BK-specific scorpion toxin IbTX (125I-IbTX-D19Y/Y36F). In earlier studies this double mutated radiolabeled toxin has been demonstrated to interact specifically with BK channels in a manner no different from native IbTX (29, 33). Labeled membranes were subsequently precipitated with antibodies raised against different kinds of voltage-gated Ca²⁺ channels. As a control, precipitation was performed using antibodies against BK channels (Alpha-18 antibody). Antibodies against both rat α₁C and α₁D L-type Ca²⁺ channels (CNC1 and CND8) precipitated IbTX binding, whereas antibodies against P-type Ca²⁺ channels (CNA-C1), denatured Ca²⁺ channel β subunits (RCP15), and rabbit L-type Ca²⁺ channels (CNCMCA-gly) hardly showed any precipitation. Measurements were performed in duplicate, and data were averaged. All data presented are specific precipitation values defined as the difference between total precipitation and precipitation obtained in the absence of antibody. Nonspecific precipitation was generally <5% of total precipitation. Remaining data are all presented in a similar manner.

Several optimization experiments were carried out to determine the most favorable solubilization conditions. Rat brain membranes labeled with 125I-IbTX-D19Y/Y36F were incubated with five different detergents. The solubilization efficiency was 10.0% for cholate, 11.4% for deoxycholate, 19.4% for Triton X-100, 12.9% for CHAPS, and 14.8% for digitonin. After solubilization, membranes were precipitated with two different concentrations (5 and 10 μl) of L-type Ca²⁺ channel-specific antibodies (Fig. 1B). The highest amount of specific precipitation was obtained for deoxycholate- and digitonin-solubilized membranes. Deoxycholate was seen to cause nonspecific precipitation using radioligands other than IbTX (e.g. ω-conotoxin; data not shown). Furthermore, digitonin has been shown previously to be a good detergent when working with ion channels (33). Therefore, digitonin was used as detergent in the following experiments. The overall digitonin solubilization efficiency for IbTX-labeled membranes was 13.6 ± 3% (n = 11) and for isradipine-labeled membranes 23.2% ± 4% (n = 5).

Membrane solubilization is in general more efficient in the presence of K⁺ compared with Na⁺. At the same time, K⁺ compromises IbTX interaction with BK channels. Optimization of the ion composition for the solubilization buffer was therefore performed and revealed 150 mM NaCl without any KCl to be most suitable for the experiments conducted in this study (data not shown).

In a final optimization step, the reactivity of applied antibodies was tested. Immunoprecipitation was performed with preimmune serum or serum from early bleeds and the results compared with precipitation performed with antisera from final bleeds. Fig 1C shows an example of 125I-IbTX-D19Y/Y36F-labeled membranes precipitated with antibodies against BK channels (Alpha-18) and L-type Ca²⁺ channels (CNC1 and CND8). Precipitation performed with preimmune serum constituted less than 5% of control precipitation performed with final bleed antibodies. For the CNC1 antibody, where no preimmune serum was available, precipitation with second bleed antibodies constituted 18% of control values. These results indicate that the antibodies used are highly specific in immunoprecipitation assays.

Immunoblotting—All antibodies applied in these experiments have proven highly specific in earlier studies (24, 30–32). Specificity was confirmed under the current experimental conditions. Western blot analysis was performed with antibodies recognizing BK channel α subunits, L-type α₁C, channels, and L-type α₁D channels, respectively. Channel proteins were recognized as specific bands with expected molecular masses of ~150 kDa for BK channels and ~200 and 240 kDa for L-type Ca²⁺ channels, respectively. Molecular masses for the standard lane are given in kDa.

![ Immunoblot analysis of BK channels and L-type Ca²⁺ channels. Rat brain membranes (~9 μg of protein/lane) were separated by 9% SDS-PAGE under reducing conditions. Immunoblotting was performed with antibodies recognizing BK channel α subunits, L-type α₁C, channels, and L-type α₁D channels, respectively. Channel proteins were recognized as specific bands with expected molecular masses of ~150 kDa for BK channels and ~200 and 240 kDa for L-type Ca²⁺ channels, respectively. Molecular masses for the standard lane are given in kDa. ](image: fig2.jpg)
Immunoprecipitation results obtained with radioactive labeled ligands and specific antibodies indicate a specific interaction between BK channels and L-type Ca$_{2+}$ channels. As seen in Fig. 5, it was possible to obtain faint but specific bands for both $\alpha_{1C}$ and $\alpha_{1D}$ subunits against BK channels as a control. Faint but distinct bands could be observed for both L-type $\alpha_{1C}$ and $\alpha_{1D}$ channels. The molecular mass for the standard is given in kDa.

Additive Immunoprecipitation—Molecular identification of Ca$_{2+}$ channels is based on the primary structure of the $\alpha_1$ subunit. Both $\alpha_{1C}$ and $\alpha_{1D}$ subunits belong to the L-type Ca$_{2+}$ channels, but the composition of the Ca$_{2+}$ channels harboring only one $\alpha_1$ subunit implies that these two kinds of L-type Ca$_{2+}$ channels could never be part of the same channel complex. In our experiments we could precipitate both $\alpha_{1C}$ and $\alpha_{1D}$ L-type Ca$_{2+}$ channels. The fact that precipitated $\alpha_{1C}$ and $\alpha_{1D}$ subunits originate from different pools of Ca$_{2+}$ channels implies that the precipitation of $^{125}$I-IbTX-D19Y/Y36F-labeled membranes with both CNC1 (recognizing $\alpha_{1C}$) and CND8 (recognizing $\alpha_{1D}$) antibodies must be completely additive to precipitation performed with either CNC1 or CND8 antibodies alone.

The amount of precipitation for CNC1 and CND8 antibodies used alone was comparable with the amount of precipitation obtained when both antibodies were applied together (Fig. 6). A

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**Fig. 3.** Saturation immunoprecipitation experiments. To obtain quantitative values of BK channel/L-type Ca$_{2+}$ channel coassembly, experiments were performed with $^{125}$I-IbTX-D19Y/Y36F-labeled membranes, precipitated with increasing concentrations of Alpha-18, CNC1, and CND8 antibodies. As demonstrated in A, saturated precipitation was obtained for all three antibodies, confirming the specificity of immunoprecipitation. The results of these experiments are summarized in B. 38.3 $\pm$ 7% of $^{125}$I-IbTX-D19Y/Y36F-labeled rat brain membranes were precipitated with CNC1 antibodies, and 16.6 $\pm$ 4% were precipitated with CND8 antibodies ($n$ = 8).

As a control, rat brain membranes were initially prelabeled with isradipine (recognizing L-type channels). Subsequently, BK/L-type channel complexes were precipitated with highly specific BK antibodies (Fig. 4). Saturated immunoprecipitation was again obtained, confirming coupling of the two types of channels in native rat brain tissue. BK precipitation was $\sim$30% of the amount obtained by precipitation of isradipine-labeled membranes with CNC1 and CND8 antibodies (data not shown).

Immunoprecipitation results obtained with radioactive labeled ligands and specific antibodies indicate a specific interaction between BK channels and L-type channels. This conclusion was validated further by column purification of solubilized membrane complexes with BK antibodies followed by immunoblotting with L-type Ca$_{2+}$ channel antibodies. As seen in Fig. 5, it was possible to obtain faint but specific bands for both $\alpha_{1C}$ and $\alpha_{1D}$ channels and against BK channels as a control. Faint but distinct bands with expected molecular masses of $\sim$200 and 240 kDa, respectively, could be observed for both L-type $\alpha_{1C}$ and $\alpha_{1D}$ channels. The molecular mass for the standard is given in kDa.
strictly additive amount of precipitation was observed for all antibody concentrations tested. The summarized values of the two antibodies used alone never differed by more than 2% compared with control values where both antibodies were used together (data not shown).

In a number of experiments similar to the saturation immunoprecipitation studies described for BK and L-type Ca\(^{2+}\) channels, we attempted to find other channels that coassemble under native conditions. These included N-type Ca\(^{2+}\) channels and BK channels, N-type Ca\(^{2+}\) channels and SK2 channels, and L-type Ca\(^{2+}\) channels and SK2 channels. However, it was not possible to demonstrate any true and specific coassembling among any of these channel combinations, at least not under the experimental conditions applied in these studies (data not shown).

Binding Studies—To determine the absolute number of channels that coassemble in a functional complex it is necessary to evaluate the overall number of channels present in the
membrane preparation. Therefore, saturation binding studies were performed with $^{125}$I-bTX-D19/1Y36F or isradipine recognizing BK and L-type Ca$^{2+}$ channels, respectively. BK channel density was 133 fmol/mg of protein, and L-type Ca$^{2+}$ channel density was 179 fmol/mg of protein. The $K_d$ values were 47 and 116 pM, respectively (data not shown).

**Immunohistochemistry**—The results obtained from additive and saturation immunoprecipitation experiments strongly favored a specific coupling of BK channels with L-type Ca$^{2+}$ channels in rat brain. Because membranes used for precipitation, immunoblotting, and binding studies were prepared from whole brain, these experiments did not provide any information about neuronal compartments in the brain where such an interaction might occur.

Therefore, immunohistochemistry was performed on serial, sagittal cut rat brain sections. These studies were undertaken to obtain an overview on the localization of BK channels as well as $\alpha_{1C}$ (CNC) and $\alpha_{1D}$ (CND) L-type Ca$^{2+}$ channels rather than a detailed expression profile of each channel type at the cellular level. Specificity of immunolabeling for all antibodies used was controlled in sections applying respective preimmune serum or in the presence of excess immunogenic peptide. No characteristic immunolabeling was observed in control sections (data not shown).

All three kinds of channel protein were widely expressed throughout the whole brain and displayed an overlapping distribution pattern (Fig. 7). Prominent immunolabeling was found for the olfactory bulb, caudate putamen, cortex, hippocampal formation, thalamus, substantia nigra, pontine nuclei, cerebellum, and the brain stem. Whereas in some areas all three types of channel protein were detected at high expression levels (e.g. olfactory bulb, pontine nuclei, and cerebellum), only BK and $\alpha_{1C}$ channels (e.g. caudate putamen and thalamus) or BK channels and $\alpha_{1D}$ channels were localized in others (e.g. globus pallidus and substantia nigra).

Although the overall distribution pattern of these channels appeared identical in various regions, differences were detected in their expression profile within regional subcompartments as shown for the cerebellum (Fig. 8). Dense immunolabeling was observed for BK channels in the Purkinje cell layer, whereas a moderate labeling was found for $\alpha_{1C}$ and $\alpha_{1D}$ channel subunits in this area. In contrast, the cerebellar granular layer displayed high expression levels for $\alpha_{1C}$ and $\alpha_{1D}$ channel subunits compared with a low expression of BK channels (Fig. 8, insets). Coincidences along with discrepancies were also detected for their expression profile in the hippocampal formation, both at the regional and the cellular level (Fig. 9). The hippocampal formation displayed high expression levels of BK and $\alpha_{1C}$ channel protein, whereas just moderate levels of $\alpha_{1D}$ channel protein were observed (Fig. 9, A–C). In some areas, all three types of channels were detected as in the CA1 pyramidal cell layer, dentate gyrus granule cell layer, and molecular layer. Conversely, only two types of channels were expressed in others. Prominent BK and $\alpha_{1C}$ immunolabeling was detected in the stratum oriens of the CA3 field and BK together with $\alpha_{1D}$ immunoreactivity was localized to the stratum lacunosum molecular. This region showed hardly any immunolabeling for $\alpha_{1C}$ channel subunits (Fig. 9, A–C). At the cellular level, differential targeting of channel protein was indicated. Different channel combinations might exist for particular subcellular sites. Exemplary, dense immunolabeling for BK as well as $\alpha_{1C}$ and $\alpha_{1D}$ protein was detected at the somatic membrane of dentate gyrus granule cells (Fig. 9, D–F). Although high $\alpha_{1D}$ immunoreactivity was restricted to granule cell somata, $\alpha_{1C}$ immunoreactivity was dense throughout the granule cell layer and the entire molecular layer, harboring the dendritic tree of granule cells. Expression of BK channels was detected at high levels in the granule cell layer and neurofil of the outer molecular layer while sparing the inner molecular layer. Therefore, BK/$\alpha_{1C}$ channel coassembly is indicated for distal dendritic sites, either pre- or postsynaptic, whereas BK/$\alpha_{1D}$ as well as BK/$\alpha_{1C}$ channel coupling is likely to exist at somatic sites of dentate gyrus granule cells.

**DISCUSSION**

The present study was carried out to investigate a possible interaction between Ca$^{2+}$-activated K$^+$ channels and voltage-gated Ca$^{2+}$ channels in rat brain. Out of several channel combinations tested, coassembly could be demonstrated for Ca$^{2+}$-activated K$^+$ channels of the BK type and voltage-gated Ca$^{2+}$ channels of the L-type.
In initial experiments, rat brain membranes were labeled with the modified toxin \( ^{125}\text{I}-\text{bTX-D19Y/Y36F} \), which binds specifically to BK channels. These membranes could be precipitated mainly with antibodies raised against rat L-type Ca\(^{2+}\) channels. The immunoprecipitation results showed significantly less precipitation. These results indicated a direct interaction between BK channels and L-type Ca\(^{2+}\) channels. Control experiments confirmed the existence of such a coassembly.

Protein complexes that are sensitive to solubilization. BK/L-type channel interaction was indicated further by the fact that precipitation experiments could be performed in reverse order. Membranes initially labeled with a probe recognizing L-type Ca\(^{2+}\) channels were precipitated with BK-specific antibodies. Also, these experiments resulted in saturable precipitation.

Because of the topology of voltage-gated Ca\(^{2+}\) channels in the hippocampus and cerebellum, comprising one single \( \alpha_1 \) subunit, it is concluded that L-type channels will never contain both \( \alpha_{1C} \) and \( \alpha_{1D} \) subunits. This therefore implies that the amount of precipitated membranes obtained from two experiments applying \( \alpha_{1C} \) and \( \alpha_{1D} \) antibodies independently should be completely equal to precipitated membranes from an experiment applying both antibodies simultaneously. Such additive results were confirmed in a number of experiments, independent of the amount of antibody used. In addition to accentuating a specific interaction between BK and L-type Ca\(^{2+}\) channels, these results favor an optimal solubilization process. Insufficient solubilization could imply that both \( \alpha_{1C} \) and \( \alpha_{1D} \) channels could come together in the same precipitate. However, the almost complete additive results from these experiments argue strongly against this possibility.

Immunoprecipitation experiments were performed on membranes prepared from the whole brain. For the localization of BK and L-type Ca\(^{2+}\) channel coassembly within the brain, immunohistochemistry was applied. Prominent immunolabeling for both BK channels and \( \alpha_{1C} \) and \( \alpha_{1D} \) L-type Ca\(^{2+}\) channels could be detected in various regions throughout the whole brain including subcompartments of the hippocampal formation and the cerebellum. Coassembly of BK channels and L-type Ca\(^{2+}\) channels in the hippocampus and cerebellum is contradictory to electrophysiological investigations in these areas.

**Fig. 9.** Comparative distribution of BK and L-type Ca\(^{2+}\) channel \( \alpha_{1C} \) and \( \alpha_{1D} \) subunit immunoreactivities on adjacent sagittal sections through the hippocampal formation. In the upper panels, the overall staining pattern for BK channel protein \( \alpha \), \( \alpha_{1C} \) subunit protein \( \beta \), and \( \alpha_{1D} \) subunit protein \( \gamma \) is displayed. BK and \( \alpha_{1D} \) immunolabeling is prominent in hippocampal regions, whereas \( \alpha_{1C} \) immunoreactivity is moderate to low in this area. In contrast, \( \alpha_{1C} \) subunit immunoreactivity is high throughout the outer and inner molecular layer as well as the granule cell layer but moderate in the area of the polymorphic layer adjacent to the granule cell layer. Note the comparatively low expression level in the inner molecular layer. The correlation between the amount of antibody applied and immunohistochemistry was applied. Prominent immunolabeling for both BK channels and \( \alpha_{1C} \) and \( \alpha_{1D} \) L-type Ca\(^{2+}\) channels could be detected in various regions throughout the whole brain including subcompartments of the hippocampal formation and the cerebellum. Coassembly of BK channels and L-type Ca\(^{2+}\) channels in the hippocampus and cerebellum is contradictory to electrophysiological investigations in these areas.
eas, demonstrating a functional coupling between BK channels and N-type Ca\(^{2+}\) channels (2), but in agreement with a number of other studies demonstrating functional coupling between BK and L-type Ca\(^{2+}\) channels. Furthermore, studies with dual coupling of BK channels to both L- and N-type Ca\(^{2+}\) channels have also been demonstrated, e.g. in neocortical pyramidal neurons (3, 5, 10). The discrepancy between studies reporting a functional BK/N-type coupling and the present data might be the result of several factors. First, BK and N-type coupling could take place in subcellular microdomains too small to reveal a BK/N-type coupling because of an insufficient solubilization approach used in the present study. In the present study, the solubilization efficiency was around 20%, and structures such as cholesterol-rich microdomains were not extracted. Therefore, possible coassembly of proteins present in structures such as lipid rafts could not be revealed.

Even though the performed experiments demonstrated coassembly of BK and L-type Ca\(^{2+}\) channels the true nature of the protein-protein interaction is still unknown. A direct interaction among the two ion channel proteins might be expected, but, more likely, a number of yet unidentified accessory proteins could be present. Such scaffolding proteins have been demonstrated for a number of other proteins functionally linked in the plasma membrane (34).

In summary, the present experiments favor coassembly of BK and L-type voltage-gated Ca\(^{2+}\) channels in certain parts of the rat brain. Further studies are needed to discover whether this is a direct interaction or if the presence of accessory proteins is required.

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REFERENCES
1. Gola, M., and Crest, M. (1993) Neuron 10, 689–699
2. Marrion, N. V., and Tavalin, S. J. (1998) Nature 395, 900–905
3. Prakriya, M., and Lingle, C. J. (1999) J. Neurophysiol. 81, 2267–2278
4. Sun, X., Gu, X. Q., and Haddad, G. G. (2003) J. Neurosci. 23, 3639–3648
5. Wu, S. N., Lo, Y. K., Li, H. F., and Shen, A. Y. (2001) Chin. J. Physiol. 44, 161–167
6. Sorensen, J. B., Nielsen, M. S., Gudme, C. N., Larsen, E. H., and Nielsen, R. (2001) J. Neurophysiol. 86, 1–11
7. O’Neill, W. C., and Steinberg, D. F. (1995) Am. J. Physiol. 269, C267–C274
8. Fagni, L., Bossu, J. L., and Bockaert, J. (1991) Eur. J. Neurosci. 3, 778–789
9. Twitchell, W. A., and Rane, S. G. (1994) Mol. Pharmacol. 46, 793–798
10. Chavitt, P., Ango, F., Michel, J. M., Bockaert, J., and Fagni, L. (1998) Eur. J. Neurosci. 10, 2322–2327
11. Herrera, G. M., Heppner, T. J., and Nelson, M. T. (2001) Am. J. Physiol. 280, C681–C690
12. Isaacson, J. S., and Murphy, G. J. (2001) Neuron 31, 1027–1034
13. Ashcroft, F. M. (2000) Ion Channels and Disease, pp. 125–134, Academic Press, San Diego
14. Laterre, R., Oberhauser, A., Labarca, P., and Alvarez, O. (1989) Annu. Rev. Physiol. 51, 385–399
15. Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y., and Aldrich, R. W. (2000) J. Biol. Chem. 275, 8453–8461
16. Knaus, H. G., Folander, K., Garcia-Calvo, M., Garcia, M. L., Kaczorowski, G. J., Smith, M., and Swanson, R. (1994) J. Biol. Chem. 269, 17274–17278
17. Uchele, V. N., Lagrutta, A., Wade, T., Figueroa, D. J., Liu, Y., McKenna, E., Austin, C. P., Bennett, P. B., and Swanson, R. (2000) J. Biol. Chem. 275, 20121–20128
18. Wallner, M., Meera, P., and Toro, L. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 4137–4142
19. Xia, X. M., Ding, J. P., Zeng, X. H., Duan, K. L., and Lingle, C. J. (2000) J. Neurosci. 20, 4980–4983
20. Catterall, W. A. (1993) Ann. N. Y. Acad. Sci. 707, 1–19
21. Birnbaumer, L., Campbell, K. P., Catterall, W. A., Harpold, M. M., Hofmann, F., Horne, W. A., Men, Y., Schwartz, A., Snutch, T. P., and Tanabe, T. (1994) Neuron 13, 503–506
22. Hille, B. (2001) Ion Channels of Excitable Membranes, 3rd Ed., Sinauer Associates, Inc., Sunderland, MA
23. Hu, H., Shao, L. B., Chavoshy, S., Gu, N., Trieb, M., Behrens, B., Laake, P., Pongs, O., Knaus, H. G., Ottersen, O. P., and Storm, J. F. (2001) J. Neurosci. 21, 9585–9597
24. Knaus, H. G., Schwarzer, C., Koch, O. L., Eberhart, A., Kaczorowski, G. J., Glossmann, H., Wunder, F., Pongs, O., Garcia, M. L., and Sperk, G. (1996) J. Neurosci. 16, 955–963
25. Linden, E., Huang, F. M., Storm, J. F., and Ottersen, O. P. (2002) Neuroscience 112, 277–288
26. Lancaster, B., and Nicoll, R. A. (1987) J. Physiol. 389, 187–203
27. Storm, J. F. (1987) J. Physiol. 385, 733–759
28. Storm, J. F. (1990) Prog. Brain Res. 83, 161–187
29. Kocsis, A., Koch, R. O., Liu, J., Kaczorowski, G. J., Feinstein, P., Garcia, M. L., and Knaus, H. G. (1997) Biochemistry 36, 1943–1952
30. Platzer, J., Engel, J., Schmitt-Fisher, A., Stephen, K., Bova, S., Chen, H., Zheng, H., and Striessnig, J. (2000) Cell 102, 89–97
31. Safaryi, H., Haase, H., Kramer, U., Bihlmayer, A., Roenfeldt, M., Ammon, H. P., Prochmayr, M., Cassidy, T. N., Morano, I., Ahlijanian, M. K., and Striessnig, J. (1997) Mol. Endocrinol. 11, 619–629
32. Sailer, C. A., Hu, H., Kaufmann, W. A., Trieb, M., Schwarzer, C., Storm, J. F., and Knaus, H. G. (2002) J. Neurosci. 22, 9088–9097
33. Grunnet, M., Knaus, H. G., Solander, C., and Klaerke, D. A. (1999) Am. J. Physiol. 277, G22–G30
34. Lesage, F., Hübner, H., and Hudspeth, A. J. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 671–675
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Morten Grunnet and Walter A. Kaufmann

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