Somatic Colocalization of Rat SK1 and D class (Ca_v 1.2) L-type Calcium Channels in Rat CA1 Hippocampal Pyramidal Neurons

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In hippocampal neurons, the firing of a train of action potentials is terminated by generation of the slow afterhyperpolarization (AHP). Recordings from hippocampal slices have shown that the slow AHP likely results from the activation of small-conductance calcium-activated potassium (SK) channels by calcium (Ca^{2+}) entry through L-type Ca^{2+} channels. However, the relative localization of these two channel subtypes is not known. The cloning and characterization of three subtypes of SK channel has suggested that SK1 may underlie generation of the slow AHP. Using a novel antibody directed against rat SK1 (rSK1), it has been determined that the rSK1 channel is primarily in the soma of hippocampal CA1 neurons. In conjunction with antibodies directed against C (Ca_v1.2) and D (Ca_v1.3) class L-type Ca^{2+} channel α1 subunits, it was observed that rSK1 channels were selectively colocalized with D class L-type channels. This colocalization supports the functional coupling of L-type and SK channels previously observed in cell-attached patches from hippocampal neurons. However, it appears contrary to the slow rise and decay of the slow AHP. Induction of delayed facilitation of L-type Ca^{2+} channels in cell-attached patches from hippocampal neurons evoked delayed opening of coupled SK channels. Generation of ensemble currents produced waveforms identical to the ionic current underlying the slow AHP (I_{sAHP}). Therefore, these data indicate that the slow AHP is somatic in origin, resulting from delayed facilitation of D class L-type Ca^{2+} channels colocalized with rSK1 channels.

Key words: calcium-activated potassium channel; slow afterhyperpolarization; SK channel; calcium channel; delayed facilitation; hippocampus

In hippocampal pyramidal neurons, a burst of action potentials is terminated by generation of a slow afterhyperpolarization (AHP) (Madison and Nicoll, 1984). It has been proposed that the slow AHP is generated by activation of SK channels (Pedarzani et al., 2001). However, the observation that afterhyperpolarizations show different sensitivities to the bee venom toxin apamin suggested the existence of more than one subtype of SK channel (Pennefather et al., 1985; Lancaster and Adams, 1986). This was confirmed by the cloning of three SK channel subtypes (SK1–3; Köhler et al., 1996). These cloned channels exhibited similar biophysical properties, but were distinguished by their sensitivity to block by apamin (Köhler et al., 1996; Hirschberg et al., 1998). Homomeric SK2 and SK3 channels were blocked by apamin with a high affinity, whereas SK1 was insensitive (Köhler et al., 1996; Ishii et al., 1997). Subsequently, it has been reported that hSK1 channels are sensitive to block by apamin. However, the block is poorly understood because in a proportion of cells expressing hSK1, a component of current was not sensitive, and the block displayed a biphasic inhibition (Shah and Haylett, 2000a; Grunnet et al., 2001). The mRNAs for SK1, 2, and 3 subunits are all present in rat hippocampus (Köhler et al., 1996). Activation of SK2/SK3 channels is proposed to underlie the apamin-sensitive medium AHP in the hippocampus (Stocker et al., 1999), suggesting that SK1 channel activation underlies generation of the apamin-insensitive slow AHP in these neurons (Köhler et al., 1996; Vergara et al., 1998).

In cultured neurons, the slow AHP is evoked by Ca^{2+} entry through L-and N-type Ca^{2+} channels, with a contribution from Ca^{2+}-induced Ca^{2+} release reported from organotypic cultured neurons (Tanabe et al., 1998; Shah and Haylett, 2000b). In hippocampal slices it appears that Ca^{2+} entry through L-type channels is the primary source of Ca^{2+} for activation of the slow AHP (Rascol et al., 1991; Moyer et al., 1992). However, the relative localization of SK and L-type channels in hippocampal neurons is not known. Using indirect methods it has been proposed that SK channels are in the soma (Lancaster and Zucker, 1994), the basal dendrites (Bekkers, 2000), or the apical dendrites (Sah and Bekkers, 1996). Patch-clamp recordings have shown that SK channels are present in the soma of hippocampal neurons (Marrion and Tavalin, 1998; Hirschberg et al., 1999). Similarly, immunohistochemical stud-

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ies have shown that L-type Ca^{2+} channels are primarily somatic (Hell et al., 1993). A novel antibody has been raised against the rat SK1 (rSK1) channel. The antibody recognized a protein of appropriate molecular weight only in tissues previously demonstrated to contain SK1 subunit mRNA. Using immunocytochemistry, rSK1 channels were shown to be primarily somatic in acutely dissociated hippocampal CA1 neurons. The subcellular location of C and D class L-type channels was resolved to examine whether a specific subtype is involved in generation of the slow AHP. In this study, rSK1 channels were found to be selectively colocalized with D class L-type calcium channels. This colocalization appears inconsistent with the slow rise and decay of the slow AHP. Using cell-attached patch recordings, induction of delayed facilitation of L-type channels evoked delayed openings of coupled SK channels. Generated ensemble currents were identical to the ionic current underlying the slow AHP. The observed somatic colocalization of rSK1 and D class L-type channels suggests that the slow AHP is somatic in origin. This is supported by the observation that induction of delayed facilitation of L-type channels evokes a slow AHP waveform from coupled SK channels.

MATERIALS AND METHODS

Antibody production. An antibody was raised in guinea pig against a 26 amino acid peptide (QAQQEELEARLAALESRLDVLGASLQ) corresponding to a unique region in the C terminus of the rat SK1 channel. Antiserum was collected, titer was determined by ELISA, and antibodies were purified by affinity chromatography (Research Genetics).

Tissue preparation. Tissues were removed from 10- to 13-d-old rat pups (Sprague Dawley) and homogenized (Polytron blender, full power, 10 sec) in ice-cold Tris buffer (25 mM Tris, pH 7.4). The homogenate was washed twice by centrifugation (25,000 × *g*, 10 min, 4°C), and the pellet was resuspended in Tris buffer. Solubilization buffer (25 mM Tris, 2 mM EDTA, 0.1 mM PMSF, and 0.5% Triton X-100, pH 7.4) was added, and the preparation was agitated for 60 min at 4°C, after which insoluble material was removed by centrifugation at 100,000 × *g* for 60 min at 4°C.

For Western blot analysis of calcium and potassium channels in rat brain (Fig. 1*A,C*), whole brain was removed from 10- to 13-d-old rat pups and stored at −80°C. Brain tissue was slowly thawed and homogenized in ice-cold HSE buffer (10 mM HEPES, 350 mM sucrose, and 5 mM EDTA, pH 7.4) containing protease inhibitors [pepstatin A (1 μg/ml), leupeptin (1 μg/ml), aprotinin (1 μg/ml), Pefabloc SC (0.2 mM), benzamidine (0.1 mg/ml), and calpain inhibitors I and II (8 μg/ml)]. The homogenate was centrifuged at 2000 × *g* for 5 min, and the supernatant was removed and centrifuged at 100,000 × *g* for 1 hr. The resulting pellet was resuspended in ice-cold HSE buffer containing protease inhibitors. For all tissue preparations, protein content was determined using the Bradford assay (Bio-Rad, Hemel Hempstead, UK).

Glutathione S-transferase fusion proteins. The cDNA encoding the C-terminal region of the rSK1 channel (amino acids 429–533) was amplified using PCR and subcloned into the glutathione *S*-transferase (GST) fusion vector pGEX-6P1 (Amersham Pharmacia Biotech, Little Chalfont, UK). The resulting plasmid was used to transform *Escherichia coli* strain BL21 (Stratagene, Amsterdam, The Netherlands). Expression of fusion proteins was induced by adding isopropylthio-β-D-galactoside (1 mM). Cells were sedimented by centrifugation and resuspended in PBS before separation of bacterial proteins by SDS-PAGE.

SDS-PAGE and Western blotting. Samples were solubilized in SDS sample buffer, incubated at 80°C for 20 min, and applied to acrylamide gels. After SDS-PAGE, protein was transferred to polyvinylidene difluoride membrane using a transfer cell (Bio-Rad). The membrane was washed in blocking buffer and incubated with solutions of primary and IgG—peroxidase-conjugated secondary. Immunoreactive proteins were visualized using an ECL detection system (ECL Plus, Amersham, UK).

Cell preparation. Acutely dissociated hippocampal CA1 neurons were obtained from the brains of 9- to 12-d-old Sprague Dawley rats as described previously (Cloues et al., 1997). Dissected hippocampi were sectioned into 300- to 400-μm-thick slices. After enzyme treatment, slices were stored in solution at room temperature under an oxygen atmosphere. Slices were removed, and the CA1 region was dissected when required. Pyramidal neurons were released by gentle trituration through fire-polished Pasteur pipettes and plated onto multiwell glass slides (ICN Biomedicals, Basingstoke, UK) for immunocytochemical studies or Primaria-coated dishes (Falcon) for electrophysiology.

Antibody labeling. Cells were fixed and permeabilized with PBS

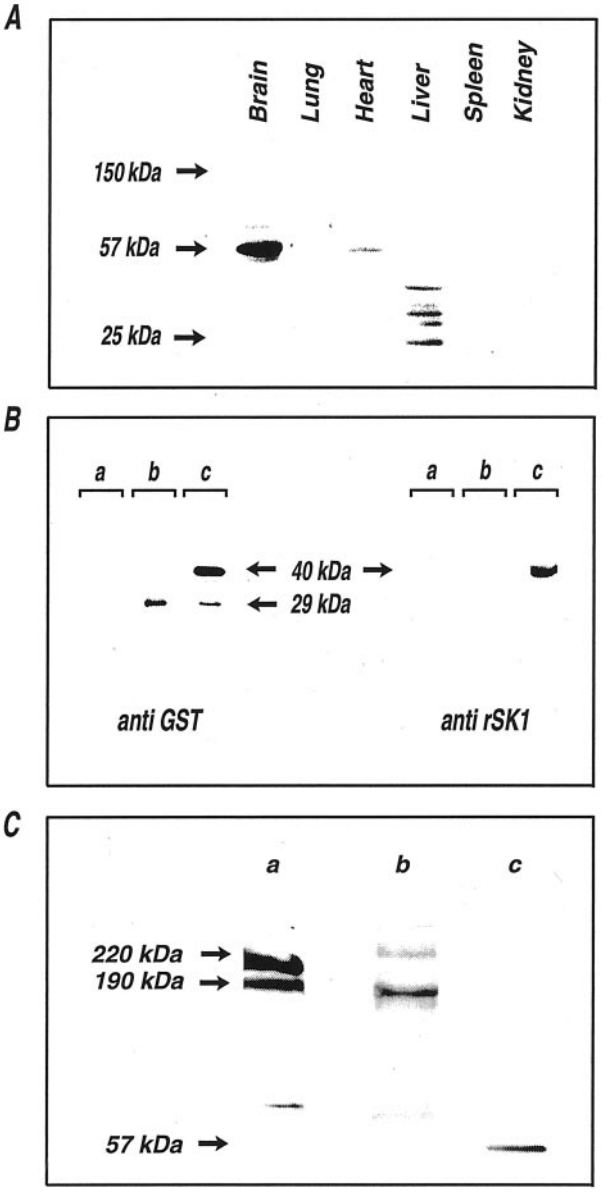

Figure 1. Characterization of the antibody to rat SK1. *A,* Western blot analysis of protein extracts from different rat tissues illustrating the distribution of proteins detected by the antibody to rSK1. A protein of ~57 kDa was recognized in brain and heart but not in lung, liver, spleen, or kidney. *B,* Western blots showing selective detection of the GST–rSK1 fusion constructs by the antibody directed against rSK1. Extracts of *E. coli* only (*a*), bacteria transformed with a plasmid encoding GST (*b*), and bacteria transformed with a plasmid encoding a fusion protein of GST and the C terminus of rSK1 (*c*) were probed with antibodies to GST and rSK1. The rSK1 antibody recognized the GST–rSK1 fusion protein but not protein extracts from bacteria expressing GST alone. *C,* Western blot probed with antisera to the Ca^{2+} channel α_{1C} subunit (*a*), the Ca^{2+} channel α_{1D} subunit (*b*), and the rSK1 subunit (*c*). Immunoblotting showed that the rSK1 antibody did not detect protein bands at the predicted molecular weights for the Ca^{2+} channel subunits. Similarly, neither of the Ca^{2+} channel antibodies detected a protein corresponding to the molecular weight for the rSK1 subunit.

containing 3% PFA and 0.1% Triton X-100 for 10 min at room temperature and then rinsed with PBS containing 1% BSA for 30 min. After incubation with the appropriate primary antibody (1:200) for 12 hr at 37°C, excess antibody was removed by washing with PBS–BSA. The appropriate fluorochrome-conjugated secondary antibody (1:100; Jackson ImmunoResearch, West Grove, PA) was then applied for 2 hr at 25°C. Finally, cells were rinsed in PBS–BSA for 20 min at room temperature, then mounted using Vectashield mount (Vector Laboratories, Orton Southgate, Peterborough, UK). Images were captured on a Leica (Nussloch, Germany) confocal laser-scanning microscope.

All standard reagents were obtained from Sigma with the exception of $CaCl_2$ (Fluka, Neu-Ulm, Germany) and HEPES (Calbiochem, La Jolla, CA).

RESULTS

Characterization of an antibody to the rat SK1 channel (anti-rSK1)

An antibody was raised to a sequence unique to rSK1. This region was selected because it displayed little sequence identity with corresponding regions in rSK2 and rSK3 (31 and 39%, respectively). Western blot analysis of protein extracts from different rat tissues showed that the novel antibody recognized a protein in brain and heart of ~57 kDa, the predicted molecular weight for the rSK1 subunit. No such protein band was recognized in lung, liver, spleen, or kidney ($n = 7$) (Fig. 1*A,C*). Specific binding was confirmed by elimination of labeling by preincubating the antibody with the peptide antigen (1:100; data not shown). This tissue distribution is comparable with that of mRNA for the rSK1 subunit (Köhler et al., 1996). Labeling was not observed at the predicted molecular weight for rSK3 (82 kDa). In addition, labeling of an 82 kDa protein was not observed in liver, a tissue rich in rSK3 (Fig. 1*A*) (Barfod et al., 2001). The predicted molecular weight for rSK2 (64 kDa) is close to that estimated for rSK1. However, the novel antibody did not recognize protein in Western blot analysis of extracts from either human embryonic kidney 293 or tSA201 cells transiently expressing rSK2 (data not shown). Therefore, the size and distribution of the protein detected are consistent with the antibody recognizing the rSK1 protein, with no cross-reactivity with either rSK2 or rSK3 subunits.

The expression of human SK1 and rat SK2 and 3 clones has been reported in both *Xenopus* oocytes and mammalian cell lines (Köhler et al., 1996; Shah and Haylett, 2000a). We and others have attempted to express a full-length clone of rat SK1 in a mammalian cell line, but have not been successful (L. Kaczmarek, personal communication). As an alternative approach to confirm the specificity of the antibody, a GST–rSK1 fusion protein was produced. Western blot analysis of extracts from bacteria expressing either GST alone or the GST–rSK1 fusion protein showed that an antibody directed against GST recognized both a 29 kDa protein corresponding to GST and a protein of 40 kDa corresponding to the GST–rSK1 fusion protein ($n = 7$) (Fig. 1*B*). In contrast, anti-rSK1 only recognized protein in extracts from bacteria expressing the GST–rSK1 fusion protein. This demonstrates that the antibody specifically recognized the rSK1 subunit epitope ($n = 6$) (Fig. 1*B*).

Subcellular distribution of class C and D L-type calcium channels in hippocampal CA1 neurons

Antibodies specific for the C and D class $\alpha 1$ subunits were used to identify and localize the L-type calcium channel subtypes in hippocampal CA1 neurons. C class L-type calcium channels were distributed at a very low density on the central region of the cell bodies. In contrast, dense immunoreactivity was observed at the base of the apical and basal dendrites and extended only into their proximal regions. The staining was further characterized by punctate clusters of intense labeling along the length of the apical dendrite (Fig. 2*a*). Visualization of the α_{1D} subunit revealed a diffuse distribution of D class channels over the surface of the cell body. This staining extended only into the proximal portions of the apical and basal dendrites and diminished along the length of the apical dendrite (Fig. 2*b*). Denser immunoreactivity was occasionally seen at the base of the basal and apical dendrites in some neurons. The observed labeling corresponded well with the expression of α_{1C} and α_{1D} subunits reported in hippocampal slices (Hell et al., 1993), confirming that channel distribution had been retained in the acutely dissociated cell preparation.

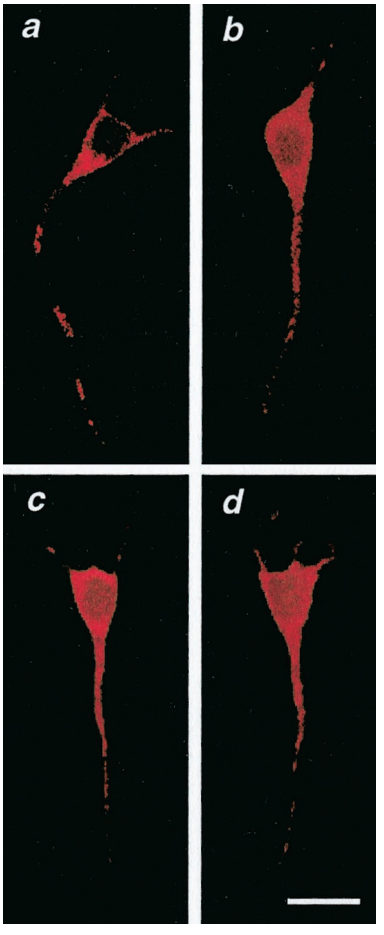

Figure 2. Subcellular distribution of L-type calcium and SK1 channels in hippocampal neurons. Confocal images show the labeling detected with specific antibodies to α_{1C} (*a*), α_{1D} (*b*), and rSK1 subunits (*c, d*) using FITC-conjugated secondary antibodies; magnification 63×. Scale bar, 10 μm. In all cases, the specificity of labeling was confirmed by elimination of immunoreactivity after preincubation of the primary antibody with the respective antigenic peptide (1:1).

Localization of rSK1 channels in hippocampal CA1 neurons

The antibody raised against a unique sequence in the rSK1 channel (anti-rSK1) was used to determine the subcellular distribution of the SK1 channel. Immunofluorescent staining of the rSK1 channel subunit detected a diffuse somatic distribution. Similar to the labeling of the α_{1D} subunit, immunoreactivity for rSK1 channels was also seen at the base of the basal and apical dendrites in some neurons. Weaker staining, which diminished along the length of the dendrites, was also observed (Fig. 2*c,d*).

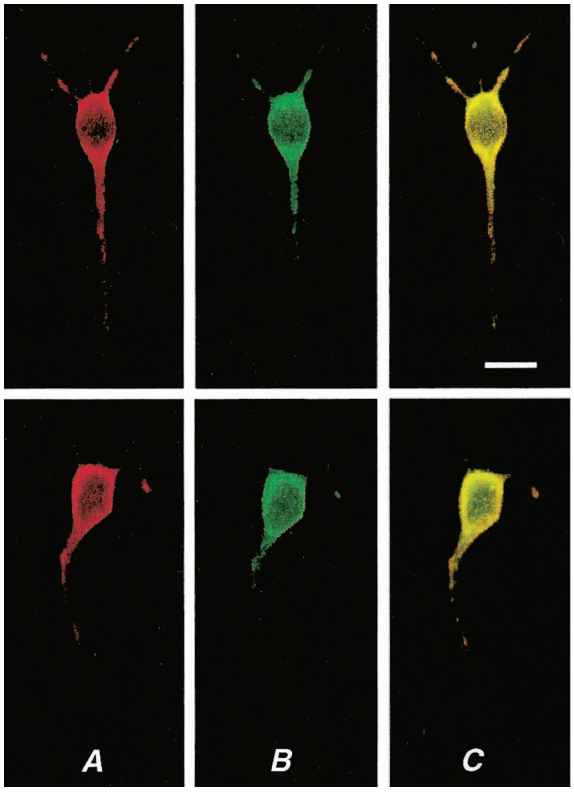

Figure 3. Colocalization of rSK1 with D class L-type calcium channels in fixed hippocampal neurons. Two examples of the pattern of immunoreactivity observed for α_{1D} (secondary antibody: donkey anti-rabbit Cy5, *red*; *A*) and rSK1 (secondary antibody: donkey anti-guinea pig FITC, *green*; *B*) subunits in acutely dissociated hippocampal neurons. In each case, areas of colocalization appear as *yellow* (*C*). Scale bar, 10 μm.

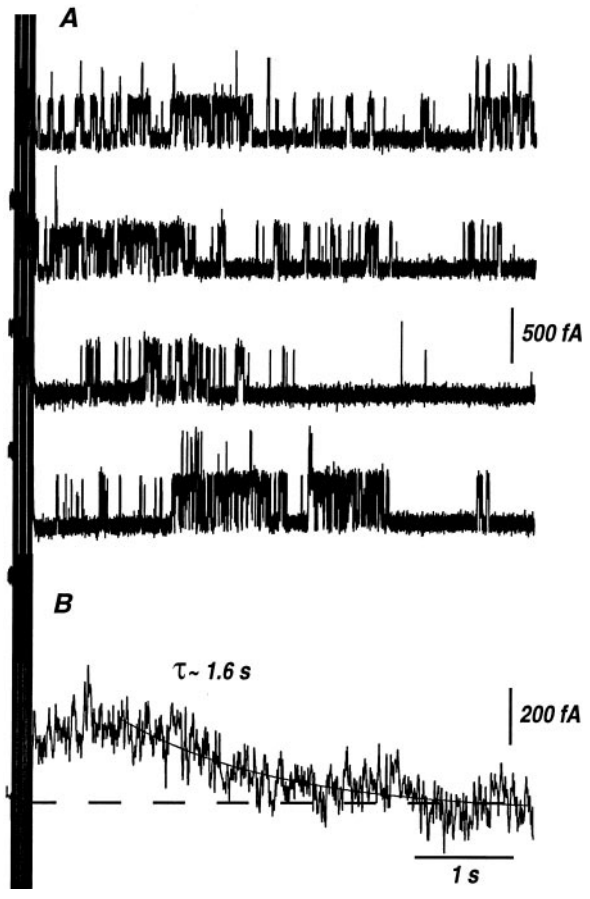

Figure 4. I_{sAHP}-like waveform of SK channel activity induced by delayed facilitation of colocalized L-type calcium. *A,* Selected sweeps show outward (upward) channel openings evoked by a train of action potential waveforms (holding potential, −60 mV; post-train voltage, 0 mV) recorded in the cell-attached configuration from acutely dissociated hippocampal neurons. Electrodes were filled with a solution containing (in mM): NMDG (150); aspartic acid (150); HEPES (10); 4-AP (1); 3–4-DAP (1); $CaCl_2$ (2); KCl (2), supplemented with α and β dendrotoxins (200 nM) and TTX (10 μM). In these conditions, individual L-type channels could not be resolved. Their presence was confirmed by subsequent block of SK channel activity by application of 3 μM nimodipine (data not shown). Evoked channels had a single-channel amplitude consistent with a conductance of 10 pS, as previously reported for hippocampal SK channels measured under these conditions (Marrion and Tavalin, 1998). Open-state kinetics were consistent with those observed for SK channels in patches excised from hippocampal CA1 neurons (Hirschberg et al., 1999). *B,* Generation of an ensemble current (average of 15 sweeps) gave a waveform resembling that of the I_{sAHP}. A slow rise of outward current was observed, peaking ~600 msec after the termination of the train. The decay of the waveform was fit with an exponential time course ($\tau \sim 1.6$ sec). The *dashed line* represents zero current.

Colocalization of rSK1 with D class L-type calcium channels in rat hippocampal CA1 neurons

Comparison of the subcellular distributions of the rSK1 and α_{1D} calcium channel subunits suggested that the two channel types may be colocalized (Fig. 2*b–d*). However, it was necessary to exclude the possibility that antibody cross-immunoreactivity could underlie the similarity in the staining patterns observed. Western blot analysis of rat brain proteins probed with antibodies to calcium channel subunits α_{1C} and α_{1D} revealed two forms for each of the L-type calcium channel subunits with apparent molecular weights of ~220 and ~190 kDa (Hell et al., 1993). The antibodies to calcium channel subunits α_{1C} and α_{1D} did not detect any reactivity in the molecular weight region corresponding to that predicted for the rSK1 channel ($n = 5$) (Fig. 1*Ca,b*). Similarly, probing with anti-rSK1 detected a single protein band of ~57 kDa, with no reactivity observed at the predicted molecular weights for the calcium channel subunits ($n = 19$) (Fig. 1*Cc*). In each case, the specific binding was eliminated by preincubation of the antibody with its corresponding peptide antigen at a ratio of 1:1 for α_{1C} and α_{1D} and 100:1 for rSK1. In addition, it was demonstrated that the immunoreactivity of the α_{1D} antibody was not affected by preincubation with the rSK1 peptide (1:1), and vice versa (100:1; data not shown). With immunocytochemistry, preincubation of the rSK1 antibody with the α_{1D} peptide (1:1) did not alter the pattern of staining compared with that observed with rSK1 antibody alone (data not shown). Taken together, these findings excluded the possibility of cross-reactivity between the antibodies to the α_{1D} and rSK1 subunits verifying the patterns observed in the single labeling immunocytochemistry studies.

Using the fact that the primary antibodies for the two channel subtypes were raised in different species (rabbit for anti-α_{1D} and guinea pig for anti-rSK1), multiple labeling studies were performed to determine if the rSK1 and D class channels were colocalized. Figure 3 shows the patterns of labeling obtained from the simultaneous acquisition of the immunoreactivity for each of the channel types within an individual neuron. Significant colocalization of the rSK1 and D class channels was detected, particularly in the cell soma and proximal region of the apical dendrite (Fig. 3*C*). It was noted that the expression patterns were not

identical, with areas of distinct labeling for each channel type also being observed. In most cases, a slight excess of somatic staining for the α_{1D} subunit could be discerned. This is consistent with previous reports from single channel studies that suggested the possibility of "spare" L-type calcium channels in the soma of hippocampal neurons (Marrion and Tavalin, 1998).

DISCUSSION

A novel antibody directed against a unique sequence in the C terminus of the rat SK1 channel showed the channels to be diffusely distributed, primarily in the soma and proximal regions of the dendrites of acutely dissociated hippocampal CA1 neurons (Fig. 2*c,d*). In contrast, C class L-type channels were found at the base of the apical and basal dendrites, around the periphery of the soma, and in punctate clusters along the length of the apical dendrite (Fig. 2*a*). D class L-type channels were more diffusely distributed in the soma with a diminishing presence along the length of the apical dendrite (Hell et al., 1993). Overlay of immunofluorescent staining of individual neurons in multiple labeling studies revealed a somatic colocalization of SK1 and D class channels.

It has been proposed that SK channel activation underlies generation of the slow AHP in hippocampal neurons (Pedarzani et al., 2001). The observed somatic colocalization of SK1 and L-type D class channels suggests that the slow AHP is generated in the soma of these neurons. Support for a somatic location for SK1 channels comes from single-channel studies, which reported the presence of SK channels in somatic patches (Marrion and Tavalin, 1998; Hirschberg et al., 1999). In addition, their somatic colocalization with L-type calcium channels identified with the low resolution of light microscopy is consistent with the functional coupling of L and SK channels observed in somatic cell-attached patches from these neurons (Marrion and Tavalin, 1998). The calcium sensitivity of SK channel gating also suggests that the two channel types must be in close proximity. Both native and cloned SK channel subtypes exhibit an open probability (P_o) of 0.5 at a calcium concentration of ~0.5–0.7 μM (Köhler et al., 1996; Hirschberg et al., 1998). The P_o of the SK channel at the peak of the slow AHP has been estimated to be 0.5–0.7 (Sah and Isaacson, 1995; Valiante et al., 1997), predicting a requirement for an intracellular calcium concentration of ~1 μM (Hirschberg et al., 1999). However, bulk increases of intracellular calcium of only 30 nM have been measured during the slow AHP (Knöpfel et al., 1990). For SK channels to experience 1 μM calcium, microdomain models predict that they must be within 150 nm of a calcium channel (Marrion and Tavalin, 1998). Therefore, the observed colocalization of L and SK channels is sufficient to account for the slow AHP.

Although close proximity of the two channel types would permit sufficient calcium to be present at the SK channels, their distribution within the soma is apparently inconsistent with the slow time course of the slow AHP in the hippocampus. It has previously been proposed that diffusion of calcium from its point of entry to the SK channels underlies the slow kinetics of the slow AHP in hippocampal cells (Lancaster and Adams, 1986). Accordingly, SK channels have always been proposed to be at some distance from calcium channels, although their exact location has been disputed. How then can a model of colocalization of the channels account for the kinetics of activation of the slow AHP? It has been proposed that the slow AHP time course may be attributable to potassium channels reacting slowly to increases in intracellular calcium (Sah and Clements, 1999). However, cloned and native SK channels have been demonstrated to activate rapidly in response to a rise in calcium (Xia et al., 1998). Furthermore, calcium-dependent channels that may underlie the slow AHP in hippocampal neurons have been shown to respond rapidly to photolytically released calcium (Lancaster and Zucker, 1994). Therefore it is apparent that SK channel kinetics alone are not sufficient to explain the time course of the slow AHP (Hirschberg et al., 1999).

An alternative explanation is that the calcium channel kinetics can account for the time course of the slow AHP in these neurons. It has been reported that a train of action potentials that would evoke the slow AHP induces an enhanced activity of L-type calcium channels at membrane potentials negative to −50 mV. This behavior is termed delayed facilitation and has been proposed to provide a prolonged source of calcium entry at negative membrane potentials (Cloues et al., 1997). Because both the time course and modulation of delayed facilitation closely resembles those of the slow AHP, delayed facilitation of L-type calcium channels has been proposed to dictate the time course of the slow AHP in hippocampal neurons (Cloues et al., 1997).

If SK channels respond to a colocalized calcium source, it would be expected that an ensemble waveform of SK channel activity after induction of delayed facilitation should mimic the macroscopic slow AHP. Figure 4 shows SK channel activity recorded in a cell-attached patch with an electrode containing 2 mM $CaCl_2$ and KCl. Induction of delayed facilitation of colocalized L-type channels by a train of action potential waveforms activated SK channels within the patch. Openings occurred after some delay and were observed to decrease in frequency during the 5 sec voltage step to 0 mV (Fig. 4*A*). Generation of an ensemble current produced a waveform that displayed a slow rise (peaking within ~0.5–1 sec) and decayed with an exponential time course ($\tau \sim 1.6$ sec) that was reminiscent of the current underlying the slow AHP (Fig. 4*B*).

In summary, our immunocytochemical studies have directly demonstrated the somatic distribution of rSK1 channels in isolated rat hippocampal CA1 neurons and their colocalization with the D subclass of L-type calcium channels. In addition, we have provided evidence for how such a channel distribution may underlie the slow AHP observed in these neurons. These findings support the view that local rather than global rises in calcium are involved in the coupling of calcium-dependent processes. They also provide direct evidence of how selective functional coupling may be achieved through specific colocalization of different channel subtypes.

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