ROMK1 (Kir1.1) Causes Apoptosis and Chronic Silencing of Hippocampal Neurons

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Received 27 March 2000; accepted in final form 4 May 2000

Nadeau, H., S. McKinney, D. J. Anderson, and H. A. Lester. ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. J Neurophysiol 84: 1062–1075, 2000. Lentiviral vectors were constructed to express the weakly rectifying kidney K+ channel ROMK1 (Kir1.1), either fused to enhanced green fluorescent protein (EGFP) or as a bicistronic message (ROMK1-CITE-EGFP). The channel was stably expressed in cultured rat hippocampal neurons. Infected cells were maintained for 2–4 wk without decrease in expression level or evidence of viral toxicity, although 15.4 mM external KCl was required to prevent apoptosis of neurons expressing functional ROMK1. No other trophic agents tested could prevent cell death, which was probably caused by K+ loss. This cell death did not occur in glia, which were able to support ROMK1 expression indefinitely. Functional ROMK1, quantified as the nonnative inward current at −144 mV in 5.4 mM external K+ blockable by 500 μM Ba2+, ranged from 1 to 40 pA/pF. Infected neurons exhibited a Ba2+-induced depolarization of 7 ± 2 mV relative to matched EGFP-infected controls, as well as a 30% decrease in input resistance and a shift in action potential threshold of 2.6 ± 0.5 mV. This led to a shift in the relation between injected current and firing frequency, without changes in spike shape, size, or timing. This shift, which quantifies function as a role of ROMK1 expression, was predicted from Hodgkin-Huxley models. No cellular compensatory mechanisms in response to expression of ROMK1 were identified, making ROMK1 potentially useful for transgenic studies of silencing and neurodegeneration, although its lethality in normal K+ channels has implications for the use of K+ channels in gene therapy.

INTRODUCTION

Potassium ion selective channels establish the neuronal resting membrane potential (RMP) and restore it after firing. We describe a method for expressing a new set of K+ channels in neurons, to alter their excitability. Neuronal RMP is often 10–20 mV depolarized relative to the K+ reversal potential, and thus more open K+ channels are expected to hyperpolarize the cell. Moreover, more open channels lead to a reduction in input resistance, rendering excitatory synaptic currents less effective in depolarizing the cell to threshold. In brief, a change in input resistance due to more open K+ channels can abolish action potential firing (“silence” the cell).

There are several reasons to silence neurons in vitro and in vivo. Selectively targeting pathways or populations of neurons has revealed the importance of interneuronal communication in developing (Murakami et al. 1992) and adult (de la Cruz et al. 1996) systems. Botulinum toxin is used to lessen muscle spasms in patients with cerebral palsy (Flett et al. 1999) and spinal cord injuries (Al-Khodairy et al. 1998), and targeted lesions are often the only possible treatment for those with intractable epilepsy (Jallon 1997; Nayel et al. 1991). Control of excitability may also be important for lessening neurological damage following ischemic injury or in degenerative disease (Rodriguez et al. 1998). A genetic approach has two major advantages over toxins and surgery: one, it can target a pathway that is not fully understood; and two, it has the potential to be fully inducible and reversible over a time course of hours.

However, to evaluate genes as silencing candidates, it is important to be able to translate alteration of excitability seen in culture into vivo long-term behavior. We use an HIV-based lentiviral vector to create an in vitro model of transgenesis, with a K+ channel as the candidate silencer. Neurons are transduced soon after plating and allowed to grow and develop for days to weeks with unopposed channel expression. The viral genome is integrated into its host at low copy number, leading to stable expression levels and no interference with host protein synthesis. Efficacy and lack of toxicity of the viral vector itself are established, and no inactivation or down-regulation of the channel is observed over a 3-wk period. However, the chronic efflux of K+ leads to apoptotic cell death unless counteracted by a raised K+ concentration in the culture medium. This imposes limits on the possible in vivo use of ROMK1, and perhaps of other K+ channels.

METHODS

Molecular biology

The Xba I fragment of pEGFP (Clontech, Palo Alto, CA), containing the complete enhanced green fluorescent protein gene, was inserted into pTRE (Clontech) in the correct orientation to give pTRE-EGFP. This was cut with EcoR I and Age I, and a 33-bp polylinker containing Pac I and Swa I sites

\[ 5\\'\text{AATT CCCCC TTAATTTA CTAG ATTTAAAT CCCA} \]
\[ 3\\'\text{GGGGG AATTAATT GATC TAAATTTA GGGTGCC} \]

was linked to the sticky ends. The cap-independent translation enhancer (CITE) (500 bp from encephalomyocarditis virus) was amplified by polymerase chain reaction (PCR) (High Fidelity PCR Kit, Boehringer Mannheim, Indianapolis, IN) from pCITE-2a (Novagen, Madison, WI) and inserted in frame into the Age I–Nco I sites. PCR was verified by complete sequencing on both strands. The fragment

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including the polylinker and CITE-EGFP was then subcloned into the EcoR I–Not I sites of pCITE-2a to give [pCITE-(CITE-EGFP)].

The ROMK1 complete cDNA (Ho et al. 1993) (1.3 kb) plus 0.9 kb of 5’ untranslated sequence was excised from pSport (Life Technologies, Gaithersburg, MD) as a single Mlu I fragment and inserted into the BbsI II site of pNEB193 (New England Biolabs, Beverly, MA) to give pNEB193-ROMK1; the orientation in which the Pac I site was located on the 5’ end of the gene was selected.

To create lentiviral constructs, sequences were cloned into the plasmid pHR’ (gift of Didier Trono, The Salk Institute), which contains a human cytomegalovirus (CMV) promoter. The 1.3-kb CITE-EGFP was removed from pCITE-(CITE-EGFP) with BamHI I and Xho I and ligated to the corresponding sites of pHR’ to give pHR’-CITE-EGFP. The EcoR I and Pac I sites on the 5’ end of this fragment serve as a unique polylinker in this vector; ROMK1 was inserted into the EcoR I and Pac I sites after removal from pNEB193-ROMK1 to give pHR’-ROMK1-CITE-EGFP (Fig. 1A).

The control construct, encoding for EGFP alone, was constructed by subcloning the Xho I fragment of pEGFP into pBluescript (Stratagene, La Jolla, CA). The gene was then excised with BamHI I and Xho I and ligated into the same sites of pHR’.

To generate the EGFP-ROMK1 fusion protein, the EGFP gene minus the final stop codon was amplified from pEGFP by PCR, generating BamHI I and Mlu I ends; this was inserted into the corresponding sites of pHR’ to give pHR’-EGFPnostop. The first 500 bp of ROMK1 was also amplified by PCR, giving an Mlu I–Bgl II fragment. The remaining 1.7 kb was excised from pSport with Bgl II and Xho I, and a three-way ligation between these two fragments and pHR’-EGFPnostop (cleaved with Mlu I and Xho I) produced the final construct, pHR’-EGFP-ROMK1 (Fig. 1B). All PCR products were verified by complete sequencing, and ligations were verified by restriction digest and partial sequencing.

Generation of lentiviruses

Plasmids were amplified using Maxi and Mega kits from Qiagen (Valencia, CA). The plasmids pHR’-ROMK1-CITE-EGFP, pHR’-EGFP-ROMK1, pH’R’-EGFP, or pHR’ alone (which encodes LacZ) were co-transfected into 293T cells with the plasmids pMD.G and pMD.R8.9 in the ratios published (Naldini et al. 1996). Transfection was performed in 15-cm tissue culture dishes (Falcon, Oxnard, CA) at 80–90% confluence, using the cationic lipophilic reagents Superfect or Effectene (Qiagen). A total of 20 μl of plasmid DNA was added per 15-cm dish when Superfect was used; a total of 4 or 8 μl was added when Effectene was used. In the latter case, the ratio of Enhancer to microgram of plasmid DNA was 20:1. The EcoR I–Not I fragment of pNEB193-ROMK1 to give pHR’-ROMK1-CITE-EGFP (Fig. 1A).

The control construct, encoding for EGFP alone, was constructed by subcloning the Xho I fragment of pEGFP into pBluescript (Stratagene, La Jolla, CA). The gene was then excised with BamHI I and Xho I and ligated into the same sites of pHR’.

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Cell culture

Pregnant Wistar rats were euthanized by inhalation of CO2, at day 18 of gestation. Embryos were immediately removed by caesarean section, and hippocampi rapidly extracted under stereomicroscopic observation under sterile conditions, cut into 1 mm pieces, and digested with 0.25% trypsin and 0.25 mg/ml DNAse (Sigma, St, Louis, MO) at 36°C for 15 min. The pieces were then gently rinsed in Hanks’ balanced salt solution without Ca2+ or Mg2+ (HBSS, Life Technologies), washed twice in plating medium, and gently triturated in 1 ml of plating medium with five passes through the 0.78 mm opening of a tip of a P-1000 Pipetman. Suspended cells were removed with a Pasteur pipette, and the remaining pieces triturated once more. The resulting suspensions were gravity-filtered through a 70-μm nylon mesh to remove large debris, and centrifuged for 2 min at 150 g to pellet the cells, which were resuspended by trituration at about 1.5 × 10^6 cells/ml. Approximately 35,000 cells were plated in an area 15 mm in diameter at the middle of a 35-mm plastic culture dish that had been coated with poly-o-lysine (PDL) and laminin. Cultures were maintained at 35°C in a 5% CO2 incubator. One half volume of medium was changed twice weekly with culture medium. Plating and feeding medium was Neurobasal with B27 supplement, with 500 μM Glutamax, 25 μM glutamate, and 5% horse serum (Life Technologies); [K+] in this medium is 5.4 mM.

HEK 293 cells were maintained with weekly passages in DME high glucose medium (GIBCO) supplemented with 10% fetal bovine serum, 200 μM glutamine, and penicillin/streptomycin. Transfections were performed with Effectene (Qiagen) in 35-mm dishes according to the manufacturer’s instructions.

Infection of hippocampal neurons

Cells were infected 1–3 d after plating by the addition of 20–30 μl of ultracentrifuged or 200–300 μl of ultrafiltered virus to the medium. Viral supernatant was not washed off, although cells continued to be fed on a weekly basis. Stocks of virus were pooled so that each dish in a preparation received the same concentration. For “high K+” cells, supplementary KCl was added to a total concentration of 15.4 mM 12–24 h after infection. At least 40 h were allowed to elapse before assaying for EGFP expression. Control neurons were matched for age (to within 1 d) and for time since application of drugs and/or EGFP-only virus. All controls were from separate dishes; nonfluorescent cells from ROMK1 dishes were never used as controls because of the difficulty of excluding faint fluorescence. Sixteen of thirty-two control cells from ROMK1 dishes were never used as controls because of the difficulty of excluding faint fluorescence. Sixteen of thirty-two control cells from ROMK1 dishes were never used as controls because of the difficulty of excluding faint fluorescence. Sixteen of thirty-two control cells from ROMK1 dishes were never used as controls because of the difficulty of excluding faint fluorescence.
Toxicity was observed with either control construct (EGFP or LacZ). Methods after fixation for 2 min in 50/50 methanol/acetone. No viral NA FITC and EGFP and a 40x Plan-Neofluar water immersion lens with NA = 0.9 (Zeiss). Beta-galactosidase was visualized by standard methods after fixation for 2 min in 50/50 methanol/acetone. No viral toxicity was observed with either control construct (EGFP or LacZ).

**Whole-cell recording**

All recordings were performed at room temperature. For hippocampal neurons, borosilicate $\Omega$-dot glass capillaries (Sutter Instruments, Novato, CA) were pulled to a tip resistance of 5–10 MΩ and filled with either a Mg$^{2+}$-containing internal solution consisting of (in mM) 100 K-glucuronate, 10 HEPES, 3 phosphocreatine, 1.1 EGTA, 3 MgATP, 0.2 NaGTP, 5 MgCl$_2$, 0.1 CaCl$_2$; or a Mg$^{2+}$-free internal solution, 100 K-glucuronate, 10 HEPES, 1.1 EGTA, 0.1 CaCl$_2$, both adjusted to pH 7.2 with KOH and 250 mM sodium succinate. The bath solution contained (in mM) 110 NaCl, 10 HEPES, 5.4 KCl, 1.8 CaCl$_2$, 1.0 MgCl$_2$, 10 glucose, adjusted to pH 7.4 with NaOH ($E_K$ = $-76 \text{ mV}$ at $25^\circ\text{C}$). Calculated junction potential for these solutions is 14 mV (Clampex 8.0), and all reported membrane potentials are corrected for this value. Tetrodotoxin (TTX) was bath-applied to a final concentration of 1 μM or perfused at 500 nM; Ba$^{2+}$ (500 μM), Co$^{2+}$ (1 mM), and a “hippocampal cocktail” consisting of bicuculline (10 μM), APV (50 μM), and CNQX (20 μM) (all from RBI, Natick, MA) were perfused continually through flow pipes of 250 μm internal diameter mounted ~500 μm from the recorded cell. The dish was washed with 4–6 ml of control saline between recordings. For HEK 293 cells, capillaries were pulled to a tip resistance of 2–5 MΩ and filled with an internal solution containing (in mM) 130 KCl, 0.8 MgCl$_2$, 5 EGTA, 5 MgATP, 10 HEPES (pH to 7.2 with KOH); the bath solution consisted of (in mM) 137 NaCl, 10 HEPES, 4.0 KCl, 1.8 CaCl$_2$, 1.0 MgCl$_2$, 10 glucose, adjusted to pH 7.4 with NaOH ($E_K$ = $-91 \text{ mV}$ at $25^\circ\text{C}$). Junction potential with this solution is 4.5 mV. Signals were recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) and sampled by a Digidata 1200 at 20 kHz (100 kHz for transients) to a Pentium PC. Series resistance was not compensated but was monitored throughout the recording. For I-V analysis, series resistance was compensated off-line (Nadeau and Lester 2000; Traynelis 1998). Current and voltage commands and data acquisition were performed using PCLAMP 6.0 and 8.0. Data were analyzed with Axograph 3.5 (Axon). Individual compiled modules were written using CodeWarrior Pro (Metrowerks Software). Cells were eliminated from the analysis if series resistance changed by more than 20% during the course of the recording, if the cell spontaneously depolarized, or if the membrane capacitance changed in either direction by more than 10%. Data from cells in TTX and cocktail were pooled for many analyses as there were no detectable differences in membrane potential or effects of ROMK1 expression or blockade. Data on threshold and silencing were unavailable for cells with TTX bath application ($n$ = 10 ROMK1 cells, 5 high $K^+$ controls).

**Immunocytochemistry**

TUNEL staining was performed with the In Situ Cell Death Detection Kit-POD (Boehringer) according to the manufacturer’s instructions; the horseradish peroxidase (HRP)-conjugated secondary was developed with nickel-diaminobenzidine reagent and visualized under brightfield. Antibody staining was with polyclonal rabbit anti-ROMK1 (Alomone Labs, Jerusalem, Israel). Cultured cells were fixed for 2 min in methanol/aceton, permeabilized for 2 min on ice with 1% Triton X-100, and incubated for 30 min in PBS with 5% goat serum. Primary antibody was diluted 1:50 in the same solution and incubated with gentle shaking for 2 h at room temperature or overnight at 4°C. The dish was washed five times and incubated with fluorescent secondary antibody (Cy-3 conjugated goat anti-rabbit, Jackson Immunoresearch, West Grove, PA) for 60 min at 37°C and visualized with a Texas Red filter (excenter 560/55; dichroic 595 LP; emitter 645/75) (Chroma). Omission of primary antibody led to weak, nonspecific staining. Fluorescence was quantified with NIH Image using confocal images to distinguish membrane-bound from cytoplasmic fluorescence.

**Results**

Transfection of bicistronic and fusion vectors

Transfected into HEK 293 cells, the vectors pHr-ROMK1-CITE-EGFP and pHr’EGFP-ROMK1 produce weakly inwardly rectifying currents that are blockable by 500 μM Ba$^{2+}$ in a time- and voltage-dependent manner. The block is reversible on Ba$^{2+}$ wash-out and reverses at $E_K$ after leak subtraction (Fig. 2). The bicistronic message produces a strong fluorescent signal throughout the cell, while the fusion protein appears as a weaker, punctate fluorescence mostly distal to the plasma membrane and completely excluding the nucleus (data not shown; see Fig. 6 for distribution in neurons).

Effects ROMK1 on neuronal morphology and survival

Hippocampal neurons infected with control virus, bearing EGFP only, become visibly fluorescent after 24–36 h and increasingly so for several days thereafter. Cell morphology, processes, and underlying glia are unchanged. Infected cells can be maintained and recorded from for up to 6 wk; their electrophysiological properties are identical to those of normal cells (Fig. 3, A and B; Table 2). Hence, there is no viral toxicity detected within the sensitivity of our experiments.

In contrast, cells infected with ROMK1-CITE-EGFP show no healthy green neurons at 48 h. Expression is limited to astrocytes, possibly activated microglia, and dead or dying cells of varying morphologies. Many dead cells are shrunken and floating, a classic indicator of apoptosis (Gibson 1999) (Fig. 3, C and D). TUNEL staining (Villalba et al. 1997) reveals no apoptotic cells 24 h post infection, but nearly 100% of the neurons are apoptotic at 48–72 h (Fig. 3, E–G). By the fourth to fifth day, few neurons remain in the dish (Fig. 3H). Glial cells can maintain stable infections with ROMK1-CITE-EGFP for weeks or months, as can CHO or HEK-293 cells. The latter may be serially passaged indefinitely without visible change in the proportion of fluorescent cells. Thus cultured hippocampal neurons but neither glia nor clonal cell lines appear susceptible to overexpression of ROMK1.

Channel block by inorganic ions is ineffective at rescuing ROMK1-expressing neurons. Dishes supplemented with 200–500 μM BaCl$_2$ show the same pattern of apoptosis as untreated cultures; however, the blockade at these concentrations affects...
mainly inward and not outward K⁺ currents (Ho et al. 1993). Higher concentrations of Ba²⁺ permit 10% of ROMK1-expressing neurons to survive but are toxic to glia and hence lead to massive deterioration in all cells.

**Elevated K⁺ prevents apoptosis**

We tested the hypothesis that K⁺ loss through ROMK1 causes apoptosis. When the neuronal growth medium K⁺ concentration is increased to 15.4 from the usual 5.4 mM (high K⁺), which shifts $E_K$ from -79 to -51 mV at 36°C, fluorescence microscopy of ROMK1-CITE-EGFP infected cells at 48–72 h reveals 50–90% green neurons with normal morphology (data not shown). Importantly, increased K⁺ has no visible effect on the fluorescence of EGFP-only cells. ROMK1-expressing neurons can be maintained with weekly feedings of medium containing 15.4 mM K⁺ for >3 wk. This life span is similar to that of controls in high K⁺. Thus this elevation of K⁺ is sufficient to prevent apoptosis and to restore
ROMK1-infected neurons to an apparently normal state of health.

The results are consistent with a specific action of K\(^+\): activation of apoptotic pathways due to K\(^+\) efflux (Yu et al. 1997). However, they may be equally explained by nonspecific trophic actions of chronic depolarization and Ca\(^{2+}\) entry. The use of Ca\(^{2+}\) agonists and antagonists is necessary to distinguish these mechanisms.

The protective effect is specific to K\(^+\)

Unlike many neuronal cells in culture, hippocampal neurons normally are not dependent on sustained depolarization for survival. They may be grown in media containing as little as 2.5 mM K\(^+\) or in 1 \(\mu\)M TTX, a concentration sufficient to block all action potentials (Table 1). On the other hand, they are highly sensitive to excitotoxicity, and increased current through L-type Ca\(^{2+}\) channels is harmful (Porter et al. 1997). It is therefore unlikely that any amount of ROMK1-mediated hyperpolarization and altered membrane conductance could lead to loss of Ca\(^{2+}\) sufficient to cause the rapid, total cell death that we observe. To eliminate this possibility, however, we treated infected dishes with combinations of trophic factors and Ca\(^{2+}\) agents: cpt-cAMP (0.1–0.5 mM); thapsigargin (100 nM), which causes the release of intracellular Ca\(^{2+}\) stores; BayK 8644 (1 \(\mu\)M), an L-type Ca\(^{2+}\) channel agonist; BayK 8644 plus nifedipine (10–100 \(\mu\)M), an L-type antagonist expected to counteract the effects of BayK; glutamate (40 \(\mu\)M); or BDNF (20 ng/ml).

None of these agents are able to maintain ROMK1-infected neurons (Table 1). Neurons in normal K\(^+\) exposed to agents that increase intracellular Ca\(^{2+}\) show 100% apoptosis by 48 h, as in untreated cells (Fig. 4A). Neither EGFP-infected controls nor ROMK1-infected neurons in high K\(^+\) are harmed by these drugs. Ca\(^{2+}\) antagonists reverse the beneficial effects of high K\(^+\) only slightly.

There is a difference in appearance between the ROMK1-infected cells with and without Ca\(^{2+}\) elevation. In the former case, nearly every neuron in the dish is fluorescent, but their morphologies are highly bizarre; they are completely depolarized, and they detach from the dish (Fig. 4B). In the latter case, the neurons disappear before many distorted forms are seen. This suggests that Ca\(^{2+}\) elevation is acting to prevent phagocytosis by astrocytes or microglia, without reversing the lethal phenotype caused by the channel; this could result either from effects of elevated Ca\(^{2+}\) on the apoptotic process (Bratton et al. 1999) or from direct effects on the glia.

These data point to K\(^+\)-efflux mediated apoptosis as the cause of cell death in long-term ROMK1 expression. Loss of K\(^+\) may impose a metabolic burden on the cell, instead of or in addition to triggering apoptotic pathways; this is a topic for future study.

K\(^+\) loss is a critical feature of ROMK1 expression that restricts its applicability for gene therapy or transgenics. However, it may still possess properties of a useful silencing gene, and understanding its effects can lead to improvements in

TABLE 1. Survival of ROMK1-infected neurons exposed to pharmacological agents

<table>
<thead>
<tr>
<th>Drug</th>
<th>KCl</th>
<th>BaCl(_2) (0.2 mM)</th>
<th>BaCl(_2) (1 mM)</th>
<th>BayK 8644</th>
<th>KCl + Nif.</th>
<th>Thap.</th>
<th>cpt-cAMP</th>
<th>BDNF</th>
<th>TTX</th>
<th>KCl + BayK</th>
<th>Glut.</th>
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<tr>
<td>Survival EGFP</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>Survival ROMK1</td>
<td>0</td>
<td>+</td>
<td>0/−</td>
<td>N/D</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>N/D</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

See RESULTS for drug concentrations. “0” survival indicates that no fluorescent neurons are found in the dish, or that those that are found are severely altered in morphology, depolarized, and cannot be patch-clamped. “(−)” survival indicates <10% of cells in the dish are fluorescent and healthy enough to yield recordings; signs of cell death or disappearance are always seen. “(+−)” survival indicates slightly reduced viability of fluorescent cells: 10–50% of the cells are fluorescent and most of these can be patch-clamped, but there is some cell death and fluorescent cells may appear less healthy than uninfected neighbors. Dishes showing “(+)” survival have no signs of cell death relative to mock-infected dishes; 50–90% of the neurons are fluorescent; no morphologic features distinguish infected from uninfected cells; and 80–90% of infected cells yield electrophysiological recordings with resting membrane potentials more hyperpolarized than −30 mV. Nif., nifedipine; Thap., thapsigargin; BDNF, brain-derived neurotrophic factor; Glut., glutamate; N/D, not determined. * Widespread glial loss and damage.
silencing strategies. In addition, outward ROMK1 currents may not occur in all neurons at all ages, especially those subjected to chronic electrical input and depolarization. It is therefore informative to examine the properties of cells maintained in high K⁺, where ROMK1-infected cells have the same growth patterns, morphology, and life span as matched EGFP-infected controls.

**Electrophysiology: Expression levels and localization**

Because this study concerns the effects of a new conductance on encoding, an accurate description of membrane parameters is important. Many neurons in culture can be successfully described by a single capacitance, input resistance, and series (pipette) resistance. The resistance of the proximal dendrites is large enough that little attenuation occurs in the signal as it travels to the soma, and the soma resistance is itself large enough to overcome most of the artifacts caused by the series resistance (typical values of input and series resistance are 1000 and 10 MΩ, respectively). However, for cells infected with a K⁺ channel, this may no longer be the case: input resistance may decrease two- to fivefold, with a corresponding increase in artifacts. We therefore use a two-compartment model to provide better exponential fits and the ability to distinguish somatic conductances from poorly space-clamped conductances on the distal dendrites (APPENDIX B; see also Nadeau and Lester 2000).

ROMK1-infected and control cells were similar in age and size of both the proximal and distal compartiments (Table 2); the apparently small size of C₁₅ in the low K⁺ controls is due to a preponderance of young cells in this group [only 2 of 9 were over 7 days in culture (dic)]. The cells are somewhat smaller than in other studies of hippocampal neurons (Mennerick et al. 1995), which primarily reflects our inclusion of young cells, although some stunting due to high K⁺ may also occur.

Blockade by 500 μM Ba²⁺ was used to quantify expression. The honeybee toxin tertiapin (Jin and Lu 1998) has been proposed as a specific blocker for inward rectifier K⁺ channels. The honeybee toxin tertiapin (Jin and Lu 1998) has been recently been synthesized (Jin and Lu 1999), its effects on other studies of hippocampal neurons (Mennerick et al. 1995), which primarily reflects our inclusion of young cells, although some stunting due to high K⁺ may also occur. On the other hand, inhibition of the channel by submillimolar Ba²⁺ is well characterized, rapid, and easily reversible (Choe et al. 1998; Ho et al. 1993). Its only drawback is incompleteness of block at depolarized potentials, following the Woodhull equation

\[ C(V) = 1 - \frac{I_{ba}}{I} = 1 - \frac{1}{1 + \frac{[Ba]}{K_d} \exp \left( \frac{-\delta V F}{RT} \right)} \]

where \( V \) represents membrane potential, \( \delta \) is the valence of the blocking ion, \( K_d \) is the electrical distance, \( K_d \) is the required [Ba²⁺] for half block at \( V = 0 \) mV, \( F \) is the Faraday constant, \( R \) is the gas constant, and \( T \) is the absolute temperature (here and elsewhere, the subscript Ba indicates quantities measured in Ba²⁺). Previously reported values of \( K_d = 10 \) mM and \( \delta = 0.41 \) (Loffler and Hunter 1997) give blocked fractions of 0.85 and 0.25 at −150 and −60 mV, respectively.

Total blocked current at a holding potential of −130 mV (corresponding to a membrane potential of −144 mV) (Fig. 5A) shows an upward trend with time in ROMK1-infected neurons, with levels of expression peaking at 6–7 d and remaining steady thereafter (Fig. 5B). In low K⁺ controls, this concentration of Ba²⁺ blocks no native channels, but after several days in high K⁺ up-regulation of native inwardly rectifying K⁺ channels (Guo et al. 1997; Knutson et al. 1997) causes high K⁺ controls to exhibit a smaller but significant Ba²⁺ block. It is not certain that the up-regulated channels are members of the Kir superfamily, but for convenience, we shall abbreviate them as EIRKs, for “endogenous inwardly rectifying K⁺” channels. We show (APPENDIX A, Fig. 8) that they display strong inward rectification. Levels of this channel(s) remain essentially constant over the period studied.

With Mg²⁺-free internal solution, the mean current at this voltage is 1.75-fold that is seen with internal solution containing Mg²⁺, ATP, and GTP (n = 5 for Mg²⁺-free cells, P < 0.15, t-test), consistent with suppression of ROMK1 by cytoplasmic ATP (Ho 1993). If blockable currents in control cells are subtracted from those in ROMK1 neurons (this slightly underestimates the amount of ROMK1 expressed, see APPENDIX A), the resulting average expression level is approximately one quarter of that previously measured with adenovirus-mediated

<table>
<thead>
<tr>
<th>Type</th>
<th>( N )</th>
<th>( C_M )</th>
<th>( C_d )</th>
<th>dic</th>
<th>dpi</th>
<th>( R_{TOT} ) (Ba²⁺)</th>
<th>( R_{TOT} ) (Ba²⁺)</th>
<th>( V_m )</th>
<th>( V_m ) (Ba²⁺)</th>
<th>I144</th>
<th>Block</th>
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<tbody>
<tr>
<td>ROM</td>
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<td>27 ± 2</td>
<td>24 ± 3</td>
<td>4–21</td>
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<td>314 ± 27*</td>
<td>459 ± 42‡</td>
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<td>C15</td>
<td>32</td>
<td>32 ± 2</td>
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<td>0–20</td>
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<td>494 ± 93</td>
<td>−65 ± 1</td>
<td>−65 ± 1</td>
<td>253 ± 34</td>
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<td>C5</td>
<td>9</td>
<td>28 ± 3</td>
<td>11 ± 2</td>
<td>4–21</td>
<td>0–20</td>
<td>952 ± 173*</td>
<td>1079 ± 257*</td>
<td>−60 ± 3*</td>
<td>−60 ± 4</td>
<td>82 ± 14*</td>
<td>4 ± 5*</td>
</tr>
</tbody>
</table>

\( N \), total number of neurons retained in each group; \( C_M \), capacitance of the first compartment (pF), corresponding to the soma and proximal dendrites; \( C_d \), capacitance of the distal compartment (pF), corresponding to narrow processes that are separated from the soma by a substantial resistance; dic, days in culture; dpi, days post infection; \( R_{TOT} \), total input resistance measured from a −10 mV step from −94 mV (MΩ); \( R_{TOT} \) (Ba²⁺), input resistance after the addition of 500 μM Ba²⁺ (MΩ); \( V_m \), resting membrane potential (mV); \( V_m \) (Ba²⁺), resting membrane potential in 500 μM Ba²⁺ (mV); I144, total current at −144 mV (pA); block, Ba²⁺-blockable current at −144 mV (pA). All values are shown ± SE. All membrane potentials are adjusted for junction potential. ROM indicates cells infected with ROMK1; C are control cells, half uninfected and half infected with EGFP only (dpi = 0 refers to uninfected cells); C15 designates cells raised in 15.4 mM K⁺ and C5, those in 5.4 mM K⁺. The relatively small value of the distal capacitance is due to the inclusion of very young cells in the experiments; \( C_d \) approximately doubled during the first week in culture. * Significantly different from high K⁺ controls (P < 0.05); ‡ significantly different in Ba²⁺ (P < 0.01).
Ehrengruber et al.; a level this high represents an unusual situation for a retrovirus and is probably due to positional effects (Schubeler et al. 1998). However, even with retroviral vectors, current densities of >12 pA/pF were not unusual, representing 11% of all infected cells.

We identify a subgroup of infected neurons that do not fire action potentials at the maximum tested level of current injection (200 pA), but exhibit normal firing patterns after blockade by Ba\(^{2+}\). These are designated “completely silenced cells” and show significantly greater blockable currents than the mean of all ROMK1-infected neurons; these cells illustrate the effects of the maximal level of expression achievable with lentiviral transduction.

Localization was investigated by antibody staining, confocal imaging of EGFP-ROMK1 fusion expression, and two-compartment calculation of currents (Fig. 6). No specific localization pattern was identified, consistent with simple diffusion of proteins throughout the cell.

Effects of ROMK1 on encoding properties: Change in RMP with Ba\(^{2+}\)

Full I-V relations with and without Ba\(^{2+}\) demonstrate voltage dependent block in both ROMK1 and control neurons (Fig. 7). While EIRK complicates the picture at hyperpolarized potentials, blockable currents near typical RMP values of −60 to −65 mV are due almost entirely to ROMK1, giving an I-V similar to that seen in HEK cells (Figs. 7E and 8). Therefore, a significant change in RMP is expected in expressing cells with the addition of Ba\(^{2+}\), but not in controls. This depolarization can be predicted, with two assumptions: that Na\(^{+}\) and K\(^{+}\) are the only ions contributing to RMP, and that Na\(^{+}\) conductance does not change with Ba\(^{2+}\). Then

\[
\Delta V_m = \frac{G_K - G_{Ba}}{G_{TOT}} (E_K - V_{nab})C(V_m) = \frac{G_{ROM}}{G_{TOT}} (E_K - V_{nab})C(V_m)
\]

where \(\Delta V_m\) is the shift in RMP, \(E_K\) is the K\(^{+}\) reversal potential, \(G_K\) represents K\(^{+}\) conductance, and \(G_{TOT}\) is the total conductance, and \(C(V_m)\) is the Woodhull equation Eq. 1 giving the fractional Ba\(^{2+}\) block at RMP. Using this formula with values of \(C(V_m)\) obtained from block at −55 mV predicts \(\Delta V_m = 8.2 \pm 0.4\) for all cells <5 dpi. The observed value in this group is −8.4 ± 0.5 mV (\(n = 6\)), giving excellent agreement between the observed and theoretical values. All cells in this age group show a change in RMP; the minimum and maximum are −7 and −10 mV, respectively. Control cells do not change by more than 1 mV in either direction (\(n = 6; -1 \pm 1\) mV, mean ± SE). Ba\(^{2+}\) blockade restores RMP of ROMK1 neurons to the level seen in low K\(^{+}\), rather than high K\(^{+}\), controls (Table 2). This suggests that less EIRK is present in ROMK1-expressing neurons than in controls (discussed in detail in APPENDIX A).

With increasing periods, postinfection changes in RMP become smaller, even in cells expressing 20–40 pA/pF of Ba\(^{2+}\) blockable current at hyperpolarized potentials. At 13 dpi, average \(\Delta V_m\) has fallen to −5.1 ± 1.0 mV (\(n = 12\)), even as ROMK1 expression has increased (Fig. 5B). There are two identifiable reasons for this. First, \(V_{nab}\) is hyperpolarized by 1 mV, from −58 ± 1 to −59 ± 2 mV, making it closer to \(E_K\); the predicted change has now decreased slightly, to −7.0 ± 1.6 mV.

In addition, there is now a discrepancy between the pre-
predicted and observed values. Its origin is a hyperpolarization resulting from Ba\(^{2+}\) wash-in in neurons in long-term high K\(^{+}\); this means that our assumption that \(G_{Na}\) does not change with Ba\(^{2+}\) is no longer correct. At 13 dpi, the mean in controls is 1.5 ± 1 mV, \(n = 10\). Subtracting this from the mean given by Eq. 2 gives an adjusted value of \(-5.5 \pm 1.9\) mV, well in line with the observations.

By 18–21 dic, average RMP change in ROMK1 cells has fallen to \(-2.4 \pm 0.8\) mV (\(n = 12\)). This can be almost entirely explained by proximity of \(V_{m,Ba}\) to \(E_{K}\); cells are now hyperpolarized to \(-66 \pm 1\) mV, and the predicted change is \(-3.5 \pm 0.6\) mV. The discrepancy of \(1.8 \pm 0.8\) mV results from 4/12 cells that show no depolarization or even hyperpolarization in Ba\(^{2+}\) (2 cells with hyperpolarization, 1.9 and 3.2 mV).

In controls (\(n = 7\)), hyperpolarization is also seen only in a subset of cells, always accompanied by the slow, stepwise block shown in the last panel of Fig. 5A. Mean change in RMP is 4.66 ± 2.2 mV, range \(-1.2\) to 15 mV, with no hyperpolarization seen in two cells and ≥10 mV in two cells. This may reflect different subpopulations of neurons with differing responses to elevated K\(^{+}\) and is a topic for future study. The degree of hyperpolarization does not appear to correlate with level of ROMK1 expression, but the numbers of neurons displaying this phenomenon are too small for meaningful statistics.

These compensatory channels may explain the extended lifetime of infected cells washed back into normal K\(^{+}\) after >7 dpi: \(4-5\) d versus <48 h for cells infected in low K\(^{+}\). However, despite the disappearance of membrane hyperpolarization, silencing is not eliminated: the effects of the channel on excitability increase with increasing levels of ROMK1 expression and are only partially dependent on RMP.

**Three changes are responsible for silencing**

In the cells we studied, spiking elicited by current injection shows a pattern typical of hippocampal neurons (Fig. 9A). There is a definite threshold potential for the occurrence of spikes (threshold was taken to be the point at which the time derivative of the current exceeded 10 V/s). In most cells, accommodation of firing rates occurs during a depolarization lasting 800 ms. Higher currents increase the firing rate, but beyond a maximum level of \(\sim 20\) Hz, spikes broaden and firing rates decrease. Plots of spike frequency versus current injection reveal that the addition of Ba\(^{2+}\) to infected cells changes the threshold of spike response without affecting the overall shape of the frequency-versus-current relation (Fig. 9B). We define this shift, with the dimensions of pA, as the measure of “silencing,” \(S\).

\(S\) can be broken down into three components, indicating the amount of current necessary to overcome the ROMK1-induced
range of voltages from $2$ to $75$ to $20$ mV.

\[ S = (T_m - V_m)(\Delta G) + G(-\Delta V_m + \Delta T) \] (3)

Each of these contributions to the curve shift can be determined from a Hodgkin-Huxley (HH) model; the only necessary measurements are cell conductance with and without Ba$^{2+}$ (corresponding to the amount of ROMK1 present), RMP, and spike threshold in Ba$^{2+}$. Then the $G_s$’s in Eq. 3 are known; the RMP shift is given by Eq. 2, and the threshold change results from the number of extra Na$^+$ channels that will have to open to compensate for the increase in $G_K$. If the Na$^+$ conductance is assumed to exhibit a voltage dependence of the form (Hille 1992)

\[ G_{Na}(V + \Delta V) = G_{Na}(V) \exp(\Delta V/3.9) \] (4)

then the extra needed Na$^+$ conductance will balance a new K$^+$ conductance when

\[ \Delta G_K = G_{Na}(V_{th}) \exp(\Delta V/3.9) - 1 \]

\[ \Delta T = 3.9 \ln \left( \frac{\Delta G_K}{G_{Na}(V_{th})} + 1 \right) \]

\[ = 3.9 \ln \left( \frac{G_{ROM}}{G_{Na}(V_{th})} + 1 \right) \]

\[ = 3.9 \ln \left( \frac{2G_{ROM}}{G(V_{th})} + 1 \right) \] (5)
since at threshold $G_{Na} = G_K$. Equation 5 predicts the observed threshold change very well for a majority of neurons: for ROMK1 cells $<18$ dic that fire both before and after Ba$^{2+}$ ($n=30$), a least-squares fit to the data gives Eq. 5 with a slope of 3.80 rather than 3.9. Beyond this age, compensatory channels confound the picture, giving a greater Na$^+$ channel conductance than that predicted by the HH equations (see APPENDIX A). Observed spike thresholds are $-50 \pm 2$ mV for ROMK1 cells, $-53 \pm 1$ mV for ROMK1 cells in Ba$^{2+}$, and $-59 \pm 3$ mV for high K$^+$ control cells. This change corresponds to a Ba$^{2+}$-induced conductance decrease of approximately 50%.

Since cells must fire both with and without Ba$^{2+}$ to yield values of $S$, the completely silenced cells are excluded from this analysis. Nevertheless, even neurons expressing an average amount of ROMK1 have significant Ba$^{2+}$-blockable conductance at depolarized potentials, and the effects on firing are well predicted by this simple ohmic model (Fig. 11).

**DISCUSSION**

K$^+$ channels have been proposed as candidates for transgenesis and gene therapy, because expression of several different types has been shown to decrease excitability in cultured neurons (Ehrengruber et al. 1997; Johns et al. 1999). In all of these experiments, however, the channels were active only in the presence of agonist or inducer. Expression was activated during or immediately before electrophysiological recording, so that long-term health effects of the gene or neuronal compensatory mechanisms could not be identified. Furthermore, adenovirus expression levels are many times those seen in transgenic animals; it is unknown whether a small change in K$^+$ conductance will have equally profound effects. In fact, mice transgenic for Shaker (AKv1.1a) (Sutherland et al. 1999) show a hyperexcitable phenotype, with spontaneous EEG discharges and lowered seizure thresholds, in apparent contradic-

![FIG. 9. Excitability changes identified by action potential analysis. A: spiking in response to current injection. Left: the upper trace shows a ROMK1-infected cell subjected to a series of depolarizing current steps in 20 pA intervals, from $-20$ to 160 pA. Lower trace: the same cell during application of Ba$^{2+}$; the membrane potential is slightly depolarized, the input resistance is increased, and a classic pattern of spiking is displayed. Right: control cells show very little difference without (above) and with (below) Ba$^{2+}$ perfusion. Infected cells display reduced input resistance with and without Ba$^{2+}$, as evidenced by smaller changes in membrane potential between each 20 pA step as compared with controls. B: spike frequency versus current injected for 2 representative ROMK1 cells and a high K$^+$, EGFP-infected control. Cell 1 (green, circles) shows a similarly shaped spike-versus-current curve with (open symbols) and without (solid symbols) perfusion of 500 μM Ba$^{2+}$. The shift in the curve is our definition of silencing, $S$. Cell 2 (red, squares) fails to fire without Ba$^{2+}$ even at the highest level of current injected (200 pA) and is designated a completely silenced cell. The value of $S$ for such a cell can be estimated by calculating the expected threshold based on the membrane potential and input resistance (see RESULTS). The control cell (black, dashed lines) shows little change in the firing peak with (open diamonds) and without (filled diamonds) Ba$^{2+}$ perfusion; mean $S < 0$ pA for controls (see Fig. 11).](http://jn.physiology.org/
based on observed conductance change, 2 pA plotted versus Ba\(^{2+}\) change, 36.7 \(\mu\)M for ROMK1 cells (open circles) and controls (open triangles), and theoretical extrapolations for completely silenced cells (gray diamonds).

C: the theoretical value for ROMK1 cells (open circles) and controls (open triangles), and theoretical extrapolations for completely silenced cells (gray diamonds).

We begin to address some of these issues by examining chronic, unopposed K\(^{+}\) conductance expressed at low levels from a viral vector with minimal toxicity. The weak inward rectifier ROMK1 results in apoptosis in 100% of dissociated hippocampal neurons, independent of age. Cell death cannot reliably be prevented by any pharmacological agents other than K\(^{+}\) channel blockers. We begin to address some of these issues by examining chronic, unopposed K\(^{+}\) conductance expressed at low levels from a viral vector with minimal toxicity. The weak inward rectifier ROMK1 results in apoptosis in 100% of dissociated hippocampal neurons, independent of age. Cell death cannot reliably be prevented by any pharmacological agents other than K\(^{+}\) channel blockers. Accordingly, K\(^{+}\) rescue is not antagonized by Ca\(^{2+}\) channel blockers.

Although increasing K\(^{+}\) to 15.4 mM is sufficient to permit survival of nearly all ROMK1-expressing cells, this manipulation has profound effects, especially after prolonged times. EGFP-infected and uninfected cells up-regulate native inward rectifiers, which we have termed EIRKs in this paper, so that they are hyperpolarized by 5 ± 2 mV when recorded in normal K\(^{+}\) medium. They also show a threefold increase in current at \(-144\) mV and a slightly more than twofold decrease in input resistance at \(-94\) mV relative to neurons in normal K\(^{+}\). After more than 2 wk in culture, additional channels begin to appear, some of which (a) are permeable to Na\(^{+}\) and (b) lead to hyperpolarization on Ba\(^{2+}\) application (details in APPENDIX A).

Application of 500 \(\mu\)M Ba\(^{2+}\) leads to block of EIRK and ROMK1 at hyperpolarized potentials, while at more depolarized potentials near threshold, the EIRK conductance disappears and a smaller fraction of ROMK1 is blocked. However, the blockade is sufficient to restore the membrane potential, spike threshold, and whole-cell conductance of ROMK1 expressing cells to near control values while they are in Ba\(^{2+}\).

Observed reduction in excitability is essentially ohmic and is due to three factors: decreased membrane resistance near RMP (1.5- to 5-fold), raised action potential threshold (2.6 ± 0.5 mV), and membrane hyperpolarization (7 ± 2 mV for all ROMK1 cells relative to precisely age-matched controls). The latter two factors are directly related to the change in resistance, identifying a single factor that is necessary for silencing and that can be used to predict neuronal response to any foreign channel.

The observed results confirm that ROMK1 is functional in the infected neurons studied and that changes in resting excitability are due to a Ba\(^{2+}\)-blockable K\(^{+}\) conductance. The...
predictability of these changes, and the normal firing patterns of infected cells in Ba²⁺ even after weeks of infection, suggest that nonspecific metabolic effects of the channel are minimal in 15.4 mM K⁺. As few as 150 open ROMK1 channels can result in silencing of 20 pA, while higher numbers (up to 1,000) lead to neurons that cannot fire in the absence of Ba²⁺.

CONCLUSION

What is the future of K⁺ channels as silencing agents? Our results confirm previous acute experiments showing hypoxcitability in response to a foreign K⁺ channel, but underline the importance of long-term expression to identify adverse affects that may occur in transgenic animals or in gene therapy. A transgenic silencing experiment may be regulated by means of inducible promoters with adjustable levels of induction, such as the tetO-CMV promoter (Huang et al. 1999) and ideally would be fully reversible. However, the time scale of induction and reversal with even the best genetic systems is days to weeks (Chen et al. 1998), several times longer than the life span of neurons expressing even the lowest levels of ROMK1. This channel therefore does not provide an alternative to all-or-nothing lesions or knockouts, at least in adult neurons.

A weak inward rectifier was chosen for these experiments partially because of its expected ability to hyperpolarize neurons with outward current, but the demonstrated minor role of membrane potential in silencing suggests that this type of channel is not the best. A strong inward rectifier may provide all of the silencing with less or none of the K⁺ loss; experiments to test long-term expression of such channels are therefore the next step.

There are also neuronal subtypes and developmental stages that require high K⁺ medium in dissociated culture, and analogously, high levels of electrical input in vivo: cerebellar granule cells (Galli et al. 1995) and retinal cells (Araki et al. 1995) are prime examples. As long as these depolarizing conditions exist, such cells may be silenced but not killed by the presence of ROMK1, as are hippocampal neurons in high K⁺. The channel may therefore be a useful tool for studying the dependence of neuronal migration and differentiation on excitability. Whether cells that require elevated K⁺ are able to survive ROMK1 expression in vitro may be easily tested before carrying out transgenic experiments. The increasingly understood link between apoptotic pathways and K⁺ loss (Padminabhan et al. 1999; Pike et al. 1996) may also make ROMK1 an excellent model of neurodegeneration.

Finally, our electrophysiological results identify the factors important for electrical silencing and suggest alternative methods of achieving this goal. K⁺ may be too intimately connected with the cell cycle to allow its balance to be altered, but any ion channel that doubles a neuron’s input conductance will be a significant silencing agent, as long as its reversal potential is more negative than RMP. An example would be Cl⁻ channels, which play important inhibitory roles in vertebrate and invertebrate nervous systems.

APPENDIX A: EFFECTS OF HIGH K⁺ CULTURE CONDITIONS

A variety of new or up-regulated channels and functional changes were identified in hippocampal neurons in response to chronic application of 15.4 mM K⁺. Apart from EIRK, these effects do not alter our analysis of ROMK1, as they occur to the same extent in control and ROMK1 neurons. Nevertheless, they are important for situations in which these cells may be exposed to depolarizing conditions.

Synaptogenesis, cation channels, and TTX-insensitive Na⁺ current

A lack of synaptogenesis due to prolonged elevation of K⁺ has been seen in neocortical neurons (Baker et al. 1991). We observe a similar effect here, where no postsynaptic potentials or currents are resolvable in high K⁺ control cells even after 14–21 dic. An identical picture occurs in the ROMK1 cells, not reversed by Ba²⁺ application (Fig. A1). The mechanisms responsible for this synaptic silence may be different in the two cases: in the ROMK1 cells, high-input conductance may shunt synaptic input. However, effects of ROMK1 on synapses are experimentally inaccessible under these conditions.

The neurons also show the development of at least one Ba²⁺-blockable nonspecific or cation current, so that by 13 dic, Ba²⁺ washout leads to membrane hyperpolarization. The effect of Ba²⁺ on neurons in long-term high K⁺ is complex and follows at least two separate time courses (see Fig. 5A): a rapid hyperpolarization, followed by a much slower increase in membrane resistance at voltages near threshold (more positive than −60 mV). On Ba²⁺ washout, the membrane potential recovers rapidly, but the resistance remains at this higher value throughout the time courses observed (1–2 min). There is no apparent difference between ROMK1-infected cells and controls.

Additionally, both the ROMK1 and high K⁺ control neurons show a TTX-insensitive Na⁺ conductance that becomes apparent with the application of 500 μM Ba²⁺ (Fig. 7, B and D); this has also been noted in brainstem motor neurons raised in elevated K⁺ (Eustache and Gueritaud 1995). The conductance is not altered by application of Co²⁺ and is therefore not Ca²⁺ dependent. It is slightly but not significantly larger in high K⁺ controls (38 ± 6 pA/pF, n = 4, occurrence in 4/5 cells in TTX) than in ROMK1 cells (25.4 ± 3.7 pA/pF, n = 9; occurrence in 9/10 cells in TTX; P = 0.2). This indicates that it is a response to the elevated K⁺, not to ROMK1, and may in fact occur to a slightly lesser degree in the latter. Nevertheless, ROMK1 cells and not controls are able to fire spikes in the presence of 1 μM TTX, 1 mM Co²⁺, and Ba²⁺ (Fig. A2).

EIRK up-regulation: Less in ROMK1 neurons

The only adaptive change that differed between ROMK1 and control neurons involved the inwardly rectifying K⁺ conductance.

![Fig. A1.](Image) Elevating K⁺ disrupts synapse formation. 10-s voltage-clamp traces (V_hold = −74 mV) showing (A) normal distribution of synaptic currents in a hippocampal neuron 14 dic; B: complete suppression of currents in an uninfected neuron 15 dic, switched from 5.4 to 15.4 mM K⁺ at 1 dic; C: a similar lack of events in a ROMK1-infected cell, before and after perfusion of Ba²⁺.
referred to in this paper as EIRK. As noted in the discussion of RMP, Ba$^{2+}$ blockade of ROMK1 restores infected neurons to a state more like that of low K$^+$ controls than high K$^+$ controls. So is it correct to subtract the high K$^+$ control I-V from that of the ROMK1 cells to quantify expression levels at very negative potentials, or is all current in ROMK1 cells due to ROMK1?

ROMK1-expressing neurons will not necessarily up-regulate EIRK as do controls, because ROMK1 prevents the hyperexcitability caused by high K$^+$ that presumably potentiates development of native inward rectifiers (Fig. A3). It is possible to distinguish the two types of conductance by their sensitivity to Ba$^{2+}$ rectifiers (Fig. A3). It is possible to distinguish the two types of conductance by their sensitivity to Ba$^{2+}$.

Examination of the currents at −144 mV (Tables 2 and A1) reveals that the Ba$^{2+}$-blockable current in completely silenced cells is significantly greater than in all ROMK1 cells, but the residual (nonblockable) current is not. Further, the percentage of current that is blockable increases with increasing expression of ROMK1. This suggests that the fractional block of EIRK differs from that of ROMK1, and that as ROMK1 expression levels increase, the percentage of current due to EIRK decreases proportionately. If the current at −144 mV is suppressed to a large degree in a ROMK1-infected cell, but application of Ba$^{2+}$ leads to uncontrollable spiking as in the control case.

**APPENDIX B: TWO-COMPARTMENT LOCALIZATION OF CURRENTS**

Electrophysiological data from all cultured neurons in our experiments, regardless of age, could be fit to a semi-empirical model with five experimentally measured parameters that describe the time dependence of the current in response to a voltage step $V_0$.

$$I(t) = I_s + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$$

where $I_s$ is the steady-state current; $A_1$ and $\tau_1$ are the amplitude and time constant of the faster component, and $A_2$ and $\tau_2$ are the amplitude and time constant of the slower component. Such a model fits the observed data to within experimental accuracy; a single exponential is insufficient, while the addition of other parameters does not improve the fit.

A current of this form is consistent with a rapidly charging cell body and distal dendrites that charge more slowly (details in Nadeau and Lester 2000). The dendrites are separated from the soma by a resistance $R_d = 175 \pm 25$ MΩ for all ROMK1 cells, and this resistance is assumed to be a property of the cell’s anatomy that does not change on Ba$^{2+}$ wash-in. The voltage at the soma as a function of time is then given by

$$V_{\text{soma}}(t) = V_0 - R_d i_{\text{m}} - R_d A_1 \exp(-t/\tau_1) - R_d A_2 \exp(-t/\tau_2)$$

while at the dendrites the charging is delayed.

**TABLE A1. Ba$^{2+}$-sensitive current and its effect on membrane properties**

<table>
<thead>
<tr>
<th></th>
<th>All ROMK1 Cells</th>
<th>High K$^+$ Controls</th>
<th>Silenced Cells (N = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block (pA)</td>
<td>131 ± 15*</td>
<td>43 ± 13</td>
<td>306 ± 22*</td>
</tr>
<tr>
<td>Residual (pA)</td>
<td>181 ± 17</td>
<td>211 ± 34</td>
<td>182 ± 23</td>
</tr>
<tr>
<td>%Block</td>
<td>39 ± 2*</td>
<td>16 ± 4</td>
<td>63 ± 3*</td>
</tr>
<tr>
<td>N total</td>
<td>166 ± 47*</td>
<td>[49 ± 13]</td>
<td>627 ± 293*</td>
</tr>
</tbody>
</table>

* "Block" indicates the current at −144 mV blockable by 500 μM Ba$^{2+}$; "Residual" indicates the current remaining while Ba$^{2+}$ is perfused; "%block" is the percentage of current block at that voltage; and $N$ total is an estimate of the number of open, blockable ROMK1 channels in the cell, assuming a single-channel conductance of 34 pS (Choe et al. 1997) (brackets in control cells indicate that these channels are not ROMK1). All values are ± SE.

* Statistically different from controls (P < .01).
\[
V_{\text{cell}}(t) = \frac{1}{1 + (R_{s}/R_{a})} \left[ V_{0} - R_{s} \ln \left( \frac{1 - \alpha}{\alpha} \exp(-t/t_{1}) \right) \right] - \frac{R_{a} A_{1}}{1 - \frac{\alpha}{\tau_{2}}} \exp(-t/t_{2}) \tag{B1}
\]

Here \( R_{s} \) is the resistance of the dendrites, and the parameter \( \alpha \) is
\[
\alpha = \frac{A_{1} \tau_{2} + A_{2} \tau_{1}}{A_{1} + A_{2}} \tag{B4}
\]

If \( t_{1} \ll \tau_{2} \), which is the case in these experiments (\( \tau_{1} = 0.57 \pm 0.04 \) ms, \( \tau_{2} = 3.0 \pm 0.2 \) ms for all ROMK1 cells and similar in controls), then at \( t = \tau_{1} \) the soma has charged appreciably and the dendrites have not. The conductance change of the soma in \( \text{Ba}^{2+} \) is then proportional to the current change at this early time
\[
G_{\text{soma}} - G_{\text{soma}0} \propto I(t_{1}) - I(0) \tag{B5}
\]

Since the total conductance change is given by
\[
G_{\text{TOT}} - G_{\text{TOT}0} = \frac{I_{a}}{V_{0}} - \frac{I_{a0}}{V_{0}} \tag{B6}
\]

the relative contributions of the soma and dendrites to the conductance change in \( \text{Ba}^{2+} \) can be evaluated.

We thank B. Khakh, G. Greif, J. Pine, and C. Lindensmith for useful suggestions and discussions.

This work was supported by Burroughs-Wellcome and by National Institute of Mental Health Grant MH-49176.

REFERENCES


