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Activation of heteromeric G protein-gated inward rectifier K^+ channels overexpressed by adenovirus gene transfer inhibits the excitability of hippocampal neurons

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ABSTRACT **G protein-gated inward rectifier K^+ channel subunits 1–4 (GIRK1–4) have been cloned from neuronal and atrial tissue and function as heterotetramers. To examine the inhibition of neuronal excitation by GIRKs, we overexpressed GIRKs in cultured hippocampal neurons from 18 day rat embryos, which normally lack or show low amounts of GIRK protein and currents. Adenoviral recombinants containing the cDNAs for GIRK1, GIRK2, GIRK4, and the serotonin 1A receptor were constructed. Typical GIRK currents could be activated by endogenous $GABA_B$, serotonin 5-HT_{1A}, and adenosine A1 receptors in neurons coinfected with GIRK1+2 or GIRK1+4. Under current clamp, GIRK activation increased the cell membrane conductance by 1- to 2-fold, hyperpolarized the cell by 11–14 mV, and inhibited action potential firing by increasing the threshold current for firing by 2- to 3-fold. These effects were not found in non- and mock-infected neurons, and were similar to the effects of muscarinic stimulation of native GIRK currents in atrial myocytes. Two inhibitory effects of GIRK activation, hyperpolarization and diminution of depolarizing pulses, were simulated from the experimental data. These inhibitory effects are physiologically important in the voltage range between the resting membrane potential and the potential where voltage-gated Na^+ and K^+ currents are activated; that is where GIRK currents are *outward*.**

G protein-gated inward rectifier K^+ channels (GIRKs) expressed in neuronal, atrial, and other cell types are formed by subunits encoded by a five-membered gene subfamily (K_{ir} 3.0), and are activated by neuromodulators acting on G protein-coupled receptors (1, 2). Although GIRKs are inward rectifiers, their usual physiological role is to permit an *outward* K^+ current near resting membrane potentials (E_M; ref. 3). This hyperpolarizing K^+ current through activated GIRKs presumably functions to decrease cellular excitability, detected, e.g., as slowing of the heart beat (*vagusstoff* response, ref. 4) and reduction of spike (i.e., action potential) train frequencies in neurons (reviewed, e.g., in refs. 5 and 6).

GIRKs normally function as heterotetrameric channels of two or more subunit isoforms (2, 7–10). The isoforms GIRK1–3 and, to a lesser extent, GIRK4 are expressed in CA1–CA3 pyramidal and dentate gyrus granule cells of the rat hippocampus (10, 11), where GIRK-type K^+ currents have previously been described (e.g., refs. 12–14). To analyze the role of cloned GIRKs in hippocampal excitation, we have developed a recombinant adenovirus system for coexpressing several GIRKs and a G protein-coupled receptor in neurons at a high per cell efficiency. Here we report a quantitative study of the inhibition of spike train initiation in cultured rat hippocampal neurons in which GIRK1 and GIRK2 have been overexpressed and activated by endogenous G protein-coupled receptors.

MATERIALS AND METHODS

Cell Culture and Reagents. Cultures of 18 day embryonic (E18) rat hippocampal neurons and 4–6 day (d) postnatal rat atrial and ventricular myocytes, pancreatic βTC3 cells (gift from S. Efrat, Albert Einstein College of Medicine), and *Xenopus* oocytes were prepared as described (8, 15, 16). Chinese hamster ovary (CHO) cells (American Type Culture Collection) were maintained at 5% CO_2/95% air in Ham's F-12 medium (Irvine Scientific) containing 10% fetal bovine serum (Irvine Scientific). Total RNA was extracted using Rneasy (Qiagen, Chatsworth, CA). Muscarinic M_2 receptor cRNA (17) was synthesized *in vitro* from *Hin*dIII-linearized plasmid (gift from E. G. Peralta, Harvard University Medical School) using Message machine (Ambion, Austin, TX). Spermine, (±)-baclofen, and serotonin (5-HT) were from Sigma; 2-chloro-N^6-cylcopentyladenosine (CCPA) and (±)-8-hydroxy-dipropylaminotetralin (8-OH-DPAT) from Research Biochemicals; [D-Ala^2-Met^5]enkephalin was from Calbiochem.

Adenoviral Constructs. By using standard techniques (18), adenoviral recombinants were constructed with the following cDNA inserts under the control of a cytomegalovirus promoter: GIRK1 (ref. 19; AdGIRK1), GIRK2 (ref. 20, gift from D. E. Clapham, McMaster University; AdGIRK2), GIRK4 (ref. 21, gift from J. P. Adelman, University of Oregon; AdGIRK4), and *Shaker* H4 K^+ channel (22). GIRK1, GIRK2, and GIRK4 were inserted into adenovirus Ad*Pac*I (gift from F. L. Graham, McMaster University); *Shaker* H4 was inserted into AdΔ309 (gift from A. J. Berk, University of California, Los Angeles). The 5-HT_{1A} receptor cDNA (23) was ligated into AdRR5 (ref. 24, gift from R. D. Gerard, University of Texas) to obtain Ad5HT_{1A}R. Adenovirus containing *Escherichia coli* LacZ cDNA (AdLacZ) was a gift from A. J. Berk. We frequently tested functionality of cDNA inserts, such as GIRK1 plus GIRK2 cloned into the pAC adenovirus transfer plasmid (18), by Lipofectamine cotransfection prior to making the recombinant viruses. Viruses were propagated in HEK293 cells (American Type Culture Collection) maintained at 5% CO_2/95% air in Dulbecco's modified Eagle's medium (Irvine Scientific), supplemented with 10% fetal bovine serum. For

Abbreviations: ACh, acetylcholine; AdGIRK, GIRK adenovirus; Ad5HT_{1A}R, serotonin 1A receptor adenovirus; AdLacZ, *E. coli* LacZ adenovirus; CCPA, 2-chloro-N^6-cylcopentyladenosine; d, day; 8-OH-DPAT, (±)-8-hydroxy-dipropylaminotetralin; E18, 18 day embryonic; E_M, membrane potential; g_{Cell}, cell membrane conductance; g_{GIRK}, GIRK conductance; GIRK, G protein-gated inward rectifier K^+ channel; 5-HT, serotonin; $I_{-40\ mV}$, current at −40 mV; I_{Th}, threshold current for action potential firing; CHO, Chinese hamster ovary.
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infection, cells plated in 35 mm Petri dishes (Corning) were incubated for 2 hr in 750 μl of conditioned medium containing virus with gentle mixing every 15 min, then washed twice and cultured for 1–7 d. β-galactosidase detection was as described (25).

Western Blots. GIRK1 and GIRK2 proteins were detected by Western blots using affinity-purified GIRK-specific antibodies. A previously described rabbit anti-GIRK1 antibody was used (15, 26). For GIRK2, a guinea pig anti-GIRK2 antibody was produced against a glutathione *S*-transferase fusion protein carrying the GIRK2-unique N terminal aa 1–24. It displayed high specificity as determined by Western blot and immunocytochemistry in transfected COS cells (data not shown). Dissected adult rat hippocampus and cultured E18 hippocampal neurons were solubilized in 62.5 mM Tris·HCl (pH 6.8), 1% SDS, 10% (vol/vol) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride (Sigma), 2 μg/ml leupeptin (Sigma), 1 μg/ml pepstatin A (Sigma), and 1 μg/ml aprotinin (Boehringer Mannheim). Lysate proteins were separated by 10% SDS/PAGE and transferred to Immobilon-P membranes (Millipore). Primary antibody binding at 0.1 (GIRK1) and 1 μg/ml (GIRK2) was carried out in phosphate-buffered saline containing 0.1% Tween-20 and 5% dried milk (blocking solution); the horseradish peroxidase-conjugated secondary antibodies anti-rabbit IgG (Boehringer Mannheim) and anti-guinea pig IgG (Jackson ImmunoResearch) were used at 40 ng/ml in blocking solution. Immunodetection was by enhanced chemiluminescence (Amersham). As a control for the specificity of immunoreactive bands, primary antibody preabsorption with the respective GIRK1 or GIRK2 immunogen was performed by overnight incubation at 4°C.

Electrophysiology. Whole-cell patch-clamp recording was performed using borosilicate glass capillaries (Sutter Instruments, Novato, CA) pulled to a tip resistance of 2–7 MΩ and coated with Sylgard. Signals were recorded with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA) interfaced to a 386 personal computer through a Digidata 1200 (Axon Instruments). Current and voltage commands, data acquisition, and analysis were performed using PCLAMP 6.0. Recordings in myocytes, CHO, and βTC3 cells were performed as described (23). For neurons, the bath solution contained 120 mM NaCl, 2.5 mM KCl, 5 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose (pH 7.3 with NaOH, $\approx$260 mOsm); the pipette solution contained 5 mM KCl, 125 mM K gluconate, 5 mM Hepes, 5 mM Na_2 phosphocreatine, 1.1 mM EGTA, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM MgATP, 0.3 mM Na_2GTP (pH 7.2 with KOH, $\approx$250 mOsm). For recordings at 25 mM $[K^+]_o$, NaCl was replaced by KCl. At low $[K^+]_o$, the calculated junction potentials (using Jpcalc 2.0 and ion mobilities given in refs. 27 and 28) were +14 mV (neurons) and +4 mV (atrial cells). In the data below, measured E_M are reported without a junction potential correction. *Shaker* currents were activated in 5.4 mM $[K^+]_o$ by depolarization pulses to -50 to +40 mV (after a 20 msec hyperpolarizing pulse at -100 mV). GIRK currents were assessed by applying 2-sec voltage ramp protocols from -140 to +20 mV before and during agonist perfusion. Holding E_M was -70 mV; signals were sampled at 0.5–2 kHz, and series resistance compensation was not employed. GIRK currents in *Xenopus* oocytes were assayed as described (8). All recordings were at room temperature. The simulations in Fig. 5 were performed using Axon Engineer Pro (Axon Instruments).

RESULTS AND DISCUSSION

Test of Adenoviral Gene Expression. In preparation for adenoviral GIRK expression, we examined the virus titer needed for high infection levels in E18 hippocampal cell cultures using AdLacZ expressing the reporter gene β-galactosidase. A titer of $\geq 5 \times 10^7$ plaque-forming units/ml resulted in β-galactosidase staining of >99% of the neurons; lower titers gave incomplete staining ($\approx$30% at 10^6 and $\approx$80% at 10^7 plaque-forming units/ml). Based on these results, we used titers of $\approx 10^8$ plaque-forming units/ml per virus to achieve complete infection in our transfection experiments. Before attempting to express heteromeric GIRKs, we first tested the adenoviral expression of homomeric *Shaker* K^+ channels. Atrial and ventricular myocytes, and βTC3 and CHO cells infected with the *Shaker* adenovirus expressed new large transient K^+ currents as soon as 1 d postinfection (120 $\pm$ 10 pA/pF in myocytes, $n = 3$, mean $\pm$ SEM of peak currents at +30 mV), persisting for the longest time span tested (5 d; 250 $\pm$ 120 pA/pF, $n = 4$). Taken together, these data suggest that adenovirus is useful for expression of K^+ channels in various cell types, including hippocampal neurons.

As an initial test of adenovirus containing GIRK1 cDNA, we infected pancreatic βTC3 cells, which have been reported to be suitable for functional GIRK1 expression (29). However, we could not detect any GIRK currents in these cells 1–2 d postinfection. By contrast, *Xenopus* oocytes injected with 0.2 ng of muscarinic M_2 receptor cRNA together with 100 ng total RNA from AdGIRK1-infected βTC3 cells, but not with M_2 receptor plus total RNA from noninfected cells, did express acetylcholine (ACh)-activated GIRK currents (520 $\pm$ 70 pA at -120 mV and 98 mM $[K^+]_o$, 3 d after injection, $n = 13$). This result shows that AdGIRK1 produces intact GIRK1 mRNA and expresses protein that coassembles with GIRK5 in *Xenopus* oocytes (2) to form functional GIRKs. In our experiments on βTC3 cells, however, an unknown mechanism, such as failure to express GIRK2 and/or GIRK4 (30), interfered with functional GIRK1 expression.

We then coinfected CHO cells with Ad5HT_{1A}R plus either AdGIRK1+2 or AdGIRK1+4. These cells showed large agonist-activated GIRK currents, whereas no GIRK currents could be detected in cells transfected with receptor plus GIRK1 alone (data not shown). The 5-HT-activated GIRK currents for Ad5HT_{1A}R plus AdGIRK1+4-coinfected cells 2 d postinfection were -50 $\pm$ 28 pA/pF (at 25 mM $[K^+]_o$ and -120 mV, $n = 3$). These results demonstrate that a single cell can be infected by at least three different adenoviruses, when useful for the functional expression of heteromeric channels. Overall, these data confirm that GIRKs function in mammalian cells as heteromers (7, 10).

Expression of GIRK1 and GIRK2 Proteins in Hippocampal Neurons. Fig. 1*a* shows Western blots of lysate from (*i*) adult hippocampus, (*ii*) E18 hippocampal neurons cultured for 4 d, and (*iii*) 4 d-cultured hippocampal neurons 3 d after coinfection with AdGIRK1+2. A single 60-kDa band, consistent with glycosylated GIRK1 protein (7), was detected in adult hippocampus but not in 4 d-cultured E18 hippocampal neurons. Coinfection with AdGIRK1+2 produced an $\approx$30-fold increase in GIRK1 immunoreactive protein compared with adult hippocampus. In addition to the putative 60-kDa glycosylated GIRK1 band, a significant 52–56-kDa doublet band was observed, consistent with unglycosylated GIRK1 (7). Bands with >100 kDa mobility were also present. Since all bands were blocked by immunogenic peptide preabsorption (data not shown), the upper bands are thought to be aggregates, including overexpressed GIRK1 protein. Densitometric comparison of the 60-kDa bands indicates a more modest, 8-fold increase in glycosylated GIRK1 by adenoviral overexpression.

GIRK2 protein was detected in adult hippocampus as an $\approx$49 kDa protein that was not found in 4 d-cultured E18 hippocampal neurons. Coinfection with AdGIRK1+2 produced an $\approx$60-fold increase in GIRK2 immunoreactive products compared with the adult hippocampus (Fig. 1*a*). An $\approx$46 kDa significant band was present in addition to the higher molecular weight bands as observed for GIRK1. All bands were blocked by preabsorption with the immunogenic fusion protein (data not shown). The higher molecular weight

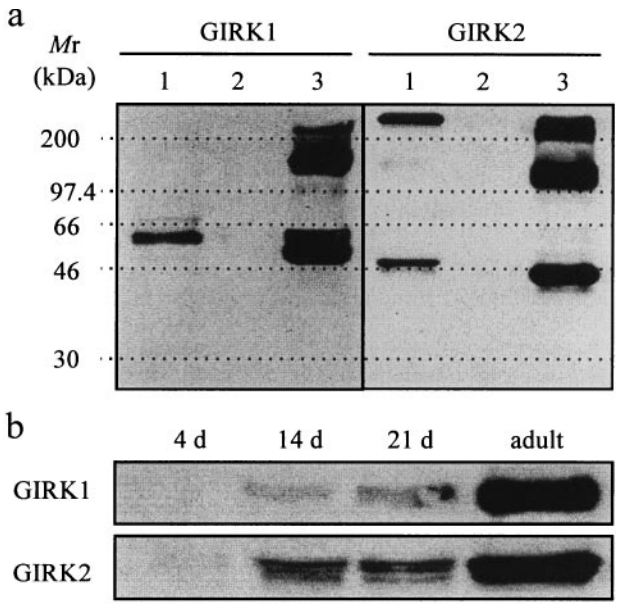

FIG. 1. Expression of GIRK proteins in cultured rat E18 hippocampal neurons. (*a*) Overexpression of GIRK1 and GIRK2 proteins in cells coinfected with AdGIRK1+2. Total cellular protein was loaded (50 μg per lane): adult hippocampus (lane 1); 4 d-cultured neurons (lane 2); 4 d-cultured AdGIRK1+2-coinfected neurons, 3 d postinfection (lane 3). Left and right blots were probed with affinity-purified anti-GIRK1 and -GIRK2 antibodies, respectively. Protein samples were separated by SDS/PAGE with prestained molecular weight markers (Amersham) in neighboring lanes. The molecular weights of the marker proteins are indicated on the left with dotted lines across the gels. (*b*) Time-dependent expression of GIRK1 (*Upper*) and GIRK2 protein (*Lower*) *in vitro*. Total cellular protein of E18 neurons cultured for 4, 14, and 21 d, and of adult hippocampus was used (100 μg per lane) and probed with affinity-purified anti-GIRK1 and -GIRK2 antibodies, respectively.

of the GIRK2 protein in adult hippocampus (49 kDa) vs. the AdGIRK2-expressed 46 kDa protein may indicate the expression of a longer, alternatively spliced GIRK2 isoform in adult hippocampus (31) or may be due to unknown posttranslational modifications. Quantitative comparison of these bands indicates an 8-fold overexpression of GIRK2 protein, equivalent to the GIRK1 data.

Our finding that in embryonic neurons cultured for 4 d little or no GIRK1 and GIRK2 protein levels were detectable (3 and 6% of adult hippocampus, respectively) is supported by previous studies reporting that GIRK expression is postnatal (32). To examine whether developmental GIRK expression *in vivo* can be mimicked *in vitro*, we analyzed cells that had been cultured for longer periods. As shown in Fig. 1*b*, we could measure low amounts of GIRK1 and GIRK2 proteins in E18 neurons after 14 d and increased amounts after 21 d in culture (4 and 11% of adult for GIRK1, respectively, and 28 and 29% for GIRK2). These results indicate that expression of GIRKs occurs in long-term cultures of E18 hippocampal neurons; however, expression levels are lower than *in vivo*.

Expression of GIRK Currents. We could not detect any GIRK currents activated by the $GABA_B$ receptor agonist baclofen or by 5-HT in uninfected E18 hippocampal neurons $\leq$11 d in culture. In older cell cultures ($\geq$13 d), however, we found small baclofen-activated GIRK currents (-1.9 ± 0.4 pA/pF, $n = 6$, at -140 mV and 2.5 mM $[K^+]_o$). This time-dependent expression of GIRK currents parallels the appearance of GIRK proteins in uninfected E18 hippocampal cell cultures (see above). In contrast to uninfected cells, large baclofen-activated GIRK currents sensitive to 0.5 mM Ba^{2+} were apparent in hippocampal neurons that had been coinfected with either AdGIRK1+2 or AdGIRK1+4 (Fig. 2). At

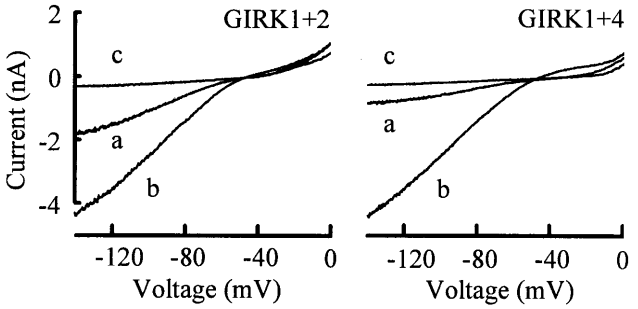

FIG. 2. Adenovirus-mediated expression of GIRK currents in hippocampal neurons. Cells cultured for 5 d were coinfected with AdGIRK1+2 or AdGIRK1+4 and analyzed 2 d postinfection in 25 mM $[K^+]_o$ and 250 nM tetrodotoxin. Currents in the presence (b) and absence (a) of 100 μM baclofen; 0.5 mM Ba^{2+} blocked basal and baclofen-activated GIRK currents (c).

2 d postinfection, 25 mM $[K^+]_o$, and -120 mV, these GIRK currents were -76 ± 12 pA/pF ($n = 5$) and -55 ± 17 pA/pF ($n = 4$), respectively, comparable to 5-HT-activated GIRK currents found in infected CHO cells (above), and 3.7–5 times larger than the endogenous ACh-activated GIRK currents in atrial cells (-15 ± 2 pA/pF, $n = 23$). These results, together with the Western blot data, demonstrate that GIRK overexpression is achievable using adenoviral delivery, thus making it practical to study GIRK function in cultured hippocampal neurons.

To examine GIRK function under more physiological conditions, measurements were performed at physiological $[K^+]_o$, 2.5 mM for neurons and 5.4 mM for atrial cells. In AdGIRK1+2-coinfected neurons, both baclofen (50 μM) and 5-HT (30 μM) induced prominent inward currents (Fig. 3 *Upper Left*), as did ACh in atrial myocytes (Fig. 3 *Lower Left*). Positive to the reversal potentials of -89.9 ± 0.8 mV for neurons ($n = 20$; $E_K = \approx -99$ mV) and -79.0 ± 0.7 mV for myocytes ($n = 17$; $E_K = -82$ mV), relatively large outward K^+

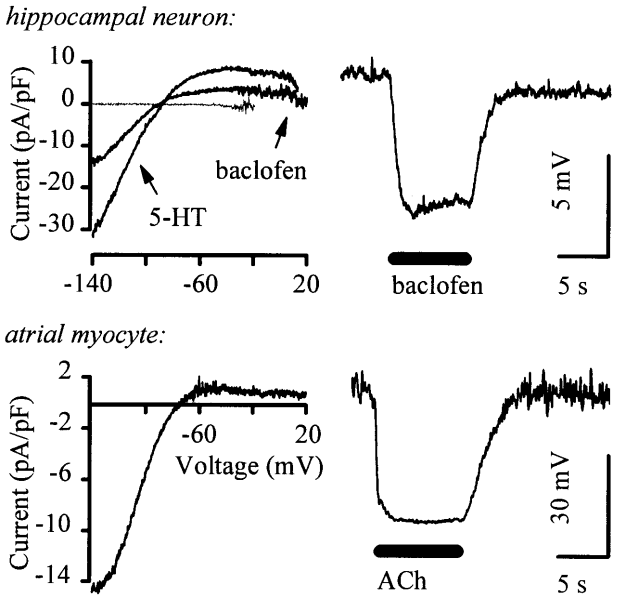

FIG. 3. Cellular responses to GIRK activation at physiological $[K^+]_o$. (*Left*) Currents activated by 30 μM 5-HT and 50 μM baclofen in AdGIRK1+2-coinfected (4 d postinfection, thick lines) and uninfected (fine line, 5-HT only) hippocampal neurons 11 d in culture (*Upper*), and by 5 μM ACh in an atrial cell (*Lower*). (*Right*) E_M changes caused by application of 50 μM baclofen and 5 μM ACh (bars); resting E_M were -68 (neuron) and -24 mV (myocyte). Similar responses were observed to 8-OH-DPAT in neurons. The mean resting E_M of 15 atrial cells was -35.8 ± 3.8 mV.

Table 1. Comparison of agonist-induced changes in atrial myocytes and AdGIRK1+2-infected hippocampal neurons

Agonist	$\Delta I_{-40\ mV}$, pA/pF	ΔE_M, mV	Δg_{Cell}, nS
Myocytes			
ACh	1.8 ± 0.2 (18)	−18.9 ± 3.8 (14)	1.2 ± 0.5 (9)
Neurons			
Baclofen	2.0 ± 0.3 (12)	−11.0 ± 1.0 (30)	2.8 ± 0.4 (30)
5-HT	4.9 ± 1.1 (11)	−14.4 ± 1.9 (20)	4.1 ± 0.5 (19)
8-OH-DPAT	1.4 ± 0.1 (2)	−10.9 ± 2.8 (8)	4.8 ± 1.9 (7)
CCPA	ND	−11.0 ± 2.3 (7)	3.9 ±1.2 (7)

Means ± SEM (n) of changes caused by 5 μM ACh at 5.4 mM $[K^+]_o$ in myocytes, and by 50 μM baclofen, 30 μM 5-HT or 8-OH-DPAT, and 3 μM CCPA at 2.5 mM $[K^+]_o$ in neurons (cultured for 9–27 d, assayed 3–7 d postinfection). ND, not determined.

currents were apparent. For neurons, the agonist-activated outward GIRK currents at −40 mV ($I_{-40\ mV}$) were 21 ± 1% (n = 12, baclofen) and 26 ± 2% (n = 11, 5-HT) of the inward currents at −140 mV, indicating conductance ratios of 4–5 × 10^{-3}. The important point, nevertheless, is that in the range between the resting E_M and ≈−40 mV, where currents due to voltage-gated channels are negligible, there is a significant agonist-activated outward K^+ current.

In AdGIRK1+2-coinfected neurons, the 50 μM baclofen-activated $I_{-40\ mV}$ was ≥5 times larger than in AdLacZ- and noninfected neurons; 30 μM 5-HT caused an even larger response (P = 0.02, t test; Table 1). Similar results were obtained using the 5-HT_{1A} receptor-specific agonist 8-OH-DPAT (30 μM; Table 1), suggesting that the 5-HT response is exerted via 5-HT_{1A} receptors. Overexpression of these receptors by coinfection with Ad5HT_{1A}R did not increase the agonist-activated $I_{-40\ mV}$ (5.1 ± 1.7 pA/pF, n = 7 vs. 3.5 ± 1.1 pA/pF, n = 6, for Ad5HT_{1A}R-coinfected neurons), indicating that GIRK channel activation by endogenous 5-HT_{1A} receptors was maximal.

The intracellular polyamines spermine and spermidine are important for the rectification of inward rectifier K^+ channels (33–35). To test whether the observed ligand-activated $I_{-40\ mV}$ was due (at least partially) to loss of intracellular polyamines during whole-cell recording, we tested whether the addition of a natural $[spermine]_i$ (0.1 mM; ref. 36) to the pipette solution altered $I_{-40\ mV}$. Both 5-HT- and baclofen-activated $I_{-40\ mV}$ in neurons and ACh-evoked $I_{-40\ mV}$ in atrial cells were unchanged by the addition of spermine (data not shown), suggesting that the outward $I_{-40\ mV}$ is not due to loss of internal blocking molecules, but instead is a result of the rectification properties of overexpressed GIRKs under physiological conditions (i.e., $[K^+]_o$, E_M).

GIRK Activation and Cellular Excitability. Under current clamp with zero-injected current, AdGIRK1+2-coinfected hippocampal neurons and atrial myocytes were hyperpolarized 10–20 mV by agonist addition (Fig. 3 *Right*; Table 1). Furthermore, the cell membrane conductance (g_{Cell}) of the neurons (Table 2) and myocytes (1.4 ± 0.4 nS, n = 10), as measured by the voltage shift due to injection of a hyperpolarizing current pulse, increased by 90–170% (Table 1). In GIRK-overexpressing neurons, application of baclofen and 5-HT hyperpolarized E_M by 11–14 mV and increased g_{Cell} by 1- to 2-fold, whereas AdLacZ- and noninfected cells showed significantly lower changes (0–4 mV and 0- to 0.2-fold, respectively; Table 3). 8-OH-DPAT similarly hyperpolarized E_M and increased g_{Cell} in GIRK-overexpressing cells (Table 1) but not in AdLacZ-infected cells (ΔE_M = −1.0 ± 0.6 mV, n = 4, Δg_{Cell} = 0.3 ± 0.3 nS, n = 4). AdGIRK1+2-coinfected neurons showed about the same resting E_M and g_{Cell} as AdLacZ- and noninfected cells (Table 2), suggesting that basal GIRK activity does not affect resting E_M.

Table 2. Properties of unstimulated infected hippocampal neurons

Infection	E_M, mV	g_{Cell}, nS	I_{Th}, pA
AdGIRK1+2	−63.0 ± 1.0 (65)	2.4 ± 0.2 (66)	25 ± 7 (46)
AdLacZ	−60.4 ± 0.9 (26)	1.7 ± 0.2 (26)	37 ± 10 (18)
Uninfected	−62.0 ± 1.2 (27)	2.4 ± 0.3 (27)	41 ± 11 (17)

Means ± SEM (n) of neurons cultured for 9–27 d and assayed 3–7 d postinfection. I_{Th}, the threshold current for action potential firing, was determined as described in Fig. 4*b* (*Inset*).

In rat E18 hippocampal cell cultures, the majority of neurons are pyramidal; however, GABAergic interneurons can comprise up to 6–36% of the cells (37, 38). Adenosine A1 receptors coupling to GIRKs are expressed in pyramidal cells but not interneurons (14, 37). To explore the nature of our GIRK-overexpressing neurons, we examined whether CCPA, an adenosine A1 receptor-selective agonist, caused excitability changes. We also tested whether such changes were induced by [D-Ala^2-Met^5]enkephalin, an agonist known to cause membrane hyperpolarization in interneurons but not pyramidal cells (39). Similarly to $GABA_B$ and 5-HT_{1A} receptor agonists, CCPA caused membrane hyperpolarization and increased g_{Cell} (Table 1), whereas 10 μM [D-Ala^2-Met^5]enkephalin was without effect (three cells tested, data not shown). These results indicate that the majority of the hippocampal cells used in our study were pyramidal neurons.

The consequences of GIRK-mediated changes in the cell electrical properties are illustrated in Fig. 4. Baclofen suppressed spike train firing in GIRK-overexpressing hippocampal neurons in records that resembled ACh inhibition of firing atrial myocytes (Fig. 4*a*). In Fig. 4*b*, current pulses were injected into GIRK-overexpressing neurons in steps of 16 or 20 pA, both in the presence and absence of baclofen or 5-HT. Without agonist, the E_M plateaued in 3–5-mV steps over a time period of ≈50 msec until it reached ≈−40 mV, at which point a slow depolarization occurred and then an action potential was fired. Larger current injections gave rise to spike trains. With agonist, the depolarizing steps resulting from equivalent current injections were significantly reduced. The I_{Th} was increased from ≈60 pA to ≈180 pA by 5-HT for the particular neuron shown in the top of Fig. 4*b*. Fig. 4*b* (*Inset*) shows that the slope of the spike frequency vs. current relationship is similar in the presence and absence of 5-HT for that cell. Overall, agonist addition did not change this slope in GIRK-overexpressing neurons (0.22 ± 0.02 Hz/pA, n = 46 vs. 0.20 ± 0.02 Hz/pA, n = 25, and 0.24 ± 0.03 Hz/pA, n = 15 for baclofen and 5-HT, respectively), indicating that GIRK channels do not further influence neuronal firing once I_{Th} has been reached.

To measure the effect of GIRK activation on hippocampal action potential firing, we estimated I_{Th} both in the presence

Table 3. Agonist-induced excitability changes of infected hippocampal neurons

	50 μM baclofen-induced changes			30 μM 5-HT-induced changes		
Infection	ΔE_M, mV	Δg_{Cell}, nS	ΔI_{Th}, pA	ΔE_M, mV	Δg_{Cell}, nS	ΔI_{Th}, pA
AdGIRK1+2	−11.0 ± 1.0 (30)	2.8 ± 0.4 (30)	68 ± 18 (25)	−14.4 ± 1.9 (20)	4.1 ± 0.5 (19)	60 ± 14 (13)
AdLacZ	−3.5 ± 0.7* (18)	0.3 ± 0.1* (18)	−3 ± 6* (11)	−1.6 ± 2.3* (7)	0.2 ± 0.2* (7)	−14 ± 11* (5)
Uninfected	−3.0 ± 0.6* (21)	0.5 ± 0.2* (18)	4 ± 16* (12)	−0.1 ± 1.2* (5)	0.0 ± 0.2* (5)	−20 ± 29* (3)

Means ± SEM (n) of cells cultured for 9–27 d and assayed 3–7 d postinfection. *, Significant differences ($P < 0.05$, t test) between this value and the corresponding value from AdGIRK1+2-coinfected cells.

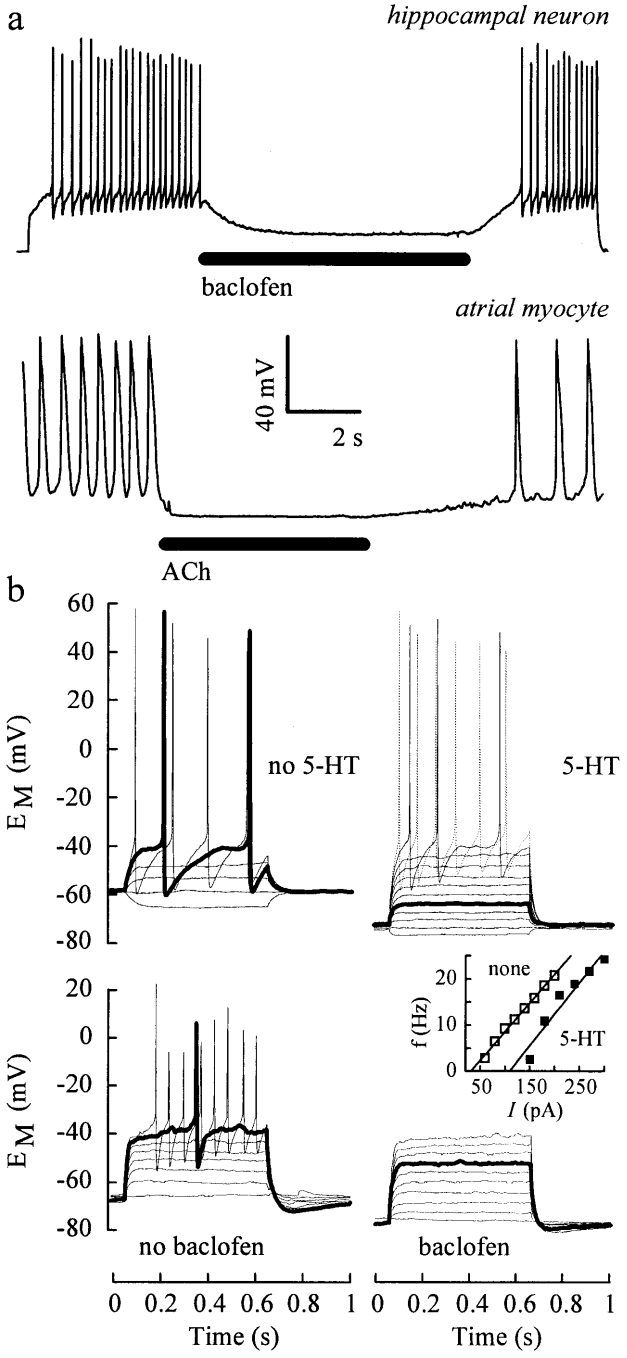

FIG. 4. GIRK activation inhibits action potential firing. (*a*) Bars indicate the application of 50 μM baclofen to a 13 d-cultured neuron (3 d after coinfection with AdGIRK1+2), and of 5 μM ACh to an atrial cell. A 15.8 sec current pulse of +60 pA was injected into the neuron to cause firing; the myocyte was firing spontaneously. Resting E_M were −70 mV (neuron) and −66 mV (myocyte). (*b*) E_M responses of AdGIRK1+2-coinfected neurons to 0.6 sec current pulses in the presence (*Right*) and absence (*Left*) of 30 μM 5-HT (*Upper*) and 50 μM baclofen (*Lower*); steps of 20 pA (*Upper*, from −20 pA) and 16 pA (*Lower*, from 0 pA). Note that the 60 and 96 pA pulses cause action potential firing in the absence but not in the presence of 5-HT and baclofen, respectively (thick lines). (*Inset*) Firing frequencies calculated from action potential intervals of the top cell in the presence (■) and absence (□) of 5-HT. Solid lines show linear fits; *x* axis intercepts indicate I_{Th}.

and absence of agonist as described in Fig. 4*b* (*Inset*). In the absence of agonist, I_{Th} was similar for all neurons (Table 2). Addition of baclofen and 5-HT increased I_{Th} by 2- to 3-fold in GIRK-overexpressing neurons, but not in AdLacZ- and non-

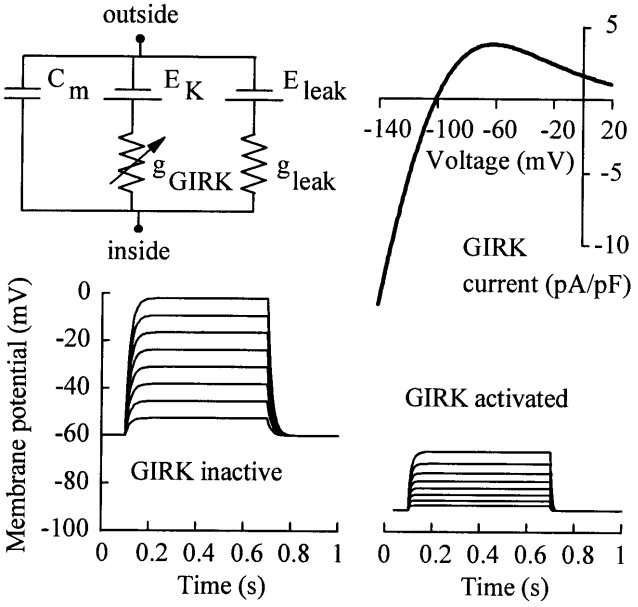

FIG. 5. Simulations of the effects of g_{GIRK} on cell membrane properties. (*Upper Left*) A simplified equivalent circuit incorporating only GIRK and leakage conductances. The membrane capacitance (C_m) is 50 pF. The leakage conductance (g_{leak}, 55 pS/pF) is linear with a reversal potential of −12 mV (E_{leak}); g_{GIRK} is as described in the text. (*Upper Right*) Current–voltage relation for the simulated g_{GIRK}. The resting potential (−60 mV in the absence of GIRK activation) is simulated by an applied current of −132 pA in both panels. (*Lower*) Voltage changes in response to current injections (starting at 20 pA, 20 pA steps) with (*Right*) and without g_{GIRK} (*Left*).

infected neurons (Table 3). 8-OH-DPAT (30 μM) and CCPA (3 μM) had a similar effect on I_{Th} in GIRK-overexpressing neurons (1.4- and 2.5-fold increase, respectively). Taken together, our data provide a quantitative demonstration of how GIRK activation inhibits neuronal action potential firing.

GIRK Function Modeling. Our results on the effect of the GIRK conductance (g_{GIRK}) on cell membrane properties were further analyzed by the equivalent circuit shown in Fig. 5 (*Upper Left*). For the model cell, we assumed a membrane capacitance of 50 pF in accordance with the average value for our cultured hippocampal neurons. For simplicity, the cell geometry was spherical and isopotential. Introduced into the model was g_{GIRK} described by an empirically derived Boltzmann relation: $I_{GIRK}(V) = g_{Max}(E_M - E_K)/[1 + \exp(0.026(E_M - E_{1/2}))]$, where the maximal g_{GIRK} (g_{Max}) was then set at 25 nS and the membrane potential for half conductance ($E_{1/2}$) at −95 mV. The resulting GIRK current–voltage relation is shown in Fig. 5 (*Upper Right*) and closely reproduces the experimental curves from AdGIRK1+2-coinfected neurons (compare with Fig. 3). E_M changes in response to current injections are shown for the model cell in the absence and presence of g_{GIRK} (Fig. 5, *Lower*). Note that introducing g_{GIRK} causes a substantial steady hyperpolarization (≈30 mV) and reduces the depolarization in response to current injection. Thus, the qualitative explanation of decreased excitability observed in GIRK-expressing cells is that GIRK activation produces both a hyperpolarization and an increased conductance.

Concluding Remarks. Because of the high GIRK expression level achieved by adenovirus-mediated gene expression, it has been possible in this study to measure the important *outward* GIRK current between the resting E_M and the membrane potential needed for action potential firing in a neuron. Our study provides quantitative data in support of the general belief that GIRK activation inhibits the excitability of a neuron, and thus extends the results of earlier investigators who have found hippocampal spike train inhibition by GIRK

activation (e.g., refs. 5, 6, 40, and 41). Our data may explain the recent observation that mice lacking GIRK2 and most of GIRK1 are more seizure-prone than normal mice (42). It remains to be determined whether adenovirus-mediated GIRK overexpression could inhibit cellular hyperexcitability in model systems of certain biological malfunctions, including epilepsy and cardiac hypertrophy.

GIRKs occur postsynaptically in hippocampal CA1 neurons (15, 26, 43, 44), where they are activated by 5-HT_{1A}, $GABA_B$, adenosine A1, and opiate receptors (12–14, 41, 45, 46). The principal serotonergic and opiate inputs are external to the hippocampus. An understanding of these interactions will be necessary for the study of synaptic function and plasticity in the hippocampus.

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