A voltage-driven switch for ion-independent signaling by ether-à-go-go K⁺ channels

Andrew P. Heglé, Daniel D. Marble, and Gisela F. Wilson*

Department of Molecular, Cellular, and Developmental Biology, University of Michigan, 830 North University Avenue, Ann Arbor, MI 48109-1048

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Voltage-gated channels maintain cellular resting potentials and generate neuronal action potentials by regulating ion flux. Here, we show that Ether-à-go-go (EAG) K⁺ channels also regulate intracellular signaling pathways by a mechanism that is independent of ion flux and depends on the position of the voltage sensor. Regulation of intracellular signaling was initially inferred from changes in proliferation. Specifically, transfection of NIH 3T3 fibroblasts or C2C12 myoblasts with either wild-type or nonconducting (F456A) eag resulted in dramatic increases in cell density and BrdUrd incorporation over vector- and Shaker-transfected controls. The effect of EAG was independent of serum and unaffected by changes in extracellular calcium. Inhibitors of p38 mitogen-activated protein (MAP) kinases, but not p44/42 MAP kinases (extracellular signal-regulated kinases), blocked the proliferation induced by nonconducting EAG in serum-free media, and EAG increased p38 MAP kinase activity. Importantly, mutations that increased the proportion of channels in the open state inhibited EAG-induced proliferation, and this effect could not be explained by changes in the surface expression of EAG. These results indicate that channel conformation is a switch for the signaling activity of EAG and suggest an alternative mechanism for linking channel activity to the activity of intracellular messengers, a role that previously has been ascribed only to channels that regulate calcium influx.

Voltage-gated ion channels generate neuronal action potentials, the primary units of information transfer in the brain, by regulating ion flux (1). Effects of ion channels on synaptic connectivity, transmitter release, plasticity, and other cellular processes are generally assumed to be a secondary consequence of ion flux. Specifically, changes in membrane potential and action potentials alter Ca²⁺ influx, and Ca²⁺ regulates multiple intracellular signaling pathways (2–7). Severe recent studies, however, have indicated that some voltage-gated ion channels are bifunctional proteins (5, 8–11). These studies show that voltage-gated channels can contribute to transcriptional regulation, protein scaffolding, cell adhesion, and intracellular signaling, and the effects appear largely independent of ion conduction.

Recent studies of Ether-à-go-go [EAG (KCNH1)] voltage-dependent K⁺ channels suggest that EAG may also be bifunctional. First, a region of Drosophila EAG with similarity to the autoinhibitory domain of Ca²⁺/calmodulin-dependent protein kinase II can associate with activated, Ca²⁺/calmodulin-bound Ca²⁺/calmodulin-dependent protein kinase II. In vitro assays indicate that, once Ca²⁺ levels decline, EAG-bound kinase retains 5–10% of its maximum Ca²⁺-stimulated activity (12). Second, human EAG has been implicated in cell-cycle regulation and cancer: transfection can induce oncogenic transformation, EAG is present in some cancer cell lines but absent in the corresponding healthy tissues, and implanting EAG-expressing cells into immune-suppressed mice results in tumor progression (13, 14). These studies implicate EAG as a component of one or more intracellular signaling pathways.

Our investigation of the involvement of EAG in intracellular signaling was prompted by experiments in which we observed an increase in NIH 3T3 fibroblast density after transient transfection with Drosophila eag. Our findings indicate that conformations changes of EAG associated with the position of the voltage sensor may be an alternative mechanism, independent of ion flux, by which ion channels can affect intracellular signaling.

Results

Transfection with EAG Stimulates Proliferation. Fig. 1A shows a representative experiment demonstrating an increase in NIH 3T3 cell density after transfection with eag. Cell density was significantly higher for coverslips transfected with eag than for controls transfected with empty vector (P < 0.01; similar results obtained for two other experiments). To determine the mechanism underlying this increase, cells were labeled with BrdUrd, a marker for proliferation. Coverslips transfected with eag displayed substantial increases in BrdUrd incorporation when compared with vector-transfected controls (Fig. 1B) (P < 0.0001; n = 3). In contrast, transfection with the gene encoding Shaker, another voltage-dependent K⁺ channel, resulted in BrdUrd incorporation that was indistinguishable from control levels, indicating that the effect was specific to EAG. Increased proliferation also was observed by using phospho-histone labeling, another marker for proliferation (data not shown). These results indicate that proliferation accounts, at least in part, for the observed increase in cell density. EAG-induced proliferation was not limited to NIH 3T3 cells because EAG also increased proliferation in C2C12 myoblasts (data not shown). Finally, increased proliferation was also observed in response to EAG when cells were “synchronized” in serum-free media before reintroduction of FBS. However, proliferation was increased even in the complete absence of FBS (Fig. 1C) (P < 0.0001; n = 3); thus, the growth factors present in serum were not required for the effect of EAG on signaling.

EAG-induced Proliferation Is Independent of Ion Flux. K⁺ currents are essential for the proliferation of numerous cell types, including T lymphocytes and Schwann cells (15, 16). The role of K⁺ channels in proliferation, as well as other cellular processes, is generally assumed to be indirect. K⁺ channels alter the membrane potential to modulate Ca²⁺ influx through voltage-dependent Ca²⁺ channels, which, in turn, affects numerous intracellular messenger pathways (17, 18). However, in the present experiments, ion conduction was not required for the

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Abbreviations: EAG, Ether-à-go-go; [K⁰⁺]₀, extracellular K⁺ concentration; MAP, mitogen-activated protein.

*To whom correspondence should be addressed. E-mail: wilsongf@umich.edu.

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effect of EAG on proliferation. Fig. 2A shows recordings from *Xenopus* oocytes expressing wild-type EAG, myc-tagged EAG, or EAG-F456A, which contains a point mutation in the selectivity filter of the channel pore. The selectivity filter sequence is conserved in all K⁺ channels (19), and point mutations in this sequence eliminate conduction in Shaker, as well as numerous other K⁺ channels (8, 20, 21). Both myc-EAG and EAG-F456A failed to produce the outward currents characteristic of the wild-type channel. Comparison of current–voltage relationships (Fig. 2A Right) revealed little difference between myc-EAG and EAG-F456A currents and currents recorded from water-injected controls, which are carried by channels endogenous to oocytes. Although the mechanism underlying the absence of current in myc-EAG is unclear, both myc-EAG and EAG-F456A produced detectable gating currents (data not shown), indicating that defects in the folding or trafficking of EAG cannot wholly account for the absence of K⁺ current.

Myc-eag and eag-F456A increased proliferation to a degree similar to wild-type channels (Fig. 2B). The increases in BrdUrd incorporation were significant in comparisons with vector-transfected controls (*P* < 0.0001; *n* = 3). Moreover, the effects of myc-eag and eag-F456A were not significantly different from the effect of the wild-type channel. In short, changes in K⁺ influx, and the changes in membrane potential and Ca²⁺ influx that are presumed to result, cannot account for the proliferation induced by EAG. Additional evidence that the signaling mechanism of EAG does not include an indirect effect on Ca²⁺ influx was obtained by incubating cells in EGTA-buffered media before and during incubation of cells with BrdUrd. EAG-induced proliferation in the presence of EGTA (1 mM, 5 h) was 90.1 ± 11.2% and 95.1 ± 3.7% of the proliferation in standard Ca²⁺-containing media for wild-type
EAG and EAG-F456A channels, respectively (n = 3; not significant). Higher concentrations of EGTA caused cells to detach and, therefore, were not assessed.

EAG-Induced Proliferation Requires the p38 Mitogen-Activated Protein (MAP) Kinase Pathway. MAP kinase signaling is central to proliferation in numerous cell types and in response to a variety of signals (22). To determine whether the proliferation induced by nonconducting EAG channels requires this pathway, cells were treated with inhibitors of MAP kinase signaling in serum-free media. The p38 MAP kinase inhibitor SB-203580 [4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4-pyridyl)-1H-imidazole; Calbiochem] (20 μM) blocked the proliferation observed in response to EAG-F456A (Fig. 2C) (P < 0.0001; n = 3), reducing proliferation to levels that were no different from the proliferation observed for controls (P > 0.05; n = 3). Similar results were obtained by using the "p38 MAP kinase inhibitor" [2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one; Calbiochem; Fig. 2D Left]. In contrast, although the control compound SB-202474 [4-ethyl-2(4-methoxyphenyl)-5-(4-pyridyl)-1H-imidazole; Calbiochem] reduced the overall level of proliferation in both vector- and eag-F456-transfected conditions, it failed to inhibit the EAG-specific increase (Fig. 2D Center) (P < 0.0001; n = 2). Finally, although PD-98059 (2′-amino-3′-methoxyflavone; Calbiochem), an inhibitor of the p44/42 extracellular signal-regulated kinases, reduced proliferation in the presence of FBS (data not shown), PD-98059 (40 μM) had little effect on the increase in proliferation specifically induced by nonconducting EAG in serum-free media (Fig. 2D Right) (P < 0.01; n = 3). These results suggest that p38, but not p44/42, MAP kinase signaling is required for the proliferation stimulated by nonconducting EAG-F456A channels.

To determine whether EAG affects p38 MAP kinase activity, we immunoblotted NIH 3T3 cell lysates with antibodies that detect either total p38 MAP kinase or, specifically, the phosphorylated, active kinase. As shown in Fig. 2E, p38 phosphorylation nearly doubled in the presence of either wild-type or nonconducting EAG (Fig. 2E) (P < 0.05; n = 4), and the magnitude of the effect appeared to approximate the average increase in BrdUrd incorporation (Fig. 2 B and C).

EAG-Induced Proliferation Is Regulated by the Position of the Voltage Sensor. The observation that the signaling activity of EAG does not depend on ion conduction predicts that changes in extracellular K+ concentration ([K+]o) should not affect EAG-induced proliferation. However, although increased [K+]o increased proliferation in vector-transfected controls, increasing [K+]o by 10 mM inhibited EAG-induced proliferation, returning proliferation to control levels. Specifically, at 15 mM[K+]o, EAG-induced proliferation was 93.9 ± 1.5% of controls compared with 151.4 ± 7.3% in normal 5.3 mM[K+]o. [Measurements were normalized to vector-transfected controls in 5.3 mM (P < 0.001)]. Similar results were observed in two additional experiments. Because increases in [K+]o will depolarize the membrane and shift the position of the voltage sensor even in nonconducting EAG channels, we hypothesized that the signaling activity of EAG might depend on voltage-sensitive conformations of the channel. Specifically, the [K+]o experiments predict that increases in the proportion of channels in the open state should decrease EAG signaling activity.

To explore the possibility that the signaling activity of EAG might be regulated by the position of the voltage sensor, we examined the effects of EAG channels containing mutations in the sixth transmembrane segment that shifted their voltage dependence of activation. Fig. 3 shows that gene expression is obtained for the wild-type channel and two mutants, EAG-TATSSA (T449S/K460S/T470A) and EAG-HEETE (H487E/T490E), when expressed in Xenopus oocytes. Comparison of the conductance–voltage (G–V) relationships (Fig. 3B) reveals that the predominant effect of both mutations was to produce hyperpolarizing shifts in the midpoints of activation from 8.0 ± 1.1 mV (wild type, n = 6) to −10.8 ± 1.2 mV (n = 9) and −31.6 ± 2.0 mV (n = 7) for EAG-TATSSA and EAG-HEETE channels, respectively. In addition, the TATSSA and HTEE mutations also produced changes in kinetics; however, these changes were in opposite directions (Fig. 3A). Comparison of the average resting potentials of oocytes expressing EAG channels (Fig. 3C) revealed that the resting potentials closely followed the changes in the V10 for activation (the voltage at which 10% of channels are activated) (Fig. 3D). This would be expected if EAG is the major channel contributing to the membrane potential. Wild-type EAG produced only a small shift in the resting potential from −44.5 ± 2.8 to −52.8 ± 1.9 mV. In contrast, EAG-TATSSA and EAG-HEETE shifted the resting potential to −82.8 ± 1.1 and −90.7 ± 0.9 mV, respectively. It is important to note that, given that K+ channel conformation and membrane potential act as a negative feedback loop, the proportion of channels in the closed state should be similar in each case, provided that each of the constructs contributes to the membrane potential to a similar extent.

The above EAG constructs were used to address whether the signaling activity of EAG is regulated by channel conformation. As shown in Fig. 4A, there was an ~2-fold increase in the proliferation of NIH 3T3 cells regardless of whether wild-type eag, eag-TATSSA, or eag-HEETE were used. Given the negative feedback of conducting EAG channels on channel conformation, these results suggest that proliferation depends on the position of the voltage sensor of EAG channels rather than on a specific resting membrane potential. Indeed, measurement of the resting potentials of NIH 3T3 cells transfected with these constructs indicated that, although EAG channels contributed to the membrane potential to a lesser degree than in oocytes, each construct shifted the resting potential closer to the respective activation threshold (Fig. 4B).

Additional evidence in support of the hypothesis that channel conformation is a "switch" for the signaling activity of EAG was obtained by using eag-TATSSA and eag-HEETE constructs that
Discussion
Our results indicate that EAG is a bifunctional protein that not only regulates K⁺ flux but also regulates intracellular signaling pathways. The effect of EAG on intracellular signaling was evident as an increase in proliferation of NIH 3T3 cells and did not appear to be due to an indirect effect of K⁺ ions because it was observed even with nonconducting channels. To date, other examples of bifunctional channels include α1C Ca channels whose carboxyl-terminal regions regulate transcription (5), a member of the transient receptor potential family of cation channels (TRP-PLIK) that contains a functional kinase domain (10), and voltage-gated sodium channels whose β-subunits not only modulate channel function but also act as cell adhesion molecules (9). EAG appears to differ from the above channels in that signaling activity is linked to channel conformations determined by the position of the voltage sensor. The signaling function of EAG is a novel mechanism that may link channel gating to intracellular messenger pathways. This role has typically been ascribed only to channels that regulate Ca²⁺ influx. Recently, however, Ci-VSP, a novel protein containing a transmembrane voltage sensor linked to a functional cytoplasmic phosphatase and tensin domain, has been reported to regulate phosphoinositide turnover in a voltage-dependent manner (23).

The signaling activity of EAG characterized in the present study appears negatively correlated with the proportion of channels in the open state or, at the single-channel level, the magnitude of the single-channel open probability (Pₒ). It is tempting to speculate that EAG-induced signaling is limited to channels in a specific conformation, presumably one of two previously characterized EAG closed states (24, 25). Comparison of NIH 3T3 cell resting potentials (Fig. 4B) to the G–V curves obtained in oocytes (Fig. 3B) predicts that >50% of the channels must be closed or that the single-channel Pₒ must be <50% of the maximum for a significant increase in proliferation to occur. However, given the reduced effect of EAG on the NIH 3T3 cell
versus oocyte resting potentials, this correlation appears to break down for more quantitative comparisons. For example, at the mean resting potentials of ~12, ~18, and ~28 mV for cells expressing wild-type, TAT-SASA, and HTEE channels (Fig. 4B), the G–V curves indicate that ~68, 56, and 49% of channels will be closed, respectively. This observation predicts that proliferation response should be highest for wild-type channels and lowest for EAG-HTEE, a trend that is not observed in our data. There are several possible explanations of this discrepancy. First, the resting potentials measured in NIH 3T3 cells may not accurately reflect the resting potentials of cells in our proliferation assays given that the cells are treated differently. Second, there are several possible explanations of this discrepancy. Fourth, EAG-induced proliferation may be limited by the availability of components of the signaling pathway. Finally, voltage-dependent enzymatic activity or protein–protein interactions may require that channels occupy a given conformation for a specific duration. Although channel conformation may serve as a switch for the signaling activity of EAG, the domain underlying signaling remains under investigation. Possibilities include an amino-terminal PER-ARNT-SIM domain, several putative nuclear localization signals in the carboxyl-terminal domain, and a region with homology to the autoinhibitory domain of Ca\(^{2+}\)/calmodulin-dependent protein kinase II that has been shown recently to regulate kinase activity in in vitro assays (26).

The proliferation induced by EAG was unaffected by changes in extracellular Ca\(^{2+}\), suggesting that increased Ca\(^{2+}\) influx is not an essential downstream component of EAG-induced signaling. Nonetheless, our experiments do not address intracellular Ca\(^{2+}\) concentrations or the possible role of Ca\(^{2+}\) released from intracellular stores. Moreover, although Ca\(^{2+}\) influx may not be a downstream component of the EAG-induced pathway, our results suggest that any mechanism that regulates EAG surface expression or voltage dependence may be an upstream regulator of EAG signaling. Indeed, Ca\(^{2+}\)/calmodulin binding to EAG decreases EAG current by shifting channel activation to more positive potentials (J. D. Clyne, X. X. Sun, D. D. Marble, L. C. Griffith, and G. F. Wilson, unpublished work) and, Ca\(^{2+}\), by increasing Ca\(^{2+}\)/calmodulin-dependent protein kinase II activity and phosphorylation of EAG, increases EAG surface expression (28). Thus, both of these Ca\(^{2+}\)-dependent mechanisms could act upstream of EAG to increase EAG signaling in an activity-dependent manner, and the role of Ca\(^{2+}\) should be further explored.

In the present study, the voltage-dependent signaling activity of EAG increased proliferation of NIH 3T3 cells and C2C12 myoblasts; however, it is unclear whether EAG-induced signaling normally regulates proliferation in vivo. No gross morphological defects have been observed in Drosophila eag mutants at any developmental stage. It should be noted, however, that a role for Drosophila ras in proliferation was only uncovered by using overexpression of mutant ras constructs (29), despite the well-established role of ras in proliferation in other systems. Although the developmental profile of EAG expression is unknown, EAG transcripts and protein appear most highly expressed in mature neurons in both Drosophila and mammals (12, 30–32). Thus, there is little evidence to suggest that EAG regulates proliferation in normal tissues at present. However, abnormally expressed EAG may have a role in proliferation and transformation given that human EAG has been suggested to have an oncogenic potential and EAG appears abnormally expressed in several tumor cell lines (13, 14, 33).

Intracellular signaling pathways typically have a variety of possible roles; the output of a pathway in any given cell at any given developmental stage will depend on context and cross-talk between other pathways. In the present study, proliferation may simply be the “read-out” of a change in the activity of one or more intracellular signaling pathways. Given that EAG appears largely neuron specific and localized at synapses (12, 26, 32) and gives that synaptic plasticity and memory acquisition are disrupted in eag mutants (34, 35), EAG signaling may normally regulate activity-dependent changes in neuronal function. Indeed, EAG-mediated proliferation was blocked by inhibitors of the p38 MAP kinase pathway, and transfection of EAG increased p38 MAP kinase activity. An important future question concerns whether the link between EAG and p38 activity is conserved in neurons. MAP kinase signaling is central to not only proliferation but also synaptic plasticity and learning (36, 37).

Materials and Methods

Plasmids and Construction. pcS2-myc-eag contains a myc tag added to the amino terminus (26). For the wild-type construct, EcoRI and XbaI sites flanking the coding sequence were used to subclone eag (without the myc tag) into the pcS2 vector. A Kozak sequence (GCCACC) was added to improve channel expression. Shaker was subcloned into pcS2 by using EcoRI sites flanking the coding sequence of pGH19-Shaker (38). Eag-F456A, eag-H487E/T490E (HTEE), eag-T449S/A460S/T470A (TATSSA), and double mutants were generated by site-directed mutagenesis using QuikChange (Stratagene). For oocyte expression, mutant constructs were subcloned into pGH19-eag (39). All constructs were verified by sequencing.

Immunocytochemistry and Proliferation Assays. NIH 3T3 fibroblasts were maintained at 37°C and 5% CO\(_2\) in DMEM (Invitrogen) supplemented with 10% FBS as described (38). For transfection, coverslips were washed with Opti-MEM and incubated for 8–10 h in 350 μl of Opti-MEM containing 0.4 μl of the indicated cDNAs and 1.5 μl of Lipofectamine (Invitrogen). Coverslips were then washed and incubated in standard media for 12 h. For serum-free experiments, this was followed by incubation in FBS-free DMEM for 12 h, with 1% FBS added to a subset of wells as a positive control. MAP kinase inhibitors or control compounds were added after washout of Lipofectamine.

For BrdUrd labeling, 10 μM BrdUrd was added to each well for ~60 min. Coverslips were washed with PBS and fixed with a 3:7 mixture of 50 mM glycine (pH 2.0)/100% ethanol for 1 h at room temperature and then denatured with 4 M HCl for 15 min. Cells were labeled with anti-BrdUrd fluorescein-conjugated antibody (Invitrogen) for 45 min at 37°C. Labeling was visualized by using an Olympus (New Hyde Park, NY) BX51W1 microscope equipped with a Qimaging Retiga Exi camera and IPLAB 3.6 software. Coverslips from the same experiment were viewed by using identical settings, and multiple representative scans were taken for each coverslip. To quantify fluorescence, scans were background subtracted, and the intensity of all pixels above background was summed across the total area of each scan. Total intensities were averaged across all scans for each condition before normalizing to the average intensity for controls. Normalized data were then averaged across experiments (n). Data were analyzed by using a two-way ANOVA with Tukey’s post hoc analysis with the condition and experiment number as variables (*, P < 0.05; **, P < 0.01; *** , P < 0.0001; N.S., not significant). Data are presented as the mean ± SEM. MAP kinase inhibitors were purchased from CalBiochem.
Biochemistry. Cells grown on culture plates were dissociated with trypsin-EDTA 48 h after transfection, washed, and resuspended in PBS. Biotinylation and precipitation of EAG was performed as described (28). Briefly, cell suspensions were incubated in 2 mM sulfo-NHS-LC-biotin (Pierce), and the reaction was quenched by washing with 100 mM glycine in PBS. Cells were lysed in PBS supplemented with 1% IGEPEAL-CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, and protease inhibitors, and the homogenate was centrifuged twice for 10 min at 20,000 × g. Protein concentrations of supernatants were determined by Bradford assay and diluted to ~0.5 mg/ml. Surface proteins were precipitated with streptavidin agarose, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, DTT, 1 mM benzamidine, and 0.001 mM microcystin-LR with inhibitors, and the homogenate was centrifuged twice for 10 min at 20,000 × g. Protein concentrations of supernatants were determined by Bradford assay and diluted to ~0.5 mg/ml. Surface proteins were precipitated with streptavidin agarose, and the precipitate was washed extensively before addition of sample loading buffer. Blots were probed with antisera directed against the carboxyl-terminal domain of EAG [EAG (CT) antisera; 1:2,000] in blocking buffer, followed by horseradish peroxidase-conjugated secondary antibody (1:2,000), and visualized with ECL (Amersham Biosciences). Protein bands were quantified by using QUANTITY ONE software (Bio-Rad). Assays of p38 MAP kinase activity were performed by using extracts prepared in buffer containing the following: 20 mM Tris, 100 mM NaCl, 50 mM NaF, 1 mM Na3VO4, 1 mM EDTA, 1 mM sulfo-NHS-LC-biotin (Pierce), and the reaction was formed as described (28). Oocytes were typically injected with 1–2 ng. The recording solution contained the following: 40 mM sodium aspartate, 100 mM NaCl, 4 mM KCl, 1.5 mM MgCl2, 1 mM CaCl2, 2 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH). The pipette solution contained the following: 35 mM potassium aspartate, 110 mM KCl, 2 mM MgATP, 1 mM NaATP, 3 mM sodium phosphocreatine, 0.1 mM NaGTP, 8 mM EGTA, and 10 mM Hepes (pH 7.4 with KOH).

Electrophysiology. Experiments using Xenopus oocytes were performed as described (28). Oocytes were typically injected with 0.1–0.2 ng of RNA; nonconducting constructs were injected at 1–2 ng. The recording solution contained the following: 140 mM NaCl, 2 mM KCl, 1 mM MgCl2, and 10 mM Hepes (pH 7.1 with NaOH). Pipettes had resistances of 0.3–0.6 MΩ. Experiments were performed at room temperature. Leak and capacitative currents were subtracted by using P/4 methods.

For NIH 3T3 cell recordings with an Axopatch200B amplifier (Molecular Devices), cells maintained on plates were cotransfected with pCDNA3-EGFP and the indicated constructs and were then replated onto coverslips. Pipette resistances ranged from 3 to 6 MΩ. The extracellular solution contained the following: 40 mM sodium aspartate, 100 mM NaCl, 4 mM KCl, 1.5 mM MgCl2, 1 mM CaCl2, 2 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH). The pipette solution contained the following: 35 mM potassium aspartate, 110 mM KCl, 2 mM MgATP, 1 mM NaATP, 3 mM sodium phosphocreatine, 0.1 mM NaGTP, 8 mM EGTA, and 10 mM Hepes (pH 7.4 with KOH).

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