Bidirectional control of BK channel open probability by CAMKII and PKC in medial vestibular nucleus neurons

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van Welie I, du Lac S. Bidirectional control of BK channel open probability by CAMKII and PKC in medial vestibular nucleus neurons, J Neurophysiol 105: 1651–1659, 2011. First published February 9, 2011; doi:10.1152/jn.00058.2011.—Large conductance K⁺ (BK) channels are a key determinant of neuronal excitability. Medial vestibular nucleus (MVN) neurons regulate eye movements to ensure image stabilization during head movement, and changes in their intrinsic excitability may play a critical role in plasticity of the vestibulo-ocular reflex. Plasticity of intrinsic excitability in MVN neurons is mediated by kinases, and BK channels influence excitability, but whether endogenous BK channels are directly modulated by kinases is unknown. Double somatic patch-clamp recordings from MVN neurons revealed large conductance potassium channel openings during spontaneous action potential firing. These channels displayed Ca²⁺ and voltage dependence in excised patches, identifying them as BK channels. Recording isolated single channel currents at physiological temperature revealed a novel kinase-mediated bidirectional control in the range of voltages over which BK channels are activated. Application of activated Ca²⁺/calmodulin-dependent kinase II (CAMKII) increased BK channel open probability by shifting the voltage activation range towards more hyperpolarized potentials. An opposite shift in BK channel open probability was revealed by inhibition of phosphatases and was occluded by blockade of protein kinase C (PKC), suggesting that active PKC associated with BK channels in complexes with patches was responsible for this effect. Accordingly, direct activation of endogenous PKC by PMA induced a decrease in BK open probability. BK channel activity affects excitability in MVN neurons and bidirectional control of BK channels by CAMKII and PKC suggests that cellular signaling cascades engaged during plasticity may dynamically control excitability by regulating BK channel open probability.

phosphorylation; firing rate potentiation; vestibulo-ocular reflex; vestibular nucleus; calmodulin-dependent kinase II; protein kinase C; large conductance potassium channels

PLASTICITY IN NEURONAL SPIKE output can occur via changes in synaptic strength but also via modulation of intrinsic excitability [for reviews, see Daoudal and Debanne (2003); Zhang and Linden (2003); Frick and Johnston (2005); Schultz (2006); Weston and Baines (2007)]. Many types of voltage-gated ion channels in mammalian neurons that determine excitability are targets for plasticity mechanisms, including hyperpolarization-activated cyclic nucleotide gated (HCN) channels (van Welie et al. 2004; Fan et al. 2005), A-type K⁺ channels (Frick et al. 2004; Kim et al. 2007; Jung and Hoffman 2009), Kv1.1 channels (Raab-Graham et al. 2006), Kv3.1b channels (Grabert and Wahle 2009; Strumbos et al. 2010), Na⁺ channels (Mee et al. 2004; Xu et al. 2005; Pratt and Aizenman 2007; Grubb and Burrono 2010; Kuba et al. 2010), and small conductance K⁺ (SK) channels (Sourd et al. 2003).

Neurons in the medial vestibular nuclei (MVN) mediate eye movements that stabilize gaze, including the vestibulo-ocular reflex (VOR), which ensures retinal image stability during head movement by controlling eye movement and the optokinetic response (OKR), which ensures image stability during image movement. Medial vestibular nucleus neurons are a site for VOR learning (Lisberger et al. 1994; du Lac et al. 1995; Mauk et al. 1998; Medina et al. 2000; Attwell et al. 2002; Ohyama et al. 2006), and intrinsic plasticity in the MVN occurs in the form of persistent increases in action potential firing termed firing rate potentiation (FRP), which is occluded by blockade of large conductance K⁺ (BK) channels (Nelson et al. 2003) as well as by inhibition of Ca²⁺/calmodulin-dependent kinase II (CAMKII; Nelson et al. 2005). BK channels are good candidates for regulation of intrinsic excitability because they are both voltage dependent and Ca²⁺ sensitive, suggesting that their activity will reflect recent activity and could serve to stabilize membrane excitability.

BK channels are targets for phosphorylation and dephosphorylation by a variety of kinases and phosphatases. Kinases that have been shown to affect BK channels include CAMKII (Braun et al. 2000; Sansom et al. 2000b; Liu et al. 2006; Liu et al. 2007), PKA (Tian et al. 2001 2003; Gong et al. 2002; Widmer et al. 2003), and PKC (Reinhart and Levitan 1995). Phosphatases that have been shown to affect BK channels include protein phosphatase 1 (Bielefeldt and Jackson 1994; Reinhart and Levitan 1995), phosphatase 2A (Zhou et al. 2006), and calcineurin (Loane et al. 2006). Most of the above studies, however, were performed in reduced preparations and at room temperature, conditions under which signaling cascades may function differently from that in more physiological preparations in which excitability and plasticity are generally studied.

Here, we therefore studied putative kinase modulation of BK channels in patches from intact MVN neurons in slices at near-physiological temperature and found that the open probability of BK channels is bidirectionally controlled by the kinases CAMKII and PKC.

MATERIALS and METHODS

Slice preparation. Male and female C57Bl6 mice (postnatal days 16–30) were decapitated under Nembutal anesthesia, and brains were immediately removed. Coronal slices of brainstem (250 μm) including medial vestibular nuclei were prepared using a Leica VT1000S vibratome. Slices were incubated at 34 °C for 30 min and then held at room temperature. Carbogenated artificial cerebrospinal fluid (aCSF)
contained the following (in mM): 124 NaCl, 5 KCl, 2.5 CaCl₂, 1.3 MgSO₄, 1 NaH₂PO₄, 26 NaHCO₃, and 11 glucose. During recordings, slices were submerged in a recording chamber that was superfused with 37°C carbogenated aCSF. For excised patch recordings, slices were cut along the midline to double the number of slices containing medial vestibular nuclei. Neurons targeted for recording were not identified with respect to their neurotransmitter content or projection pattern. After the presence of an active channel in the inside-out patch configuration was determined, the bath was perfused with a HEPES-based solution containing the following (in mM): 130 KCl, 10 HEPES, 2 MgCl₂, and Ca²⁺ and EGTA in appropriate concentrations to give desired free Ca²⁺ concentrations. The concentration of the free Ca²⁺ concentration in the bath solution was estimated using MaxChelator (www.stanford.edu/~cpatton/maxc.html). All experiments were performed in accordance with the standards of the Salk Institute Institutional Animal Care and Use Committee.

Electrophysiological recordings. Whole cell and cell-attached recordings were made with a Multiclamp 700B amplifier and ITC-18 acquisition board and were acquired using Patchmaster 2.2 software run on a G4 Mac. Pipette solutions for whole cell recordings contained the following (in mM): 140 K-glucolone, 10 HEPES, 8 NaCl, 0.1 EGTA, 2 MgATP, and 0.03 NaGTP. Pipette solutions for cell-attached recordings contained the following (in mM): 140 KCl, 10 HEPES, 2 MgCl₂, and during initial recordings 2.5 Ca²⁺. BK channel activity was similar with (n = 71) and without 2.5 mM Ca²⁺ (n = 31) in the pipette solution, and results were thus pooled. Patch pipettes were pulled from flame-polished borosilicate glass (Sutter) with resistances of 5–7 MΩ. Whole cell data were sampled at 40 kHz and filtered at 10 kHz. Cell-attached data were sampled at 40 kHz and filtered at 2 kHz. All standard chemicals for bath and pipette solutions were purchased from Sigma or Fluka. Drugs were bath applied to inside-out patches at a flow rate of 100 μl/h for 10 min following 10 min of control recordings in which BK channel activity was allowed to stabilize. CAMKII was purchased from New England Biolabs and was prepared minutes before use by following the manufacturer’s suggested protocol in which CAMKII was diluted in reaction buffer and incubated in 200 μM ATP, 1.2 μM calmodulin, and 2 mM CaCl₂ to reach maximal levels of autophosphorylated CAMKII. Alkaline phosphatase was purchased from Invitrogen, microcystin LR was from Sigma and from the University of Dundee (Dundee, Scotland), PKA peptide inhibitor 5–24 was from Calbiochem, PKC peptide fragments 19–36 was from Sigma, and PMA was from Alexis Biochemicals.

Data analyses. BK channel open probability and amplitude were determined using a custom-made algorithm in Igor Pro. Data recorded during experimental conditions were integrated and binned in 0.25-pA bins over 2-s time periods, resulting in all-points histograms consisting of two Gaussian peaks. From these histograms, peak amplitudes were determined and the cut-off between peaks was set at the median value between the peaks. Open probability was calculated as the area under the Gaussian peaks divided by the total area under the curve, where one peak represents the open channel state and the other the closed channel state. Patches with a maximum of two active channels were used for analysis. Channel openings in response to negative voltage steps are indicated as negative and vice versa. The statistical significance of results was tested using paired and unpaired t-tests and nonparametric Wilcoxon matched pairs test where appropriate (Instat 3.0). Statistical significance is indicated as P < 0.05 and P < 0.01.

RESULTS

BK channel activity during action potential firing of MVN neurons. Whole cell BK channel mediated conductance has both a transient and a sustained component in MVN neurons (Gittis and du Lac 2007). To determine whether both components were readily observed in single BK channel activity in patches of MVN neurons, we performed double-patch recordings on MVN neurons in slices with one electrode in whole cell current clamp mode to monitor action potential firing and the other in cell-attached voltage clamp mode to monitor single channel activity (Fig. 1A). For cell-attached patches, the reversal potential for K⁺ was set at 0 mV by a 140 mM K⁺ solution in the pipette. Active large conductance single channel activity was seen in about half of the cell-attached patches and channel activity reversed at 0 mV, indicating these channels mediated a potassium conductance. Overlaying traces from the whole cell and cell-attached electrode revealed that the majority of these channels opened on both the falling phase of the action potential and during interspike intervals (Fig. 1B). Occasionally, large conductance channels were observed that opened only during the falling phase of the action potential.

Biophysical properties of MVN BK channels in excised patches. Single BK channels were further studied in inside-out patches excised from MVN neurons in slices. After cell-attached seals >5 GΩ were obtained, patches were pulled from MVN neurons; only excised patches with a seal resistance >1 GΩ were used for recording. The presence of a BK channel could be quickly determined, as the excised patch was pulled in standard aCSF containing low K⁺ and 2 mM Ca²⁺, and BK channels present in the patch typically displayed high activity within seconds of patch excision. Once the presence of a BK channel was thus determined, a high K⁺, HEPES-based solution was washed in, setting the K⁺ reversal potential at 0 mV and the free Ca²⁺ concentration at 1 μM. After the channel
activity was allowed to stabilize for at least 10 min in the new bath solution, current-voltage relationships were determined in voltage clamp mode by stepping from a holding potential of 0 mV to a range of hyperpolarizing and depolarizing potentials (Fig. 2A). In 1 μM free Ca\(^{2+}\), the mean single channel conductance of BK channels was 267 ± 5 pS (n = 25; Fig. 2B), which is in the range of single conductance values typically reported for BK channels in symmetrical K\(^+\) (Salkoff et al. 2006).

Sensitivity to Ca\(^{2+}\) was tested in separate patches using solutions with different Ca\(^{2+}\) concentrations exposed to the inside of patches. Higher Ca\(^{2+}\) concentrations shifted the voltage range of activation of BK channels to more hyperpolarized potentials, resulting in a maximal voltage of half-maximal activation in 0.1 μM Ca\(^{2+}\) of 101 ± 5 mV (n = 4), −58 ± 2 mV (n = 18) in 1 μM Ca\(^{2+}\), and −66 ± 8 mV (n = 4) in 10 μM Ca\(^{2+}\) (Fig. 2C). The slope factor in 0.1, 1, and 10 μM Ca\(^{2+}\) was 11 ± 2 mV (n = 4), 15 ± 1 mV (n = 18), and 15 ± 2 mV (n = 4), respectively. BK channel properties in 1 μM Ca\(^{2+}\) were also determined at room temperature (data not shown), yielding a single channel conductance of 218 ± 2 pS (n = 5), a voltage of half-maximal activation of −45 ± 2 mV (n = 4), and a slope factor of 10 ± 1 mV (n = 4); all three parameters were significantly different from those at near physiological temperature (P ≤ 0.0001; P = 0.001, and P = 0.04, respectively).

Phosphorylation of BK channels by CAMKII shifts the voltage range of activation to more hyperpolarized potentials. The intrinsic excitability of MVN neurons exhibits activity-dependent plasticity, which relies on both BK channels (Nelson et al. 2003) and inhibition of constitutively active CAMKII (Nelson et al. 2005). We therefore set out to test directly whether CAMKII phosphorylates BK channels and, if so, what effect this phosphorylation might have on BK channel open probability. BK channel activity was assessed in inside-out patches as described above. For pharmacological experiments, we chose to study BK channel open probability at a voltage of −50 mV and chose a free Ca\(^{2+}\) concentration of 1 μM. BK channel open probability under these baseline conditions displayed considerable variability across patches and ranged between 0.01 and 0.94 (n = 65); all drug effects were therefore compared with control data within individual patches. After BK channel activity was recorded under control conditions for 10 min, activated CAMKII (500 U/ml; see MATERIALS AND METHODS for details on activation procedure) was applied to the inside of patches by bath perfusion at the same flow rate as perfusion of standard external. After 10 min of perfusion, CAMKII had significantly increased the open probability of BK channels in every patch by ~50%, regardless of the baseline open probability (0.35 ± 0.08 before CAMKII, 0.67 ± 0.05 after αCAMKII; n = 11; P = 0.022; Fig. 3A). Full current-voltage curves recorded before and after CAMKII application revealed that CAMKII induced an average leftward shift towards more hyperpolarized values in the voltage of half-maximal activation of 56 ± 10 mV (n = 6; P = 0.002; Fig. 3B). In addition, CAMKII induced a small change in the slope factor of 4.6 ± 0.8 mV (n = 6; P = 0.05). This result indicates that active CAMKII affects BK channels by shifting the range of voltages over which the channel is activated towards more hyperpolarized potentials.

To control for potential rundown effects of BK channel activity in time, we also recorded BK channel activity in separate patches for a similar time period but without application of CAMKII. BK channel open probability exhibited a small, nonsignificant run-up of ~10% (0.65 ± 0.04 control early to 0.70 ± 0.05 control late; n = 8; P = 0.08; Fig. 3C).

BK channel activity could potentially be affected by the concentrations of Ca\(^{2+}\), calmodulin, and ATP that were required to activate the CAMKII used in the above experiments. In control experiments, bath application of the kinase activation solution without the kinase did not significantly affect BK channel open probability (0.57 ± 0.06 before activation solution to 0.54 ± 0.13 after activation solution; n = 6; P = 0.71). These results show that phosphorylation by CAMKII affects MVN BK channel activity by shifting its voltage range of activation to more hyperpolarized potentials.

![Image](http://jn.physiology.org/content/105/4/1653/F2.large.jpg)
Dephosphorylation of BK channels in excised patches does not affect open probability. To test whether any endogenously active CAMKII was already associated with BK channels in inside-out patches resulting in phosphorylation of the channels, alkaline phosphatase, a broad spectrum phosphatase that will remove any phosphate groups bound to the channels, was applied to the inside of patches. Because a potential decrease in phosphatase activity could unmask the activity of CAMKII bound to BK channels in inside-out patches, we bath applied alkaline phosphatase (80 U/ml) to channels recorded at −30 mV. Alkaline phosphatase had little effect on BK channel open probability [from 0.82 ± 0.03 before alkaline phosphatase (●) to 0.79 ± 0.04 after alkaline phosphatase (○); n = 6; P = 0.05], suggesting that either little active CAMKII is bound to these channels or that there already is strong phosphatase activity in the inside-out patches.

Phosphorylation of BK channels by PKC shifts voltage range of activation to more depolarized potentials. If the activity of endogenous CAMKII in inside-out patches was counteracted by phosphatases, one would expect that inhibition of such phosphatases could unmask the activity of CAMKII and, thus, an increase in BK channel open probability would be observed. We therefore tested the effect of the broad-spectrum phosphatase inhibitor microcystin LR on BK channels in inside-out patches, which surprisingly revealed that inhibition of phosphatases decreased BK channel activity. In all patches tested, application of 1 μM microcystin LR (final concentration of 0.02–0.2% DMSO) caused a ~30% decrease in open probability (0.48 ± 0.07 before microcystin LR, 0.35 ± 0.08 after microcystin LR; n = 7; P = 0.01, Fig. 5A). Full current-voltage curves recorded before and after microcystin LR application revealed that microcystin LR induced an average rightward shift towards more depolarized values in the voltage range of activation to more depolarized potentials.

Fig. 4. Dephosphorylation of BK channels in inside-out patches does not affect open probability. To test for the presence of any endogenously active CAMKII bound to BK channels in inside-out patches, we bath applied alkaline phosphatase to (60–80 U/ml) to channels recorded at −30 mV. Alkaline phosphatase had little effect on BK channel open probability [from 0.82 ± 0.03 before alkaline phosphatase (●) to 0.79 ± 0.04 after alkaline phosphatase (○); n = 6; P = 0.05], suggesting that either little active CAMKII is bound to these channels or that there already is strong phosphatase activity in the inside-out patches.

Fig. 5. Inhibition of phosphatase activity decreases the open probability of BK channels. A. left: example traces show that inhibition of endogenous phosphatases by the phosphatase blocker microcystin LR (MC-LR; 1 μM) induced a decrease in the open probability of BK channels recorded at −50 mV. A. right: within patch comparisons showed a highly significant effect of MC-LR on BK channel open probability [from 0.48 ± 0.07 before MC-LR (●) to 0.35 ± 0.08 after MC-LR (○); n = 7; P = 0.01]. B: example of the voltage-dependence of a BK channel before (●) and after application of activated CAMKII (○). Active CAMKII induced a mean shift of 56 ± 0.03 mV (n = 6; P = 0.002) towards more hyperpolarized potentials, with a small change in the slope factor of 4.6 ± 0.5 mV (n = 6; P = 0.05). C: control recordings of active BK channels in time showed a slight, but nonsignificant, run-up in BK channel open probability [from 0.65 ± 0.04 control early (●) to 0.70 ± 0.05 control late (○); n = 8; P = 0.08]. **P < 0.01.

Dephosphorylation of BK channels in excised patches does not affect open probability. To test whether any endogenously active CAMKII was already associated with BK channels in inside-out patches resulting in phosphorylation of the channels, alkaline phosphatase, a broad spectrum phosphatase that will remove any phosphate groups bound to the channels, was applied to the inside of patches. Because a potential decrease in open probability was expected, patches were held at a potential of −30 mV for this experiment to ensure a high baseline open probability. In all patches tested, 10 min of alkaline phosphatase (80 U/ml) treatment had no significant effect on the open probability of BK channels (0.82 ± 0.03 before alkaline phosphatase to 0.79 ± 0.04 after alkaline phosphatase; n = 6; P = 0.05; Fig. 4). This result suggests that there is not much active CAMKII bound in excised inside-out patches or that endogenously active CAMKII is already actively counteracted by phosphatase activity.

Phosphorylation of BK channels by PKC shifts voltage range of activation to more depolarized potentials. If the activity of endogenous CAMKII in inside-out patches was counteracted by phosphatases, one would expect that inhibition of such phosphatases could unmask the activity of CAMKII and, thus, an increase in BK channel open probability would be observed. We therefore tested the effect of the broad-spectrum phosphatase inhibitor microcystin LR on BK channels in inside-out patches, which surprisingly revealed that inhibition of
probability was recorded during application of 0.2% DMSOperformed control experiments in which BK channel open
H11006
0.83
PKA by peptide inhibitor 2–24 (0.45
peptide inhibitors of PKA and PKC (Fig. 6
A
are blocked, we applied microcystin LR in the presence of
for the decrease in BK channel probability when phosphatases
decrease in open probability. To identify the kinase responsible
endogenous active kinase that is closely associated with the
channel is able to phosphorylate the channel resulting in a
more depolarized potentials.
activity shifts the voltage range of activation of BK channels to

PKA, by peptide inhibitor 2–24 (0.45
MK-LR (10 μM) occluded the effect of microcystin LR on BK channel
activity (open probability changed from 0.69
0.05 before MC-LR
0.06 after microcystin LR; n = 10; P = 0.05; Fig. 6A). This result suggested that PKC is
the kinase responsible for the decrease in open probability
following inhibition of phosphatase activity. To confirm this
prediction, we applied an activator of PKC, PMA (10 μM), to
patches, which significantly decreased BK channel open
probability by ~23% (from 0.71 ± 0.06 before PMA to 0.55 ±
0.09 after PMA; n = 7; P = 0.02; Fig. 6B).

Taken together, these results suggest that in inside-out
patches active PKC that is closely associated with BK channels
is counteracted by phosphatase activity and that activated PKC
affects BK channels in the opposite direction from activated
CAMKII. As BK channel activity affects the spontaneous
firing rate and gain in MVN neurons (Smith et al. 2002; Nelson
et al. 2003; Gittis et al. 2010), bidirectional modulation by
CAMKII and PKC is expected to also bidirectionally regulate
the excitability of MVN neurons (Fig. 7).

Fig. 6. Activated PKC activity decreases BK channel open probability
A: blockade of phosphatase activity by microcystin LR in the presence of
a peptide-inhibitor of PKA (0.45 μM) did not prevent the decrease in open
probability in 5 out of 5 patches tested [from 0.68 ± 0.09 before MC-LR +
PKA inhibitor (●) to 0.22 ± 0.10 after MC-LR + PKA inhibitor (○); n = 5;
P = 0.004; left], while blockade of phosphatases in the presence of a
peptide-inhibitor of PKC (1 μM) did prevent the decrease in open probability
[from 0.69 ± 0.05 before MC-LR + PKC inhibitor (●) to 0.67 ± 0.06 after
MC-LR + PKC inhibitor (○); n = 10; P = 0.55; right], thus identifying PKC
as the active kinase that is unmasked by inhibition of endogenous phosphatase
activity. B, left: example traces show that application of the PKC agonist
PMA (10 μM) to patches with intact phosphatase activity reduced BK channel open
probability. On the right, within patch comparisons showed a significant effect
of PMA on BK channel open probability [from 0.71 ± 0.06 before PMA (●) to
0.55 ± 0.09 after PMA (○); n = 7; P = 0.02]. *P < 0.05; **P < 0.01.
of half-maximal activation of 51 ± 11 mV (n = 5; P = 0.009;
Fig. 5B) with no significant change in the slope factor (~0.9 ±
0.5 mV; n = 5; P = 0.12). As microcystin LR had to be
dissolved in a relatively high final concentration of DMSO, we
performed control experiments in which BK channel open
probability was recorded during application of 0.2% DMSO
without microcystin LR and found no significant effects on BK
channel open probability (0.79 ± 0.04 before 0.2% DMSO to
0.83 ± 0.04 after 0.2% DMSO; n = 4; P = 0.09; data not
shown). These results indicate that inhibition of phosphatase
activity shifts the voltage range of activation of BK channels to
more depolarized potentials.

It is plausible that when phosphatases are inhibited, an
endogenous active kinase that is closely associated with the
channel is able to phosphorylate the channel resulting in a
decrease in open probability. To identify the kinase responsible
for the decrease in BK channel probability when phosphatases
are blocked, we applied microcystin LR in the presence of
peptide inhibitors of PKA and PKC (Fig. 6A). Inhibition of
PKA by peptide inhibitor 2–24 (0.45 μM) did not occlude the
effect of microcystin LR on BK channel activity (open prob-
ability changed from 0.68 ± 0.09 before microcystin LR to
0.22 ± 0.10 after microcystin LR; n = 5; P = 0.004, Fig. 6A).
In contrast, inhibition of PKC by the peptide inhibitor 19–36 (1
μM) occluded the effect of microcystin LR on BK channel
open probability (open probability changed from 0.69 ± 0.05

Fig. 7. Schematic of bidirectional phosphorylation of BK channels by CAMKII
and PKC. The effect of phosphorylation of MVN BK channels by CAMKII
and PKC is bidirectional: active CAMKII increases open probability, while
active PKC decreases open probability. As a decrease in active CAMKII
increases gain, an effect that is occluded by blockade of BK channels (Nelson
et al. 2005) and blockade of BK channels increases the input-output gain of
MVN neurons (Smith et al. 2002), it can be concluded that in MVN neurons
CAMKII phosphorylation of BK channels decreases firing rates while PKC
phosphorylation of BK channels increases firing rates. This bidirectional
BK channel modulation by CAMKII and PKC thus constitutes a powerful and
dynamic mechanism to regulate the excitability of MVN neurons.

DISCUSSION

Here, we studied kinase modulation of BK channels at
near-physiological temperature in membrane patches excised
from MVN neurons in brainstem slices. We found that the
open probability of endogenous BK channels can be modified
by CAMKII, which shifts the voltage range over which BK
channels are activated to more hyperpolarized potentials. Inhi-
bition of phosphatases associated with BK channel complexes
in patches produced a decrease, rather than a predicted in-
crease, in BK channel open probability, suggesting the presence
of another active kinase with opposite effects on BK

BIDIRECTIONAL KINASE CONTROL OF BK CHANNELS

J Neurophysiol • VOL 105 • APRIL 2011 • www.jn.org
channels. Inhibition of phosphatases with simultaneous block of PKA or PKC revealed that PKC was responsible for the decrease in BK channel activity. Accordingly, activation of endogenous PKC in patches with intact phosphatase activity also decreased BK channel activity. Although we cannot exclude that the effects of CAMKII and PKC on BK channel open probability were mediated through phosphorylation of regulatory proteins associated with BK channels, together, these results indicate that CAMKII and PKC control BK channel open probability by shifting the voltage range of activation in opposite directions (Fig. 7).

**CAMKII modulation of BK channel open probability.** CAMKII is a versatile kinase that has been implicated in a variety of neuronal and behavioral plasticity mechanisms (Griffith et al. 2003). Both αCAMKII and βCAMKII are implicated in plasticity and learning in the vestibulo-cerebellar system (Hansel et al. 2006; van Woerden et al. 2009; Nelson et al. 2005). CAMKII is thought to function as a molecular switch due to its capacity for autophosphorylation that renders it constitutively active and independent of Ca$^{2+}$/CaM activity. In MNV neurons, a large proportion of CAMKII protein is autophosphorylated, in contrast to the low level of autophosphorylated CAMKII in silent neurons such as hippocampal and cortical neurons (Nelson et al. 2005). Here, we show that such active CAMKII can increase the open probability of BK channels.

Modulation of BK channel activity by CAMKII has previously only been described in nonneuronal tissues such as glomerular mesangial kidney cells (Sansom et al. 2000) and in the neuromuscular junction, where CAMKII in the presynaptic terminal affects neurotransmitter release by shifting the voltage dependence of activation of slo-1 channel activity to more hyperpolarized potentials (Liu et al. 2007), similar to the effects of CAMKII on endogenous channels we describe here. CAMKII modulation of BK channels also affects channel sensitivity to alcohol, switching responses from inhibited to activated (Liu et al. 2006). To our knowledge, we are reporting the first example of CAMKII modulation of endogenous mammalian neuronal BK channels at near-physiological temperatures.

In the present study, we did not explicitly address which phosphatase may be counteracting endogenous CAMKII modulation of BK channels, given that the alkaline phosphatase experiment suggested that no endogenous active CAMKII was bound to our patches. A variety of phosphatases including PP1, PP2A, PP2B, and PP2C [reviewed in Skelding and Rostas (2009)] could be counteracting CAMKII modulation of BK channels by either direct dephosphorylation of autophosphorylated CAMKII or by dephosphorylation of the BK channels themselves or their associated regulatory proteins.

**PKC modulation of BK channel open probability.** Modulation of BK channels by PKC has also been described predominantly in nonneuronal cell types, where contrasting effects on BK channel activity have been reported. In artery smooth muscle cells, PKC can inhibit the activity of single BK channels (Minami et al. 1993, Schubert et al. 1999; Taguchi et al. 2000) but can also activate BK channels (Barman et al. 2004; Zhu et al. 2006). PKC also increases the activity of BK channels that are reconstituted in lipid bilayers (Reinhart and Levitan 1995) and in COS7 cells (Kim and Park 2008). In contrast, in pituitary tumor cells, single channel recordings revealed that inhibition of PKC increased the open probability of BK channels and shifted the voltage range of activation to more hyperpolarized potentials. This finding suggested that active PKC serves to shift the voltage range of activation towards depolarized potentials (Wu et al. 2007), a similar result to what we show here.

Recently, two PKC phosphorylation sites were identified in cloned α-subunits from bovine trachea (Zhou et al. 2010), where PKC inhibited BK channels by both decreasing channel open time and prolonging the closed state of the channel, with no effects on voltage range of activation. We did not analyze the kinetics of our channel recordings, as incomplete channel openings, which preclude straightforward kinetic analyses, are very frequent at physiological temperature, and we therefore cannot exclude effects on channel kinetics. However, as our observed effects of PKC on the voltage range of activation in defined calcium are very evident, it may be that endogenous mammalian BK channels have additional phosphorylation sites that are targeted by PKC.

BK channels with low open probability in cerebellar Purkinje cells are sensitive to PKA modulation during blockade of protein phosphatase 2A (Widmer et al. 2003). Furthermore, PKA modulation of these channels is counteracted by PKC phosphorylation that itself is counteracted by protein phosphatase 1. In our experiments, blocking both phosphatase 1 and 2A by microcystin resulted in a decrease in channel activity that was completely occluded by the PKC inhibitor, suggesting that PKC is solely responsible for the observed decrease. Given that PKA should be maximally activated by block of both phosphatases and PKC under these circumstances, it seems likely that MNV BK channels are not readily modulated by PKA. To summarize, it appears that BK channels in reduced preparations are differentially modulated by PKC. Part of these differential effects might be due to isoform specificity, as 12 different isoforms for PKC have been described that may differ in their Ca$^{2+}$ and DAG dependence and sensitivity (Dai et al. 2009).

In MNV neurons, previous experiments have shown that the PKC inhibitor chelerythrine chloride does not affect MNV neuron excitability but that inhibition of phosphatases by microcystin LR induced a decrease in gain (Smith et al. 2002). These results are consistent with our findings that PKC activity is normally counteracted by phosphatase activity and is only revealed after inhibition of phosphatases by microcystin LR.

**Physiological relevance of bidirectional regulation of BK channel activity.** In the experiments presented here, BK channel open probability under baseline conditions displayed considerable variability across patches. We suggest that the baseline variability may be due to differences in phosphorylation state of the channels at the time that patches were pulled or due to differences in regulatory proteins that stay associated with the channels in the patches. Furthermore, patches analyzed in this experiment were obtained from unidentified MNV neurons. Although all MNV neurons express BK currents (Gittis et al. 2007), the contributions of BK currents to excitability (Smith et al. 2002) and to action potential repolarization (Gittis et al. 2010) vary across neurons. In fast firing projection neurons, Kv3 currents dominate repolarization at all firing rates, whereas in slower firing GABAergic interneurons, BK currents dominate repolarization at higher firing rates (Gittis et al. 2010). It will be of interest to determine whether PKC and
CAMKII activities are differentially engaged in local vs. projection neurons.

Previous work (Nelson et al. 2003, 2005) also showed that in spontaneously firing MVN neurons blockade of BK channels prevents firing rate and gain potentiation, which is furthermore occluded by CAMKII blockade. In silent neurons in contrast, application of activated CAMKII caused a decrease in excitability. This suggests that high levels of active CAMKII in spontaneously firing neurons may function to increase the open probability of BK channels and that, thus, decreases in CAMKII phosphorylation of BK channels may underlie FRP in MVN neurons.

Individual examples of phosphorylation of BK channels by CAMKII and PKC have been described before, but only in nonneuronal cells at nonphysiological temperatures, and never have two kinases been described to bidirectionally control the voltage range of activation in defined calcium concentrations of BK channels within a given cell type. Although these two kinases are both activated by signaling cascades, PKC is engaged by G-protein channel activation and requires both diacylglycerol and Ca\(^{2+}\) for its activation, while CAMKII is activated by any Ca\(^{2+}\) increase in the cell. This suggests that different signaling cascades can affect the open probability of BK channels in opposite directions.

Both the voltage sensor and the Ca\(^{2+}\) sensitivity determine the voltage activation range of BK channels, and from our experiments we cannot conclude whether the shifts in activation range are due to changes to the voltage sensor or the Ca\(^{2+}\) sensitivity. Nevertheless, as the shifts in activation range occurred under fixed Ca\(^{2+}\) concentrations, a direct result will be that the effective Ca\(^{2+}\) concentration required to evoke a certain BK-mediated conductance at a given voltage will be different before and after channel phosphorylation. In other words, the phosphorylation state of BK channels will influence BK currents activated by the Ca\(^{2+}\) influx during spontaneous firing and plasticity. This indicates that bidirectional regulation of BK channel open probability ensures a highly dynamic control of BK channel conductance based on cellular state.

Although other kinases may additionally affect MVN BK channel open probability, CAMKII and PKC are two kinases of particular interest, as they have been implicated in several learning and adaptive paradigms. PKC is involved in vestibular adaptation (de Zeeuw et al. 1998), vestibular compensation (Sansom et al. 2000a), eye-blink conditioning (Bank et al. 1988; Alkon et al. 1998; Schreurs et al. 1996, 1997; van der Zee et al. 1997), spatial learning and memory (Olds et al. 1990; Paylor et al. 1991, 1992; Colombo et al. 1997; Bonini et al. 2007), and contextual fear memory (Ahi et al. 2004; Levenson et al. 2004). A role for CAMKII in learning paradigms has been shown for spatial learning and memory (Silva et al. 1992a,b; Giese et al. 1998; Mayford et al. 1996a; Frankland et al. 2001, 2004; Wang et al. 2003) and in vestibulo-ocular adaptation (Hansel et al. 2006). BK channel activity itself has also been implicated in learning and memory processes (Matthews et al. 2008, Matthews and Disterhoft 2009).

Activity-dependent modulation of voltage-gated ion channels can serve as a powerful form of plasticity and here we show that ion channels are major targets of kinases, whereas roles for kinases in plasticity have mainly been associated with synaptic function. Bidirectional modulation of BK channel open probability by two different kinases may represent a dynamic mechanism for context-specific regulation of neuronal excitability.

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DISCLOSURES

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