Structural Requirements for Differential Sensitivity of KCNQ K⁺ Channels to Modulation by Ca²⁺/Calmodulin

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Calmodulin modulation of ion channels has emerged as a prominent theme in biology. The sensitivity of KCNQ1–5 K⁺ channels to modulation by Ca²⁺/calmodulin (CaM) was studied using patch-clamp, Ca²⁺ imaging, and biochemical and pharmacological approaches. Coexpression of CaM in Chinese hamster ovary (CHO) cells strongly reduced currents of KCNQ2, KCNQ4, and KCNQ5, but not KCNQ1 or KCNQ3. In simultaneous current recording/Ca²⁺ imaging experiments, CaM conferred Ca²⁺ sensitivity to KCNQ4 and KCNQ5, but not to KCNQ1, KCNQ3, or KCNQ1/KCNE1 channels. A chimera constructed from the carboxy terminus of KCNQ4 and the rest KCNQ1 displayed Ca²⁺ sensitivity similar to KCNQ4. Chimeras constructed from different lengths of the KCNQ4 carboxy terminal and the rest KCNQ3 localized a region that confers sensitivity to Ca²⁺/CaM. Lobe-specific mutations of CaM revealed that its amino-terminal lobe mediates the Ca²⁺ sensitivity of the KCNQ/CaM complex. The site of CaM action within the channel carboxy terminus overlaps with that of the KCNQ opener *N*-ethylmaleimide (NEM). We found that CaM overexpression reduced NEM augmentation of KCNQ2, KCNQ4, and KCNQ5, and NEM pretreatment reduced Ca²⁺/CaM-mediated suppression of M current in sympathetic neurons by bradykinin. We propose that two functionally distinct types of carboxy termini underlie the observed differences among this channel family.

INTRODUCTION

The sensitivity of KCNQ1–5 channels to intracellular Ca²⁺ ([Ca²⁺]_i) and the physiological relevance of such sensitivity have been highly debated questions in the physiology of this K⁺ channel family that underlie neuronal (M current; KCNQ2, 3, 5), cardiac (I_{Ks}; KCNQ1), epithelial (KCNQ1) and other important K⁺ currents. Although M-type channels have been shown to be sensitive to [Ca²⁺]_i (Marrion *et al.*, 1991; Selyanko and Brown, 1996a; Gamper and Shapiro, 2003), muscarinic stimulation of superior cervical ganglion (SCG) sympathetic neurons does not raise [Ca²⁺]_i (Beech *et al.*, 1991; Cruzblanca *et al.*, 1998; del Rio *et al.*, 1999) and intracellular Ca²⁺ signals do not mediate muscarinic modulation of SCG M current. However, in the same cells, other G_{q/11}-coupled receptors that also modulate the M current do act via [Ca²⁺]_i signals (Cruzblanca *et al.*, 1998; Bofill-Cardona *et al.*, 2000). Receptor specificity seems to arise from differences in ability to raise [Ca²⁺]_i, with this determined by whether receptors are intimately colocalized with inositol trisphosphate (IP₃) receptors (Delmas *et al.*, 2002). In SCG neurons, muscarinic M₁ and IP₃ receptors do not colocalize and thus do not raise [Ca²⁺]_i; in contrast, bradykinin and probably P₂Y receptors are coupled to IP₃-sensitive Ca²⁺ signals, and their action on M channels is blunted by any maneuvers that disrupt [Ca²⁺]_i rises (Cruzblanca *et al.*, 1998; Bofill-Cardona *et al.*, 2000; Scholze *et al.*, 2002; Gamper and Shapiro, 2003). Finally, calmodulin (CaM) has been found to interact with all KCNQ1–5 channels (Wen and Levitan, 2002; Yus-Najera *et al.*, 2002; Gamper and Shapiro, 2003) and to

mediate high Ca²⁺ sensitivity of KCNQ2/3 heteromultimers (Gamper and Shapiro, 2003); thus, overexpression of mutant calmodulin that cannot bind Ca²⁺ in sympathetic neurons blunts bradykinin-induced M current suppression.

This seemingly consistent concept is, however, complicated by solid evidence obtained in amphibian sympathetic neurons showing that Ca²⁺ sensitivity of M currents in those cells to be more complex. In those studies, increases of [Ca²⁺]_i in the range of 60–120 nM caused augmentation of M current and only further rises above 200 nM had an inhibitory effect (Marrion *et al.*, 1991; Yu *et al.*, 1994; Marrion, 1996). It was suggested that calcineurin mediates this Ca²⁺ modulation of that M current (Marrion, 1996). Similarly conflicting are data on the Ca²⁺ sensitivity of KCNQ1 or the cardiac I_{Ks} K⁺ current (formed by KCNQ1 with its auxiliary subunit KCNE1), and it has been suggested that KCNQ1 can be inhibited (Shen and Marcus, 1998) or augmented (Kerst *et al.*, 2001) by [Ca²⁺]_i, or indeed wholly Ca²⁺ insensitive unless coexpressed with KCNE1 (Boucherot *et al.*, 2001). These striking differences in the Ca²⁺ sensitivity of KCNQ channels in different physiological systems suggest that Ca²⁺ sensitivity is not an intrinsic property of KCNQ channels but rather the result of their interaction with Ca²⁺ sensors, and so may depend on the nature of the available sensor, channel subunit composition and other physiological conditions.

In this work, we use patch-clamp electrophysiology, Ca²⁺ imaging, and a variety of molecular biological, biochemical, and pharmacological approaches to study the subunit specificity of KCNQ channels to CaM-mediated Ca²⁺ modulation. We find that CaM confers high Ca²⁺ sensitivity to KCNQ2, KCNQ4, and KCNQ5, that this, sensitivity is determined by the channel carboxy terminus and mediated by the amino-terminal lobe of CaM. We also show competition for CaM binding and resultant Ca²⁺/CaM actions on the channels with the KCNQ-channel opener *N*-ethylmaleimide (NEM) (Roche *et al.*, 2002), which alkylates a carboxy-termi-

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nal cysteine (Li *et al.*, 2004) located near a site of CaM binding (Wen and Levitan, 2002; Yus-Najera *et al.*, 2002; Gamper and Shapiro, 2003). Our results suggest a mechanism to explain the subunit specificity that we observe that links Ca^{2+} /CaM and NEM actions, and we propose the existence of two types of KCNQ-channel carboxy terminus.

MATERIALS AND METHODS

cDNA Constructs, Cell Culture, and Transfection

Plasmids encoding human KCNQ1, human KCNE1, human KCNQ2, rat KCNQ3, human KCNQ4, and human KCNQ5 (GenBank accession nos. NM000218, NM000219, AF110020, AF091247, AF105202, and AF249278, respectively) were kindly given to us by Michael Sanguinetti (University of Utah, Salt Lake City, UT; KCNQ1 and KCNE1), David McKinnon (State University of New York, Stony Brook, NY; KCNQ2 and KCNQ3), Thomas Jentsch (Zentrum für Molekulare Neurobiologie, Hamburg, Germany; KCNQ4), and Klaus Steinmeyer (Aventis Pharma, Frankfurt am Main, Germany; KCNQ5). Plasmids containing wild-type (wt) (Persechini *et al.*, 1989), dominant-negative (DN), and amino-lobe or carboxy-lobe mutant vertebrate CaM were given to us by Trisha Davis (University of Washington, Seattle, WA). DN CaM has an alanine substitution in each of the four Ca^{2+} -binding EF hands (D20A, D56A, D93A, and D129A). The amino-lobe or carboxy-lobe mutants have the first two, or last two, of these mutations, respectively. The wt and DN CaM coding regions were subcloned by PCR into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA) by using *Bam*H1 and *Xba*I. KCNQ2 and KCNQ3 were subcloned into pcDNA3 as described previously (Shapiro *et al.*, 2000). The KCNQ1_N/4_C chimera was generated by restriction cutting wild-type KCNQ1 and KCNQ4 with *Xcm*I and *Xba*I. *Xcm*I cuts KCNQ1 at V418 and cuts KCNQ4 at Q454, and *Xba*I cuts after the stop codon in both cases. Thus, the chimeras contain most, but not all, of the carboxy terminus downstream of S6. The junction site is just downstream of the highly conserved "IQ" domain (Gamper and Shapiro, 2003). The KCNQ3_N/4_C chimera, KCNQ3_N/4_C-1, is the same chimera examined by us previously as "KCNQ3_N/4_C" (Li *et al.*, 2004) and was generated by cutting both clones with *Xcm*I and *Xba*I. *Xcm*I cuts KCNQ3 at Q385 and cuts KCNQ4 at Q454, and *Xba*I cuts after the stop codon in both cases. KCNQ3_N/4_C-2 was generated by introducing silent-site mutations for *Nae*I at G456 in KCNQ3, and for *Eco*RV at D525 in KCNQ4, and then cutting KCNQ3 or KCNQ4 with *Nae*I/*Xho*I or *Eco*RV/*Xho*I, respectively. *Nae*I and *Eco*RV have blunt ends, and *Xho*I cuts after the stop codon in both cases.

Electrophysiology

Cells were cultured and transfected with Polyfect reagent (QIAGEN, Valencia, CA) as described previously (Gamper and Shapiro, 2003; Gamper *et al.*, 2003). Sympathetic neurons were isolated from the SCG of 3- to 14-d-old male rats (Sprague-Dawley) as described previously (Gamper and Shapiro, 2003) and cultured for 2–4 d. The perforated patch configuration of the patch-clamp technique was used to voltage clamp and dialyze cells at room temperature (22–25°C) by using amphotericin B (0.5 mg/ml). Pipettes were pulled from borosilicate glass capillaries (IB150F-4; WPI, Sarasota, FL) by using a Flaming/Brown micropipette puller P-97 (Sutter Instruments, Novato, CA) and had resistances of 2–3 MΩ when filled with internal solution and measured in standard bath solution. Membrane current was measured with pipette and membrane capacitance cancellation, sampled at 5 ms and filtered at 200 Hz by an EPC-9 amplifier, and PULSE software (HEKA/Instrutech, Port Washington, NY). The whole-cell access resistance was typically 6–15 MΩ. Cells were placed in a 500-μl perfusion chamber through which solution flowed at 1–2 ml/min. Inflow to the chamber was by gravity from several reservoirs, selectable by activation of solenoid valves (ValveLink 8; Automate Scientific, San Francisco, CA). Bath solution exchange was complete by < 30 s.

To evaluate the amplitude of KCNQ currents, Chinese hamster ovary (CHO) cells were held at 0 mV, and 500-ms hyperpolarizing steps to –60 mV, followed by a 600-ms pulse back to 0 mV, were applied every 3 s. The amplitude of the current in CHO cells was usually defined as the outward current at the holding potential sensitive to 50 μM linopirdine. In the case of KCNQ1/KCNE1 we used 2-s pulses to –60 mV followed by a 4-s pulse back to 0 mV. M currents in SCG cells were studied by holding the membrane potential at –25 mV and applying a 500-ms hyperpolarizing pulse to –60 mV every 3 s. M-current amplitude was measured at –60 mV from the decaying time course of the linopirdine-sensitive deactivating current. All results are reported as mean ± SEM. The open probability (P_o) of the KCNQ3_N/4_C-2 chimera was evaluated in cell-attached configuration as described previously (Li *et al.*, 2004), except that recordings were always filtered at ≥500 Hz.

Ca^{2+} Imaging

For simultaneous patch-clamp recording and Ca^{2+} imaging, CHO cells were bath-loaded with 2 μM fura-2 acetoxymethyl ester for 30 min at 37°C in the presence of 0.01% pluronic acid. After gigaseal formation, single images at 340

and 380 nm were acquired for background correction. After dialysis for 5–10 min, the imaging protocol was triggered in synchrony by PULSE software. Fluorescent microscopy was performed with an inverted Nikon Eclipse TE300 microscope with an oil-immersion 40×/1.30 numerical aperture objective. A Polychrome IV monochromator (T.I.L.L. Photonics, Martinsreid, Germany) was used as the excitation light source and FURA2 71000 and FITC HQ 96170M filter cubes (Chroma Technology, Brattleboro, VT) were used for fura-2 and green fluorescent protein (GFP) imaging, respectively. Cells were excited alternatively at 340 and 380 nm (50–200 ms every 2 s), and the fluorescence emission collected by an IMAGO 12-bit cooled charge-coupled device camera, and images were stored/analyzed with TILLvisION 4.0 software. Ratiometric data were calibrated to $[\text{Ca}^{2+}]_i$ by using the equation $[\text{Ca}^{2+}]_i = K^* (R - R_{\min}) / (R_{\max} - R)$, where R is the 340/380 nm fluorescence ratio and $R_{\min}$ and $R_{\max}$ are the ratios of Ca^{2+} -free and Ca^{2+} -bound dye, respectively (Grynkiewicz *et al.*, 1985). $R_{\min}$ was measured from cells perfused with a 10 mM EGTA-buffered Ca^{2+} -free solution containing 20 μM ionomycin for 15 min; $R_{\max}$ was measured in the normal 2 mM Ca^{2+} -containing buffer with saturating concentration of ionomycin, and K^* was measured from cells perfused with 10 mM EGTA-buffered 100 μM free $[\text{Ca}^{2+}]_i$ solution containing 20 μM ionomycin for 15 min. The calculated values for $R_{\min}$, $R_{\max}$, and K^* were 0.12, 1.6, and 0.6 μM, respectively (n = 4–5 cells for each).

Biotinylation of Cell Surface Protein and Immunoblotting

Cells were grown in 100-mm culture dishes and individually transfected with myc-tagged KCNQ2–5 and either wt or DN CaM. Biotinylation assays were performed as described previously (Li *et al.*, 2004).

Protein Purification and Glutathione S-Transferase (GST) Pull-Down Assay

KCNQ2ct (KCNQ2 322–841) was generated by PCR and inserted into the pGEX-KG bacterial expression vector by using *Eco*R1/*Tth*III, and transformed into BL21 bacteria. Bacterial pellets were lysed in 10 ml of lysis buffer (100 mM KCl and 50 mM Tris, pH 7.6) with a 60 Sonic Dimembrator (Fisher Scientific, Pittsburgh, PA) five times (1 min on, 1 min off) on ice. Before each sonication, 20 μl of each of the following protease inhibitors was added to the cultures: pepstatin (1.4 mg/10 ml; Sigma-Aldrich, St. Louis, MO) and leupeptin (0.95 mg/10 ml; Sigma-Aldrich) made fresh in methanol, phenylmethylsulfonyl fluoride (17 mg/ml; Sigma-Aldrich) made fresh in acetone, for a total for each inhibitor of 100 μl per 10 ml of bacterial culture. After lysing with the sonicator, Triton X-100 (1%; Sigma-Aldrich) was added. Proteins were collected by spinning down at 16,000 rpm for 45 min at room temperature (RT), and the supernatant containing the proteins titrated to pH 7.6. Supernatants were incubated with 2 ml of immobilized glutathione beads (Pierce Chemical, Rockford, IL) on a rotator at 4°C overnight. Beads were spun down at 4000 rpm for 5 min and the supernatant discarded, and the beads washed three times with lysis buffer. Protein expression and protein-bound glutathione beads were confirmed using 10% SDS-PAGE. GST pull-down experiments were performed using 30 μl of the protein/bead slurry, to which was added 50 μM NEM, or only buffer, in 400 μl of pull-down buffer (50 mM HEPES, 50 mM NaCl, 10% glycerol, 1 mM EDTA, and 2 mM CaCl_2). Samples were rotated for 30 min at RT, after which 0.5 μg of purified calmodulin (kindly given to us by Bettie Sue Masters, Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX) was added and the incubations placed on a rotator for 2–3 h at 4°C. The beads were rinsed 3 × with pull-down buffer, run on a 15% SDS-PAGE gel, transferred to nitrocellulose, and immunoblotted with mouse anti-calmodulin (Research Diagnostics, Flanders, NJ).

Solutions and Materials

The external solution used to record KCNQ currents in CHO cells contained 160 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , and 10 mM HEPES (pH 7.4 with NaOH). In experiments in which we lowered $[\text{Ca}^{2+}]_i$, Ca^{2+} was omitted from the bathing solution and 10 mM EGTA added. The regular pipette solution for perforated patch contained 160 mM KCl, 5 mM MgCl_2 , 5 mM HEPES, and 100–120 ng/ml amphotericin B (pH 7.4 with KOH). Reagents were obtained as follows: fura-2 AM, ionomycin, and Pluronic acid (Molecular Probes, Eugene, OR); DMEM, fetal bovine serum, nerve growth factor, and penicillin/streptomycin (Invitrogen); linopirdine and oxotremorine-M (Sigma-Aldrich); and bradykinin and amphotericin B (Calbiochem, San Diego, CA). Anti-myc and anti-Fra-2 antibodies were purchased from BD Biosciences Clontech (Palo Alto, CA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

RESULTS

Calmodulin-mediated Calcium Sensitivity Is Not Uniform among the KCNQ Channel Family

To investigate the Ca^{2+} /CaM sensitivity of different KCNQ homomultimers, we individually overexpressed KCNQ1–5

Figure 1. Effect of calmodulin overexpression on the current density of KCNQ channels. Bars show the mean current density (pA/pF) of KCNQ1–5 measured at 0 mV in cells transfected with channels only (Control) or together with wild-type (wt CaM) or dominant-negative CaM (DN CaM). For each of these three conditions, $n = 20$, 11, and 4 for KCNQ1; 22, 13, and 9 for KCNQ2; 16, 13, and n.d. for KCNQ3; 21, 23, and 13 for KCNQ4; and 14, 14, and 9 for KCNQ5. In all figures *, **, and *** indicate significance at the $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively (two-tailed Student's *t* test). § Taken from Gamper and Shapiro (2003).

subunits in CHO cells with or without wt or DN CaM and performed perforated patch recordings to evaluate two measures of sensitivity to $[Ca^{2+}]_i$: 1) tonic current density at resting $[Ca^{2+}]_i$ and 2) the response of whole-cell currents to manipulations of $[Ca^{2+}]_i$ induced by bath perfusion of 5 μ M ionomycin. Similar to its effect on KCNQ2/3 heteromultimers (Gamper and Shapiro, 2003), wt CaM significantly reduced the current density of KCNQ2, KCNQ4, and KCNQ5 (Figure 1). The most dramatic effect was observed for the case of KCNQ2 for which the current density in cells overexpressing wt CaM was 0.7 ± 0.2 pA/pF ($n = 13$) compared with 4.1 ± 0.7 pA/pF ($n = 22$, $p \leq 0.001$) in cells without any CaM overexpression. For KCNQ4 and KCNQ5, wt CaM reduced the current density from 62.3 ± 6.9 ($n = 23$) and 77.2 ± 9.6 pA/pF ($n = 14$) to 36.7 ± 6.9 ($n = 25$, $p \leq 0.01$) and 37.4 ± 6.2 pA/pF ($n = 14$, $p \leq 0.01$), respectively. In contrast, there was no effect of wt CaM overexpression on KCNQ1 and KCNQ3. For KCNQ1, the current densities without and with CaM were 11.6 ± 1.6 pA/pF ($n = 20$) and 10.0 ± 2.2 ($n = 10$), respectively. For KCNQ3, the current density of 6.7 ± 1.3 pA/pF ($n = 16$) in control cells was not further reduced by wt CaM coexpression (6.1 ± 0.7 pA/pF, $n = 13$). Coexpression of DN CaM had no effect on current densities for any of the channels (Figure 1).

Previous results have demonstrated that neither changes in $[Ca^{2+}]_i$ nor overexpression of calmodulin changes the voltage dependence of activation (Wen and Levitan, 2002) or activation/deactivation kinetics (Gamper and Shapiro, 2003) of KCNQ2/3 heteromultimers. Here, we analyzed the effect of wt CaM overexpression on these parameters for KCNQ1 and KCNQ4, which display differing sensitivity to wt CaM overexpression (wt CaM overexpression reduced the current density of KCNQ4 but not KCNQ1). In accord with previous findings, wt CaM overexpression did not induce significant changes in the voltage-dependent parameters measured. Thus, the time constants (τ) of activation (at 0 mV) and deactivation (at -60 mV) and the half-maximum activation voltage ($V_{1/2}$) for KCNQ4 expressed alone were 124 ± 19 ms ($n = 10$), 114 ± 8 ms ($n = 9$), and -28.2 ± 0.6 mV ($n = 8$), respectively. For KCNQ4 expressed together with wt CaM, these parameters were 127 ± 24 ms ($n = 17$), 113 ± 12 ms ($n =$

17), and -24.4 ± 1.0 mV ($n = 15$). For KCNQ1 expressed alone, the τ for activation and deactivation and $V_{1/2}$ were 206 ± 15 ms ($n = 10$), 365 ± 55 ms ($n = 10$), and -17.2 ± 0.6 mV ($n = 10$), respectively, and for KCNQ1 together with wt CaM, they were 218 ± 15 ms ($n = 10$), 413 ± 56 ms ($n = 10$), and -15.1 ± 1 mV ($n = 7$), respectively.

We combined whole-cell recording with simultaneous Ca^{2+} imaging to directly evaluate the Ca^{2+} sensitivity of KCNQ1–5 channels. Because many KCNQ homomultimers, and especially KCNQ1 (Loussouarn *et al.*, 2003), display significant rundown during whole-cell recording, we used perforated patch measurements on CHO cells bath loaded with fura-2 as the AM ester. Given that calibration of esterified indicator dyes is often imprecise (Zhou and Neher, 1993), we here report Ca^{2+} signals as the difference in the 340/380 nm fluorescence emission ratio. A calibration estimated elevations of R340/380 from 0.25 to 0.5, routinely observed with bath application of 5 μ M ionomycin, as an increase of $[Ca^{2+}]_i$ from 57 to 203 nM (see *Materials and Methods*). The magnitude of the elevation $[Ca^{2+}]_i$ by 5 μ M ionomycin was consistent with that observed earlier (Gamper and Shapiro, 2003).

Shown in Figures 2 and 3 are the results of such recordings from CHO cells expressing different KCNQ channels together with wt CaM. We applied 5 μ M ionomycin in either regular bath solution containing 2 mM Ca^{2+} , or in an EGTA-buffered "0 Ca^{2+} " solution, to raise or lower $[Ca^{2+}]_i$ in a controlled manner. Experiments revealed little or no response to $[Ca^{2+}]_i$ rises for KCNQ1 and KCNQ3 (Figure 2) but a significant response for KCNQ4 and KCNQ5 (Figure 3). Thus, the ionomycin-induced $[Ca^{2+}]_i$ rise of the fluorescence 340/380 nm ratio from 0.15 ± 0.02 to 0.40 ± 0.06 (KCNQ1, Figure 2A, $n = 7$) and from 0.22 ± 0.04 to 0.51 ± 0.04 (KCNQ3, Figure 2C, $n = 8$) failed to produce any significant inhibition of KCNQ1 or KCNQ3 currents. It was recently suggested that KCNQ1 channels expressed in *Xenopus* oocytes can be activated by $[Ca^{2+}]_i$, but only when coexpressed with its auxiliary subunit KCNE1 that reconstitutes the cardiac I_{Ks} K^+ current (Boucherot *et al.*, 2001). To ask whether the sensitivity of KCNQ1 to Ca^{2+} /CaM depends on the presence of KCNE1 in a mammalian expres-

Figure 2. CaM does not confer Ca^{2+} sensitivity to KCNQ1 or KCNQ3. CHO cells were cotransfected with wt CaM and either KCNQ1 alone (A), KCNQ1 together with KCNE1 (B), or KCNQ3 (C). Currents were recorded as described in *Materials and Methods* from pulses delivered every 3 s, whereas $[\text{Ca}^{2+}]_i$ was simultaneously monitored from fluorescence of fura-2 bath loaded as the AM ester. Left, plots show the current amplitudes (filled circles) and the ratio of fluorescence intensities at 340/380 nm ($R_{340/380}$, red line). Bath solutions containing 5 μM ionomycin in either the 2 mM (black bar) or 0 Ca^{2+} (open bar) solutions, or the KCNQ-channel blocker 50 μM linopirdine (LP, hatched bar) were applied during the periods indicated by the bars. Representative current traces taken at the times indicated by the arrows are shown in the insets. Bars on the right summarize relative changes in current amplitude (black columns) induced by the application of the 2 Ca^{2+} ionomycin solution. Gray columns represent changes in $R_{340/380}$.

sion system, we coexpressed KCNQ1, KCNE1, and wt CaM in CHO cells (Figure 2B). For KCNQ1/KCNE1, $[\text{Ca}^{2+}]_i$ rises from $R_{340/380}$ 0.15 ± 0.01 – 0.40 ± 0.04 ($n = 4$) did not produce any measurable changes in current amplitude.

In contrast, KCNQ4 or KCNQ5, when coexpressed with wt CaM, displayed significant sensitivity to ionomycin-induced $[\text{Ca}^{2+}]_i$ rises (Figure 3). For KCNQ4, rises of $[\text{Ca}^{2+}]_i$ from $R_{340/380}$ 0.20 ± 0.03 to 0.46 ± 0.09 decreased the current amplitude by $66.8 \pm 14.2\%$ ($p \leq 0.01$, $n = 6$), and for KCNQ5, rises of $[\text{Ca}^{2+}]_i$ from $R_{340/380}$ 0.24 ± 0.04 to 0.59 ± 0.06 decreased the current amplitude

by $52.6 \pm 15.5\%$ ($p \leq 0.01$, $n = 6$). Coexpression of KCNQ4 and KCNQ5 with DN CaM resulted in channels that were insensitive to the ionomycin-induced $[\text{Ca}^{2+}]_i$ rises (data not shown). We found the reversibility of the inhibition of the KCNQ4 or KCNQ5 currents induced by rises in $[\text{Ca}^{2+}]_i$ to be variable and strongly dependent upon our ability to “washout” $[\text{Ca}^{2+}]_i$ upon switching to the 0 Ca^{2+} bathing solution for any given experiment. Possibly, prolonged exposure of the channels to abnormally high $[\text{Ca}^{2+}]_i$ may induce a secondary effect of unknown mechanism.

Figure 3. CaM confers high Ca^{2+} sensitivity to KCNQ4 and KCNQ5. CHO cells were cotransfected with wt CaM and KCNQ4 (A) or KCNQ5 (B). All other conditions and abbreviations are as in Figure 2.

Because we wished to ask whether lowering $[\text{Ca}^{2+}]_i$ from resting levels would cause an expected increase in the current in cells coexpressed with wt CaM (which might have tonic Ca^{2+} /CaM-mediated inhibition), we asked, in a separate set of experiments, if lowering resting $[\text{Ca}^{2+}]_i$ by the perfusion of the 0 Ca^{2+} solution could increase current from a Ca^{2+} /CaM-sensitive channel in cells either expressing wt CaM or no exogenous CaM (Figure 4). In accord with the above-mentioned experiments, perfusion of the cells expressing KCNQ4 and wt CaM with the 0 Ca^{2+} solution induced a run-up of the current to $138 \pm 18\%$ ($n = 5$) of control. No significant change was observed in cells expressing KCNQ4 only. These data are in agreement with recent work (Chambard and Ashmore, 2005) showing that clamping $[\text{Ca}^{2+}]_i$ in CHO cells near 100 nM induces rapid run-down of KCNQ4 current, whereas this run-down was not observed if $[\text{Ca}^{2+}]_i$ was clamped at <1 nM. For KCNQ2, direct measurements of Ca^{2+} sensitivity, similar to those shown in Figures 2 and 3, were severely compromised: overexpressed in the cell, the channels need exogenous CaM overexpressed as well to acquire Ca^{2+} sensitivity (Gamper and Shapiro, 2003), because there is likely little unbound CaM freely available (Persechini and Stemmer, 2002). However, coexpression of wt CaM with KCNQ2 induced tonic inhibition of the current to such an extent that it became impossible to further investigate its Ca^{2+} dependence. When coexpressed with wt CaM, KCNQ2 channels produced very small currents (well under 100 pA) that were not feasible to directly evaluate using these kinds of experiments.

The Ca^{2+} /CaM Sensitivity of KCNQ Channels Is Determined by the Channel Carboxy Terminus

All five KCNQ channel subunits contains two IQ-like domains in their carboxy termini and can bind CaM in vitro

Figure 4. Lowering of resting $[\text{Ca}^{2+}]_i$ in CHO cells induces run-up of KCNQ4 current. Cells transfected with KCNQ4 alone (open circles, $n = 6$) or with KCNQ4 together with wt CaM (black circles, $n = 5$) were perfused with 0 Ca^{2+} solution during the time indicated by the bar.

(Wen and Levitan, 2002; Yus-Najera *et al.*, 2002; Gamper and Shapiro, 2003), although interactions of CaM with KCNQ1 are apparently less prominent (Yus-Najera *et al.*, 2002; Lousouarn *et al.*, 2003). Because KCNQ1 and KCNQ3 do not display significant Ca^{2+} sensitivity, it is of interest to know what parts of the channel confer this subunit-specific sensitivity. We investigated the structural parts of the channels that confer sensitivity to Ca^{2+} /CaM by using a chimeric approach (Figures 5 and 6). The first chimera, KCNQ1_N/4_C (Figure 5A), has most of the carboxy terminus (including the second IQ-like domain) of the highly Ca^{2+} -sensitive KCNQ4 attached to the rest of KCNQ1, which does not display sensitivity to physiologically relevant rises in $[\text{Ca}^{2+}]_i$ (Figure 2). The chimeric channels displayed large macroscopic currents, similar to wt KCNQ4, but manifested the slight inactivation at positive voltages (Figure 5B) diagnostic of KCNQ1 (Seebold *et al.*, 2001). However, unlike KCNQ1, the chimeric channels displayed a sensitivity to ionomycin-induced $[\text{Ca}^{2+}]_i$ rises similar to that observed for wt KCNQ4 (Figure 5, B and C). Rises of $[\text{Ca}^{2+}]_i$ from 0.22 ± 0.02 to 0.48 ± 0.05 decreased the current amplitude by $46.8 \pm 6.4\%$ ($p \leq 0.001$, $n = 7$). The decrease in the current upon raising $[\text{Ca}^{2+}]_i$ was mostly reversed when the 0 Ca^{2+} solution was subsequently perfused. The second set of chimeras contained the backbone of KCNQ3 (that also seems to be Ca^{2+} insensitive) with parts of the KCNQ3 carboxy terminus substituted by two homologous parts of the KCNQ4 carboxy terminus of different lengths (Figure 6). The first chimera, KCNQ3_N/4_C-1, is the same as that examined by us at the single-channel level previously, contains most of the KCNQ4 carboxy terminus, and displays many properties of KCNQ4, including large whole-cell currents and low maximal P_o in cell-attached patches ($P_o = 0.02 \pm 0.01$ at 0 mV; Li *et al.*, 2004). Accordingly, this chimera displayed sensitivity to ionomycin-induced $[\text{Ca}^{2+}]_i$ rises similar to that of KCNQ4, unlike KCNQ3 (Figure 6A). Rises of $[\text{Ca}^{2+}]_i$ from 0.29 ± 0.04 to 0.53 ± 0.07 decreased the current amplitude by $61.8 \pm 15.7\%$ ($p \leq 0.001$, $n = 6$). The decrease

Figure 5. The carboxy terminus of KCNQ4 confers Ca^{2+} /CaM sensitivity to otherwise insensitive KCNQ1. (A) Diagram of the KCNQ1_N/4_C chimera, consisting of most of the KCNQ4 C terminus grafted onto the rest of KCNQ1. The thick bars in the amino and carboxy termini indicate regions strongly conserved among KCNQ1–5 channels. Below shown are typical currents recorded from a cell cotransfected with wild-type KCNQ1 (left) or chimeric KCNQ1_N/4_C channels (right). Shown are superimposed traces of the currents evoked by a family of 500-ms voltage pulses from -80 to 40 mV in 10 -mV increments from a holding potential of -60 mV.

in the KCNQ3_N/4_C-1 current upon raising $[\text{Ca}^{2+}]_i$ was nearly fully reversed upon subsequent perfusion of the cell with the 0 Ca^{2+} solution. The second chimera, KCNQ3_N/4_C-2, contains a more distal part of the KCNQ4 carboxy terminus (see *Materials and Methods*). It displayed properties more similar to that of KCNQ3, with very small whole-cell currents and a high maximal P_o of 0.65 ± 0.18 at 0 mV ($n = 4$; Figure 6B). The KCNQ3_N/4_C-2 chimera, like wt KCNQ3, displayed no response to ionomycin-induced $[\text{Ca}^{2+}]_i$ rises. Thus, rises of $[\text{Ca}^{2+}]_i$ from $R_{340/380}$ 0.30 ± 0.02 to 0.57 ± 0.05 failed to change current amplitudes (current amplitude at the elevated $[\text{Ca}^{2+}]_i$ was $102 \pm 7.5\%$ of control, $n = 6$; Figure 6B). Together, these results from the chimeric channels suggest that the Ca^{2+} /CaM sensitivity of a given KCNQ channel is determined by the central part of the carboxy terminus, between the first and second regions of high sequence similarity. The results from KCNQ3, KCNQ4, and the two KCNQ3/Q4 chimeras further suggest that the sensitivity of the channels to Ca^{2+} /CaM correlates with their maximal P_o (see *Discussion*).

The Amino-Lobe of CaM Is Critical for KCNQ Ca^{2+} Sensitivity

CaM has two functionally distinct Ca^{2+} -binding regions that are in its amino- and carboxy-terminal lobes that have been shown to have distinct roles in channel modulation (De-Maria *et al.*, 2001; Liang *et al.*, 2003). To test for lobe specificity of CaM actions on KCNQ channels, we used mutant CaM with selectively impaired Ca^{2+} binding to its amino- (D20A and D56A) or carboxy-terminal (D93A and D129A) lobes. We tested for lobe specificity in CaM-mediated modulation of KCNQ4 in these experiments because we find KCNQ4 to be highly Ca^{2+} /CaM sensitive and as a homomultimer has the most robust expression in CHO cells. In cells coexpressed with KCNQ4 and amino-lobe mutant CaM, the channels were virtually insensitive to $[\text{Ca}^{2+}]_i$ rises (Figure 7). Rises of $[\text{Ca}^{2+}]_i$ from $R_{340/380}$ 0.23 ± 0.02 to 0.45 ± 0.08 produced no change in current amplitude, with a mean current at elevated $[\text{Ca}^{2+}]_i$ that was $97.0 \pm 13.4\%$ of control (Figure 7A; $n = 7$). Thus, amino-lobe mutant CaM was as ineffective at conferring Ca^{2+} sensitivity to KCNQ4 as is DN CaM (with all four EF hands mutated) to KCNQ2/3 heteromultimers (Gamper and Shapiro, 2003). In sharp contrast with these results, KCNQ4 currents in cells coexpressing carboxy-lobe mutant CaM (Figure 7B) displayed a Ca^{2+} sensitivity similar to that observed in the presence of wt CaM in Figure 3A. Rises of $[\text{Ca}^{2+}]_i$ from $R_{340/380}$ 0.24 ± 0.03 to 0.56 ± 0.10 resulted in the inhibition of current amplitude by $64.2 \pm 9.5\%$ ($p \leq 0.01$, $n = 6$). These data suggest that the amino lobe of CaM is required to confer Ca^{2+} sensitivity to KCNQ channels and that Ca^{2+} binding to the carboxy lobe of CaM is likely not required.

Ca^{2+} /CaM Inhibition of KCNQ Channels Does Not Involve Reduction in Channel Surface Abundance

It has been shown that the reduction of channel P_o underlies the effects of $[\text{Ca}^{2+}]_i$ on M channels in sympathetic neurons (Selyanko and Brown, 1996a,b). We have interpreted the effect of wt CaM overexpression on the tonic current density

Figure 5 (cont). (B) Simultaneous patch-clamp/ Ca^{2+} imaging experiment, similar to those shown in Figures 2 and 3 recorded from the cell transfected with KCNQ1_N/4_C together with wt CaM. (C) Bars summarize relative changes in current amplitude (black columns) induced by the application of the 2 Ca^{2+} ionomycin solution. Gray columns represent the changes in $R_{340/380}$.

Figure 6. KCNQ3/4 chimeras reveal a central region in the carboxy terminus to determine Ca^{2+} /CaM sensitivity. KCNQ3_N/4_C-1 chimera (A) with carboxy terminus of KCNQ4 starting at Q454 and KCNQ3_N/4_C-2 chimera (B) with carboxy terminus of KCNQ4 starting at D525 are tested for Ca^{2+} /CaM-sensitivity in simultaneous patch-clamp/ Ca^{2+} imaging experiments, similar to those shown in Figures 2 and 3. Cells were transfected individually with each chimera together with wt CaM. Bars on the right summarize relative changes in current amplitude (black columns) induced by the application of the 2 Ca^{2+} ionomycin solution. Gray columns represent the changes in $R_{340/380}$ for both chimeras. Insets show single channel recordings done in cell-attached configuration at 0 mV from CHO cells transfected with chimeras only. Data shown in the top inset are taken from Li *et al.* (2004).

Figure 7. The amino-terminal lobe of CaM mediates Ca^{2+} sensitivity of the KCNQ/CaM complex. CHO cells were cotransfected with KCNQ4 and either the amino-terminal (A) or carboxy-terminal (B) lobe-specific mutants of CaM (see text). All other conditions and abbreviations are as in the Figures 2 and 3.

of Ca^{2+} -sensitive KCNQ channels as resulting from the mass action of increased cytoplasmic $[\text{CaM}]$ at resting $[\text{Ca}^{2+}]_i$ (Gamper and Shapiro, 2003). This conclusion is consistent with the lack of effect of the Ca^{2+} -insensitive CaM mutant (DN CaM; Figure 1) and with current run-up induced by the perfusion of 0 Ca^{2+} solution (Figure 4), and it is likely that the effect of wt CaM overexpression is a tonic voltage-independent reduction of channel P_o . However, an alternative possibility is that CaM overexpression could reduce expression of the channels at the plasma membrane. To investigate this, we performed biotinylation assays to specifically label KCNQ channels in the cell membrane. We compared biotinylated (cell surface) KCNQ channel protein in control cells coexpressed with wt CaM to that in cells coexpressed with DN CaM. Myc-tagged KCNQ2–5 channels were individually expressed in CHO cells. After labeling by biotin, cell surface proteins were isolated by allowing them to bind to streptavidin-coated beads. The biotinylated proteins were separated by SDS-PAGE and transferred to nitrocellulose. Anti-myc antibodies specifically labeled the channels at the molecular masses of ~100 kDa for KCNQ2, 110 kDa for KCNQ3, 80 kDa for KCNQ4, and 125 kDa for KCNQ5. Shown in Figure 8A are immunoblots of cell surface (top row) and total lysate (bottom row) from cells coexpressed with myc-tagged channels and either wt CaM or DN CaM (indicated by the bar on the top). The inset shows that no biotinylation of the cytosolic protein Fra-2 is detected using this method, showing specificity of the labeling for membrane proteins. Figure 8B summarizes the ratios

Figure 8. CaM coexpression does not change the plasma membrane abundance of KCNQ channels. Surface expression of homomeric myc-tagged KCNQ2–5 channels coexpressed with either wt CaM or DN CaM (as indicated by the top bar) was evaluated by biotinylation labeling. (A) Shown are the biotinylated proteins (top) and total protein (bottom). Inset shows labeling of the cytosolic protein Fra-2 used as a control. Bars summarize ratios of biotinylated KCNQ protein abundance in cells coexpressing wt CaM divided by that of cells coexpressing DN CaM; $n = 3$.

of biotinylated KCNQ protein abundance in cells coexpressing wt CaM divided by that of cells coexpressing DN CaM. The ratios for KCNQ2–5 were 0.79 ± 0.15 , 1.00 ± 0.36 , 1.07 ± 0.13 , and 1.03 ± 0.20 , respectively, revealing no difference in relative cell surface expression of the channels between cells coexpressed with wt CaM and those coexpressed with DN CaM. We conclude that the effect of wt CaM overexpression on KCNQ channels is not due to reduced surface abundance at the cell membrane but more likely due to a voltage-independent reduction in channel P_o .

Calmodulin Competes for the Site of Action with a KCNQ Channel Opener

The cysteine-alkylating agent NEM has been shown to augment opening of KCNQ channels in a subunit-specific way (Roche *et al.*, 2002; Li *et al.*, 2004). Sequence alignment indicates that the cysteine that is the site of action of NEM alkylation (shown for KCNQ4; Li *et al.*, 2004), and a binding site of CaM (shown for KCNQ2 and KCNQ3; Wen and

Levitan, 2002; Yus-Najera *et al.*, 2002; Gamper and Shapiro, 2003) to be in proximity. Thus, we used NEM as a probe for the site of CaM action on KCNQ channels and asked whether NEM augmentation and Ca^{2+} /CaM inhibition of their currents are competitive. We treated CHO cells coexpressed with KCNQ2 and wt CaM with 50 μM NEM, a concentration that strongly augments currents of KCNQ2, KCNQ4, and KCNQ5 (but not KCNQ1 or KCNQ3). NEM had only a modest effect on the KCNQ2 current in such cells. Even less NEM action was observed in cells coexpressed with KCNQ2 and DN CaM, but in control cells expressed only with KCNQ2 channels, NEM potently augmented the current (Figure 9, A–D). NEM increased the KCNQ2 current 3.9 ± 0.4 -fold ($n = 10$), whereas in cells cotransfected with wt or DN CaM, the increase was only 2.2 ± 0.3 -fold ($p \leq 0.01$, $n = 12$) or 1.6 ± 0.1 -fold ($p \leq 0.001$, $n = 9$), respectively. If NEM alkylation and CaM binding are competitive, then we might expect the NEM dose-response relation to be altered in cells overexpressed with CaM, and this was indeed the case. In control cells, NEM augmented the KCNQ2 current with an EC_{50} of 14.4 ± 6.3 μM ($n = 4$ – 6 for each NEM concentration) and a Hill coefficient of 1.1 ± 0.4 (data are from Li *et al.*, 2004), whereas when KCNQ2 was coexpressed with wt CaM, the EC_{50} of NEM action was shifted to 75.2 ± 12.3 μM (Hill coefficient 0.9 ± 0.1 , $n = 4$ – 6). This shift suggests that, at least in part, the actions of CaM and NEM are competitive.

We also tested whether CaM overexpression would similarly reduce NEM action for the other NEM-sensitive channels, KCNQ4 and KCNQ5 (Figure 9, F and G). These two channels are also Ca^{2+} /CaM sensitive (Figure 3). In control cells, NEM increased the KCNQ4 and KCNQ5 current 1.9 ± 0.3 - ($n = 8$) and 2.1 ± 0.3 -fold ($n = 5$), respectively, whereas in cells in which KCNQ4 and KCNQ5 were coexpressed with wt CaM, they were only 1.3 ± 0.1 ($p \leq 0.05$, $n = 8$) and 1.1 ± 0.3 ($p \leq 0.05$, $n = 5$), and in the case when channels were coexpressed with DN CaM, they were 1.1 ± 0.1 ($p \leq 0.05$, $n = 8$) and 0.8 ± 0.1 ($p \leq 0.01$, $n = 5$).

Interestingly, the patterns of NEM and Ca^{2+} /CaM sensitivity are apparently the same among KCNQ channels, with KCNQ1 and KCNQ3 being insensitive to both. We asked whether the NEM sensitivity of the KCNQ1_N/4_C, KCNQ3_N/4_C-1, and KCNQ3_N/4_C-2 chimeras that we used to localize the structural determinants of Ca^{2+} /CaM sensitivity also correlates with their Ca^{2+} /CaM sensitivity. Ca^{2+} -sensitive KCNQ3_N/4_C-1 was previously shown to be also NEM sensitive (Li *et al.*, 2004), so we tested the other two chimeras. Consistent with this pattern, the Ca^{2+} -sensitive chimera, KCNQ1_N/4_C also displayed NEM sensitivity, with an increase of current amplitudes (at 0 mV) to $166.5 \pm 17.2\%$ of control ($p \leq 0.01$, $n = 6$) after 5 min of 50 μM NEM treatment (data not shown). In contrast, the Ca^{2+} -insensitive chimera KCNQ3_N/4_C-2, similar to wt KCNQ3, displayed no NEM sensitivity, with NEM treatment resulting in current amplitude only $112.6 \pm 13.1\%$ of control ($n = 6$; data not shown). We therefore conclude that Ca^{2+} /CaM and NEM sensitivity, as well as the P_o of a given KCNQ channel, are likely determined by the same part of channel carboxy terminus. We also tested whether the mutation of C519A of KCNQ4 that abolishes most of the NEM effect would reduce Ca^{2+} /CaM sensitivity of KCNQ4, but this seemed to not be the case. For KCNQ4 C519A, rises of $[\text{Ca}^{2+}]_i$ from $R340/380$ 0.21 ± 0.02 to 0.68 ± 0.15 decreased the current amplitude by $53.2 \pm 10.1\%$ ($p \leq 0.01$, $n = 5$).

In the experiments shown in Figure 9, NEM action was protected by an excess of exogenous CaM. We thus sought an experimental protocol in which we could address the

opposite question: can NEM treatment protect the channel from Ca^{2+} /CaM action? We reasoned that bradykinin modulation of the M current in SCG neurons would be an appropriate test of this possibility, because the action is via IP_3 -sensitive $[\text{Ca}^{2+}]_i$ rises (Cruzblanca *et al.*, 1998; Delmas *et al.*, 2002) in concert with CaM (Gamper and Shapiro, 2003). We tested whether treatment of SCG neurons with NEM would reduce bradykinin-induced M current inhibition. As a control, we also tested the effect of NEM pretreatment on stimulation of muscarinic M_1 receptors, which does not rise $[\text{Ca}^{2+}]_i$ in these cells and inhibits M current via a distinct pathway that most likely involves phosphatidylinositol biphosphate depletion (Suh and Hille, 2002; Zhang *et al.*, 2003). Perforated patch recordings were made from rat SCG neurons cultured for 2–5 d, and bradykinin or muscarinic modulation of M currents evaluated. In a control neuron, application of bradykinin caused a strong suppression of the M current, with a slightly greater suppression by the muscarinic agonist oxotremorine (oxo-M) (Figure 10A). Figure 10B shows an experiment in which the SCG neuron was pretreated with 50 μM NEM for 2.5 min before bradykinin application. As previously reported (Roche *et al.*, 2002), NEM increased the M-current amplitude. Subsequent application of bradykinin induced only a very modest inhibition of the current, but that produced by subsequent application of oxo-M was large. In non-NEM-treated control cells, 150 nM bradykinin inhibition was $84 \pm 5\%$, and subsequent application of oxo-M increased this suppression to $94 \pm 2\%$ ($n = 6$; Figure 10C). However, for cells pretreated with NEM, bradykinin inhibition was only $40 \pm 8\%$ ($p \leq 0.01$, $n = 12$), whereas muscarinic modulation was still robust, $84 \pm 6\%$ ($n = 12$).

These results suggest that NEM alkylation interferes with the actions of CaM on the channel. Because we suggest that this may be due to a physical competition for sites very near each other on the channel protein, we turned to biochemical experiments to probe whether NEM alkylation would block binding of CaM to the relevant carboxy-terminal part of the channels. A GST-fusion protein of the carboxy terminus of KCNQ2 (Q2ct) was prepared and used in pull-down assays to evaluate its ability to bind purified CaM. The GST-fusion proteins were either preincubated with 50 μM NEM for 30 min, or with only buffer, before incubation with 0.5 μg of purified CaM protein. The samples were then run under denaturing SDS-PAGE, and the immunoblots probed with anti-CaM antibodies. Densitometry of the immunoblots revealed that NEM pretreatment reduced CaM binding to Q2ct to $33 \pm 16\%$ of control ($p \leq 0.01$, $n = 6$; data not shown). GST itself did not significantly bind CaM. Although NEM might interfere with Q2ct binding to CaM in a non-specific way, unrelated to its alkylation at the putative site of NEM action, our data suggest that NEM alkylation interferes with CaM binding. This would be consistent with CaM and NEM competing for the same or overlapping binding sites on the carboxy terminus of KCNQ2.

DISCUSSION

We here explore the specificity of the family of KCNQ K^+ channels to modulation by Ca^{2+} /CaM. Previous work indicates that overexpression of CaM has two related effects on KCNQ2/3 heteromultimers: 1) a tonic decrease in current density at resting $[\text{Ca}^{2+}]_i$, and 2) inhibition of whole-cell currents by ionomycin-induced $[\text{Ca}^{2+}]_i$ rises (Gamper and Shapiro, 2003). Therefore, we used these two criteria to evaluate the Ca^{2+} sensitivity of KCNQ subunits. We find that homomeric currents from KCNQ2, KCNQ4, and

Figure 9. CaM blunts NEM action on KCNQ2, KCNQ4 and KCNQ5. (A–C). Current traces evoked by a 500-ms voltage pulses from -60 to 0 mV before (*control*) or after 5-min $50 \mu\text{M}$ NEM treatment (NEM) and after $50 \mu\text{M}$ linopirdine block (LP) recorded in cells transfected with KCNQ2 only (A), KCNQ2 with wt CaM (B) or KCNQ2 with DN CaM (C). (D) The data for 10 experiments similar to that shown in A (black circles), 12 experiments as in B (open circles), and nine experiments as in C (gray circles) were pooled and temporarily aligned for the onset of NEM action (start point of NEM treatment is indicated by the arrow). (E) Dose-response curve for NEM action on KCNQ2 currents in wt CaM-overexpressing cells (squares), pooled from four to six experiments for each NEM concentration. The data were fitted by a Hill equation with the values given in the text. Data for KCNQ2 without CaM coexpression (circles) are from Li *et al.* (2004). (F) Pooled time course of NEM action, similar to that in D, but for KCNQ4; $n = 8$ for each condition, labeling as in D. (G) Pooled time course of NEM action, similar to that in D and F, but for KCNQ5; $n = 5$ for each conditions, labeling as in D.

KCNQ5, but not from KCNQ1 and KCNQ3, are reduced when coexpressed with wild-type, but not dominant-negative CaM. In the simultaneous patch-clamp/ Ca^{2+} imaging experiments, currents from KCNQ4 and KCNQ5, but not KCNQ3, KCNQ1, or KCNQ1/KCNE1 channels were sensitive to $[\text{Ca}^{2+}]_i$ rises when coexpressed with wild-type CaM.

Because expression of KCNQ channels is highly tissue- and cell-type specific (Jentsch, 2000), such differential Ca^{2+} sensitivity among the channels generating otherwise very similar currents may provide a basis for the tissue, cell, or even subcellular specificity of Ca^{2+} signaling. The Ca^{2+} /CaM modulation of KCNQ channels is not due to reduced mem-

Figure 10. NEM treatment reduces CaM-mediated modulation of neuronal M channels by bradykinin. M currents were recorded from cultured SCG neurons by using the pulse protocol described in *Materials and Methods*. (A and B) Plotted are the amplitudes of the deactivating time-dependent relaxations at -60 mV from pulses

brane abundance of the sensitive channels. Together with the lack of effect of DN CaM on current density, this result suggests that CaM actions are unlikely to affect assembly or trafficking of KCNQ channels and more likely to result from a voltage-independent regulation of channel P_o .

We then analyzed in further detail the structural requirements of the functional interaction between KCNQ channels and CaM. We find the functional amino-terminal lobe of CaM to be necessary for the Ca^{2+} sensitivity of the channel-CaM complex, whereas mutation of the carboxy-terminal lobe was without effect. The role of CaM as $[Ca^{2+}]_i$ -sensor of voltage-gated Ca^{2+} channels and SK-type Ca^{2+} -activated K^+ channels have been particularly well studied. In the former case, it has been shown that the higher affinity carboxy lobe of CaM mediates regulation dependent on the "local" Ca^{2+} concentration, which in this case reflects Ca^{2+} ions passing through the channel pore, whereas the lower affinity amino lobe detects integral activities of more distal Ca^{2+} sources, thus serving as a "global" Ca^{2+} sensor (DeMaria *et al.*, 2001; Liang *et al.*, 2003). This bifurcation in the role of CaM lobes in regulation of Ca^{2+} channels is in accord with the need to distinguish between feedback by $[Ca^{2+}]$ from opening of the channel/CaM complex itself and by $[Ca^{2+}]$ from global events (Mori *et al.*, 2004). Similar to the case of KCNQ channels found here, the amino lobe of CaM was shown to mediate Ca^{2+} sensing for SK channels (Keen *et al.*, 1999). Because there are no Ca^{2+} ions moving through the pores of K^+ channels, the major role of the CaM amino lobe in mediating modulation of K^+ channels by Ca^{2+} is consistent with the function of CaM in sensing rises of Ca^{2+} released from stores or from global Ca^{2+} channel currents.

In addition, the use of the lower affinity lobe of CaM as the sensor of M channels makes sense as another mechanism of specificity. Thus, incidental Ca^{2+} release, unrelated to receptor stimulation, or nearby transient rises of Ca^{2+} from adjacent voltage-gated Ca^{2+} channels, should be ignored to maintain fidelity in signaling. The mediation of M-channel regulation by the amino-terminal lobe of CaM as the end point of $G_{q/11}$ activation, then, is in accord with those physiological objectives. Importantly, the meaning of "local" and global Ca^{2+} for the case of Ca^{2+} channels is different from that in the context of $G_{q/11}$ signaling in which local Ca^{2+} rises are Ca^{2+} released from intracellular stores colocalized with certain $G_{q/11}$ -coupled receptors (Delmas and Brown, 2002).

In the literature, three groups have shown that CaM can bind to two carboxy-terminal domains (Wen and Levitan, 2002; Yus-Najera *et al.*, 2002; Gamper and Shapiro, 2003) present in all five KCNQ channels. However, we here suggest that only KCNQ2, KCNQ4, and KCNQ5 display CaM-mediated Ca^{2+} sensitivity. What is the origin of this deviation? Intriguingly, the subunit-specificity for the modulation by Ca^{2+} /CaM mirrors that seen for augmentation of KCNQ currents by NEM (Table 1). Thus, currents of KCNQ2, KCNQ4, and KCNQ5, but not of KCNQ1 and KCNQ3 are potentially augmented by NEM (Roche *et al.*, 2002; Li *et al.*, 2004) due to its effect on the channel P_o . The site of NEM action was localized to a cysteine at position 519 in KCNQ4

Figure 10 (cont). given every 3 s for control neurons (A) or NEM-treated neurons (B). NEM ($50 \mu M$), 150 nM bradykinin, or $10 \mu M$ oxo-M were bath applied during the periods shown by the bars. Shown in the insets are representative current traces at the indicated times from these experiments. (C) Bars show mean inhibitions by bradykinin or oxo-M for control cells (black columns, $n = 6$) or NEM-treated cells (gray columns, $n = 12$).

Table 1. NEM- and Ca^{2+} /CaM sensitivity of KCNQ channels display a similar pattern

	Q1	Q2	Q3	Q2/3	Q4	Q5	Q1 _N /4 _C	Q3 _N /4 _C -1	Q3 _N /4 _C -2
Effect of CaM overexpression on current density	–	+++	–	++*	+	+	N/A	N/A	N/A
Ca^{2+} sensitivity in the presence of CaM	Low	?(High)	Low	High*	High	High	High	High	Low
NEM sensitivity	–	+++	–	+	+	+	+	+	–
Effect of CaM overexpression on NEM sensitivity	N/A	+	N/A	N/A	+	+	N/A	N/A	N/A
Maximal open probability (0 mV)	?	Low [†]	High [†]	Low [†]	Low [†]	Low [†]	?	Low [†]	High

N/A, not analyzed.

* Data are from Gamper and Shapiro (2003).

† Data are from Li et al. (2004).

with the cysteines at analogous positions found only in KCNQ2 and KCNQ5, but not in KCNQ1 or KCNQ3. Single-channel recordings showed that KCNQ2, KCNQ4, and KCNQ5 have the relatively low maximal P_o of 0.1–0.2 and that KCNQ3 uniquely has a maximal P_o near unity (Li *et al.*, 2004), suggesting that NEM would have little effect on KCNQ3 even if this channel were to have this cysteine. Indeed, we have mutated KCNQ3 to introduce the appropriate cysteine (N467C) and found that such channels are still NEM insensitive (our unpublished observations).

Among KCNQ2–5, KCNQ3 and KCNQ4 have opposing maximal P_o values (Li *et al.*, 2004). Thus, we tested the hypothesis that maximal channel P_o and Ca^{2+} /CaM sensitivity are correlated using two KCNQ3/KCNQ4 chimeras that contain differing amounts of the KCNQ4 carboxy tail. KCNQ3_N/Q4_C-1, whose maximal P_o was found to be even lower than wt KCNQ4 (Li *et al.*, 2004), is found here to be highly Ca^{2+} /CaM sensitive, whereas we here find KCNQ3_N/Q4_C-2 to have a maximal P_o more like wt KCNQ3 and to be Ca^{2+} /CaM insensitive. Thus, the structural determinants that confer Ca^{2+} /CaM sensitivity seem to overlap with the region that determines maximal P_o . Together, these results suggest two different functional types of KCNQ carboxy termini. The first type results in stabilization of the channel in the open state, yielding a channel that energetically strongly favors opening, and a maximal P_o that cannot be significantly increased by NEM nor decreased by Ca^{2+} /CaM action. This type of carboxy terminus is possessed by KCNQ3. Unfortunately, there are no available data on the P_o of KCNQ1 because this channel has a single-channel conductance <1 pS (Yang and Sigworth, 1998; Pusch *et al.*, 2000). Due to its lack of sensitivity to NEM and Ca^{2+} /CaM, it may be that KCNQ1, like KCNQ3, has a high maximal P_o , a high energy of opening, and a similar functional type of carboxy terminus (conversely, KCNQ1 may have unique gating mechanisms). The second type of carboxy terminus does not stabilize the channel in the open state, and results in channels that much less energetically favor opening. That would be the case for KCNQ2, KCNQ4, and KCNQ5. Thus, the P_o of these channels can be increased severalfold by NEM or decreased by Ca^{2+} /CaM binding. This analysis is summarized in Figure 11, in which we depict the functional roles of these two proposed types of carboxy termini and illustrate their predicted effects.

The observed competition between NEM and CaM suggests that the CaM/KCNQ channel interaction mediating Ca^{2+} inhibition may not be constitutive, especially in the Ca^{2+} -free (“apo-”) form of CaM. We suppose that the typically strong Ca^{2+} dependence of the affinity of CaM for its substrate (Jurado *et al.*, 1999) allows apoCaM to unbind from

a site or sites on the channels when $[\text{Ca}^{2+}]_i$ is low, permitting NEM alkylation to block subsequent Ca^{2+} /CaM action when $[\text{Ca}^{2+}]_i$ rises. Recent work from the Persechini laboratory indicates that there is intense competition for CaM, especially as $[\text{Ca}^{2+}]_i$ increases (Tran *et al.*, 2003; Black *et al.*, 2004). This predicts our observations that CHO cells not cotransfected with any CaM to have insufficient endogenous CaM to partner with the overexpressed channels, leaving most channels to not be CaM bound, and upon $[\text{Ca}^{2+}]_i$ rises, competition for CaM to preclude most channels from binding Ca^{2+} /CaM at the functional modulatory site. However, in cells overexpressing wt or DN CaM, there is sufficient expressed CaM to bind to the channels, resulting in robust tonic Ca^{2+} /CaM inhibition and Ca^{2+} sensitivity in cells expressed with wt CaM, and blockade of Ca^{2+} modulation in those expressed with DN CaM (Gamper and Shapiro, 2003). Heterologously expressed SK K⁺ channels do not require coexpression of wt CaM for Ca^{2+} sensitivity, and this is likely due to their higher affinity for apoCaM than have KCNQ channels (reported harsh denaturing conditions are required to separate CaM from SK channels—not the case for KCNQ). However, SK channels with two residues mutated in their constitutive CaM-binding domain will retain their normal Ca^{2+} sensitivity only if wt CaM is coexpressed (like Ca^{2+} -sensitive KCNQ), presumably because the mutant channels have a lower affinity for apoCaM (Lee *et al.*, 2003).

Another parallel with SK channels seems to be a dual role for CaM in mediating KCNQ channel assembly or expression (Wen and Levitan, 2002; Lee *et al.*, 2003), although we do not observe this phenomenon in our heterologous or SCG neuron systems. The existing biochemical data are inconsistent on whether CaM binding to KCNQ channels is constitutive and, thus, Ca^{2+} independent or, conversely, transient and Ca^{2+} dependent. The coimmunoprecipitation and yeast two-hybrid experiments suggest the former type of interaction (Wen and Levitan, 2002; Yus-Najera *et al.*, 2002; Gamper and Shapiro, 2003), whereas binding of CaM to the channel IQ domains was definitely Ca^{2+} sensitive, because it was demonstrated in gel-shift experiments (Wen and Levitan, 2002; Gamper and Shapiro, 2003). Perhaps KCNQ channels have multiple CaM-binding sites, one of very high affinity that is required for expression of functional channels, and one or more with lower affinity that mediate Ca^{2+} sensing. In that scenario, our CHO cell system has sufficient endogenous CaM to fulfill the first role but requires coexpression of CaM to fulfill the Ca^{2+} -sensing role; and for both CHO cells and SCG neurons, cellular competition for CaM and mass action lead to increased tonic Ca^{2+} /CaM inhibition of KCNQ currents when wt CaM is overexpressed. CaM con-

KCNQ2, 4, 5 **KCNQ1, 3**

Figure 11. Schematic illustration of the functional roles of two types of KCNQ carboxy termini. Depicted are two classes of KCNQ channels, with differing types of carboxy terminus (*ct*) conferring distinct functional properties to the channels. The *ct* is shown as black bars projected down from the body of the channel, and shown in the *ct* in green the region common to binding by Ca^{2+} /CaM (orange spheres and blue horseshoes), and alkylation by NEM. For KCNQ2, KCNQ4, and KCNQ5 (top), we suppose those channels to have a *ct* that confers a relatively low maximal P_o and allows for a robust inhibition by Ca^{2+} /CaM and augmentation by NEM. For KCNQ3 and KCNQ1, we suppose those channels to have a *ct* that confers high P_o and results in channels with little functional response to Ca^{2+} /CaM action or NEM alkylation. Also illustrated is competition between Ca^{2+} /CaM and NEM. We are here depicting such competition at the site(s) on the channel responsible for Ca^{2+} modulation and do not intimate that there may not be another CaM-binding site, perhaps required for assembly, that is unaffected by NEM. NEM action is shown as occurring when $[\text{Ca}^{2+}]_i$ is low, and thus, the site of NEM alkylation is available and does not imply that NEM causes unbinding of CaM from the channel.

centrations in SCG neurons are presumably precisely tuned to allow M-current inhibition by Ca^{2+} /CaM by physiological stimuli such as hormonal stimulation, without undue tonic inhibition.

We do not here attempt to quantitatively model the effects of the observed competition between Ca^{2+} /CaM and NEM, but we do conclude that their competition confirms the proximity of their sites of action. The interplay between NEM and CaM actions observed in the present study is not unique, because modification of cysteines located close to IQ domains or CaM binding sites have been shown to prevent CaM binding to neurogranin (Huang *et al.*, 2000) and the calcium-release ryanodine receptor RYR1 (Porter Moore *et al.*, 1999; Sun *et al.*, 2001). The case of RYR1 seems to be a close analogy to the phenomena observed here because alkylation of a specific cysteine at position 3635 of RYR1 is blocked by CaM binding and, as for the case of KCNQ channels, alkylation of C3635 disrupts CaM binding to the channel. Interestingly, oxidation of C3635 of RYR1 activates the channel by promoting formation of intersubunit disulfide bonds, an effect that can be prevented by CaM binding to the channel (Porter Moore *et al.*, 1999). Thus, the findings presented in this article uncover a novel perspective that may generalize to further understanding the pleiotropic functional roles of many regulatory domains of voltage-gated and ligand-gated ion channels.

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REFERENCES

- Beech, D. J., Bernheim, L., Mathie, A., and Hille, B. (1991). Intracellular Ca^{2+} buffers disrupt muscarinic suppression of Ca^{2+} current and M current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. USA* 88, 652–656.
- Black, D. J., Tran, Q. K., and Persechini, A. (2004). Monitoring the total available calmodulin concentration in intact cells over the physiological range in free Ca^{2+} . *Cell Calcium* 35, 415–425.
- Bofill-Cardona, E., Vartian, N., Nanoff, C., Freissmuth, M., and Boehm, S. (2000). Two different signaling mechanisms involved in the excitation of rat sympathetic neurons by uridine nucleotides. *Mol. Pharmacol.* 57, 1165–1172.
- Boucherot, A., Schreiber, R., and Kunzelmann, K. (2001). Regulation and properties of KCNQ1 ($\text{K}_v\text{LQT1}$) and impact of the cystic fibrosis transmembrane conductance regulator. *J. Membr. Biol.* 182, 39–47.
- Chambard, J. M., and Ashmore, J. F. (2005). Regulation of the voltage-gated potassium channel KCNQ4 in the auditory pathway. *Pflügers Arch.* 450, 34–44.
- Cruzblanca, H., Koh, D. S., and Hille, B. (1998). Bradykinin inhibits M current via phospholipase C and Ca^{2+} release from IP_3 -sensitive Ca^{2+} stores in rat sympathetic neurons. *Proc. Natl. Acad. Sci. USA* 95, 7151–7156.
- del Rio, E., Bevilacqua, J. A., Marsh, S. J., Halley, P., and Caulfield, M. P. (1999). Muscarinic M_1 receptors activate phosphoinositide turnover and Ca^{2+} mobilisation in rat sympathetic neurones, but this signalling pathway does not mediate M-current inhibition. *J. Physiol.* 520, 101–111.
- Delmas, P., and Brown, D. A. (2002). Junctional signaling microdomains: bridging the gap between the neuronal cell surface and Ca^{2+} stores. *Neuron* 36, 787–790.

- Delmas, P., Wanaverbecq, N., Abogadie, F. C., Mistry, M., and Brown, D. A. (2002). Signaling microdomains define the specificity of receptor-mediated $\text{InsP}(3)$ pathways in neurons. *Neuron* 34, 209–220.
- DeMaria, C. D., Soong, T. W., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001). Calmodulin bifurcates the local Ca^{2+} signal that modulates P/Q-type Ca^{2+} channels. *Nature* 411, 484–489.
- Gamper, N., and Shapiro, M. S. (2003). Calmodulin mediates Ca^{2+} -dependent modulation of M-type K^+ channels. *J. Gen. Physiol.* 122, 17–31.
- Gamper, N., Stockand, J. D., and Shapiro, M. S. (2003). Subunit-specific modulation of KCNQ potassium channels by Src tyrosine kinase. *J. Neurosci.* 23, 84–95.
- Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3450.
- Huang, K. P., Huang, F. L., Li, J., Schuck, P., and McPhie, P. (2000). Calcium-sensitive interaction between calmodulin and modified forms of rat brain neurogranin/RC3. *Biochemistry* 39, 7291–7299.
- Jentsch, T. J. (2000). Neuronal KCNQ potassium channels: physiology and role in disease. *Nat. Rev. Neurosci.* 1, 21–30.
- Jurado, L. A., Chockalingam, P. S., and Jarrett, H. W. (1999). Apocalmodulin. *Physiol. Rev.* 79, 661–682.
- Keen, J. E., Khawaled, R., Farrens, D. L., Neelands, T., Rivard, A., Bond, C. T., Janowsky, A., Fakler, B., Adelman, J. P., and Maylie, J. (1999). Domains responsible for constitutive and Ca^{2+} -dependent interactions between calmodulin and small conductance Ca^{2+} -activated potassium channels. *J. Neurosci.* 19, 8830–8838.
- Kerst, G., Beschorner, U., Unsold, B., von Hahn, T., Schreiber, R., Greger, R., Gerlach, U., Lang, H. J., Kunzelmann, K., and Bleich, M. (2001). Properties and function of KCNQ1 K^+ channels isolated from the rectal gland of *Squalus acanthias*. *Pflügers Arch.* 443, 146–154.
- Lee, W. S., Ngo-Anh, T. J., Bruening-Wright, A., Maylie, J., and Adelman, J. P. (2003). Small conductance Ca^{2+} -activated K^+ channels and calmodulin: cell surface expression and gating. *J. Biol. Chem.* 278, 25940–25946.
- Li, Y., Gamper, N., and Shapiro, M. S. (2004). Single-channel analysis of KCNQ K^+ channels reveals the mechanism of augmentation by a cysteine-modifying reagent. *J. Neurosci.* 24, 5079–5090.
- Liang, H., DeMaria, C. D., Erickson, M. G., Mori, M. X., Alseikhan, B. A., and Yue, D. T. (2003). Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. *Neuron* 39, 951–960.
- Loussouarn, G., Park, K. H., Bellocq, C., Baro, I., Charpentier, F., and Escande, D. (2003). Phosphatidylinositol-4,5-bisphosphate, PIP_2 , controls KCNQ1/KCNE1 voltage-gated potassium channels: a functional homology between voltage-gated and inward rectifier K^+ channels. *EMBO J.* 22, 5412–5421.
- Marrion, N. V. (1996). Calcineurin regulates M channel modal gating in sympathetic neurons. *Neuron* 16, 163–173.
- Marrion, N. V., Zucker, R. S., Marsh, S. J., and Adams, P. R. (1991). Modulation of M-current by intracellular Ca^{2+} . *Neuron* 6, 533–545.
- Mori, M. X., Erickson, M. G., and Yue, D. T. (2004). Functional stoichiometry and local enrichment of calmodulin interacting with Ca^{2+} channels. *Science* 304, 432–435.
- Persechini, A., Blumenthal, D. K., Jarrett, H. W., Klee, C. B., Hardy, D. O., and Kretsinger, R. H. (1989). The effects of deletions in the central helix of calmodulin on enzyme activation and peptide binding. *J. Biol. Chem.* 264, 8052–8058.
- Persechini, A., and Stemmer, P. M. (2002). Calmodulin is a limiting factor in the cell. *Trends Cardiovasc. Med.* 12, 32–37.
- Porter Moore, C., Zhang, J. Z., and Hamilton, S. L. (1999). A role for cysteine 3635 of RYR1 in redox modulation and calmodulin binding. *J. Biol. Chem.* 274, 36831–36834.
- Pusch, M., Bertorello, L., and Conti, F. (2000). Gating and flickery block differentially affected by rubidium in homomeric KCNQ1 and heteromeric KCNQ1/KCNE1 potassium channels. *Biophys. J.* 78, 211–226.
- Roche, J. P., Westenbroek, R., Sorom, A. J., Hille, B., Mackie, K., and Shapiro, M. S. (2002). Antibodies and a cysteine-modifying reagent show correspondence of M current in neurons to KCNQ2 and KCNQ3 K^+ channels. *Br. J. Pharmacol.* 137, 1173–1186.
- Scholz, T., Moskvina, E., Mayer, M., Just, H., Kubista, H., and Boehm, S. (2002). Sympathoexcitation by bradykinin involves Ca^{2+} -independent protein kinase C. *J. Neurosci.* 22, 5823–5832.
- Seebach, G., Scherer, C. R., Busch, A. E., and Lerche, C. (2001). Identification of specific pore residues mediating KCNQ1 inactivation. A novel mechanism for long QT syndrome. *J. Biol. Chem.* 276, 13600–13605.
- Selyanko, A. A., and Brown, D. A. (1996a). Intracellular calcium directly inhibits potassium M channels in excised membrane patches from rat sympathetic neurons. *Neuron* 16, 151–162.
- Selyanko, A. A., and Brown, D. A. (1996b). Regulation of M-type potassium channels in mammalian sympathetic neurons: action of intracellular calcium on single channel currents. *Neuropharmacology* 35, 933–947.
- Shapiro, M. S., Roche, J. P., Kaftan, E. J., Cruzblanca, H., Mackie, K., and Hille, B. (2000). Reconstitution of muscarinic modulation of the KCNQ2/KCNQ3 K^+ channels that underlie the neuronal M current. *J. Neurosci.* 20, 1710–1721.
- Shen, Z., and Marcus, D. C. (1998). Divalent cations inhibit IsK/KvLQT1 channels in excised membrane patches of strial marginal cells. *Hear. Res.* 123, 157–167.
- Suh, B. C., and Hille, B. (2002). Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* 35, 507–520.
- Sun, J., Xin, C., Eu, J. P., Stamler, J. S., and Meissner, G. (2001). Cysteine-3635 is responsible for skeletal muscle ryanodine receptor modulation by NO. *Proc. Natl. Acad. Sci. USA* 98, 11158–11162.
- Tran, Q. K., Black, D. J., and Persechini, A. (2003). Intracellular coupling via limiting calmodulin. *J. Biol. Chem.* 278, 24247–24250.
- Wen, H., and Levitan, I. B. (2002). Calmodulin is an auxiliary subunit of KCNQ2/3 potassium channels. *J. Neurosci.* 22, 7991–8001.
- Yang, Y., and Sigworth, F. J. (1998). Single-channel properties of IKs potassium channels. *J. Gen. Physiol.* 112, 665–678.
- Yu, S. P., O'Malley, D. M., and Adams, P. R. (1994). Regulation of M current by intracellular calcium in bullfrog sympathetic ganglion neurons. *J. Neurosci.* 14, 3487–3499.
- Yus-Najera, E., Santana-Castro, I., and Villarroel, A. (2002). The identification and characterization of a noncontinuous calmodulin-binding site in noninactivating voltage-dependent KCNQ potassium channels. *J. Biol. Chem.* 277, 28545–28553.
- Zhang, H., Craciun, L. C., Mirshahi, T., Rohacs, T., Lopes, C. M., Jin, T., and Logothetis, D. E. (2003). $\text{PIP}(2)$ activates KCNQ channels, and its hydrolysis underlies receptor-mediated inhibition of M currents. *Neuron* 37, 963–975.
- Zhou, Z., and Neher, E. (1993). Mobile and immobile calcium buffers in bovine adrenal chromaffin cells. *J. Physiol.* 469, 245–273.