Single-Channel Analysis of KCNQ K⁺ Channels Reveals the Mechanism of Augmentation by a Cysteine-Modifying Reagent

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The cysteine-modifying reagent *N*-ethylmaleimide (NEM) is known to augment currents from native M-channels in sympathetic neurons and cloned KCNQ2 channels. As a probe for channel function, we investigated the mechanism of NEM action and subunit specificity of cloned KCNQ2–5 channels expressed in Chinese hamster ovary cells at the whole-cell and single-channel levels. Biotinylation assays and total internal reflection fluorescence microscopy indicated that NEM action is not caused by increased trafficking of channels to the membrane. At saturating voltages, whole-cell currents of KCNQ2, KCNQ4, and KCNQ5 but not KCNQ3 were augmented threefold to fourfold by 50 μ M NEM, and their voltage dependencies were negatively shifted by 10–20 mV. Unitary conductances of KCNQ2 and KCNQ3 (6.2 and 8.5 pS, respectively) were much higher than those of KCNQ4 and KCNQ5 (2.1 and 2.2 pS, respectively). Surprisingly, the maximal open probability (P_o) of KCNQ3 was near unity, much higher than that of KCNQ2, KCNQ4, and KCNQ5. NEM increased the P_o of KCNQ2, KCNQ4, and KCNQ5 by threefold to fourfold but had no effect on their unitary conductances, suggesting that the increase in macroscopic currents can be accounted for by increases in P_o . Analysis of KCNQ3/4 chimeras determined the C terminus to be responsible for the differential maximal P_o , channel expression, and NEM action between the two channels. To further localize the site of NEM action, we mutated three cysteine residues in the C terminus of KCNQ4. The C519A mutation alone ablated most of the augmentation by NEM, suggesting that NEM acts via alkylation of this residue.

Key words: *N*-ethylmaleimide; K⁺ channel; patch clamp; ion channel modulation; M current; KCNQ channel; signaling; gating; biotinylation

Introduction

KCNQ K⁺ channels have achieved considerable prominence since their identification as the molecular correlates of several important K⁺ currents in numerous tissues of the body (Robbins, 2001). In the heart, intestine, and inner ear, KCNQ1 subunits assemble with auxiliary KCNE subunits to make K⁺ currents important for repolarization and K⁺ transport (Barhanin et al., 1996; Sanguinetti et al., 1996; Neyroud et al., 1997; Schroeder et al., 2000a). KCNQ2, KCNQ3, and KCNQ5 make “M-type” K⁺ currents in a variety of neurons (Wang et al., 1998; Lerche et al., 2000; Schroeder et al., 2000b; Cooper et al., 2001; Roche et al., 2002; Shah et al., 2002), where they play a dominant role in regulating neuronal excitability (Jones et al., 1995; Cooper et al., 2001), and KCNQ4 primarily localizes to the inner ear (Kubisch et al., 1999). Single-channel analysis reveals homomeric KCNQ2 and KCNQ3 and heteromeric KCNQ2/3 channels to have relatively low conductance and voltage dependence of their open probability expected from macroscopic behavior (Selyanko et al.,

2001). The KCNQ family of channels was recently included in the K_v nomenclature as K_v7.1–7.5 (Gutman et al., 2003).

Given the importance of KCNQ channels in determining neuronal excitability and cardiac pacemaking, and the epileptic and arrhythmia syndromes resulting from their malfunction (Wang et al., 1996; Steinlein, 2001), much attention has focused on drugs that might augment KCNQ channel activity. Most of the compounds characterized so far augment currents mainly by shifting their voltage dependence of activation to less depolarized potentials (Rundfeldt and Netzer, 2000; Wickenden et al., 2000; Schroeder et al., 2001; Wu et al., 2003), although a few augment currents in a voltage-independent manner (Dupuis et al., 2002). It is thought that by augmenting KCNQ channel activity, these drugs may be antiepileptic agents, or could otherwise strongly modify nervous function.

To understand how K⁺ channels work, numerous investigators have used cysteine-modifying reagents as probes to study permeation and gating (del Camino et al., 2000; Karlin, 2002). At low concentrations, the cysteine-alkylating reagent *N*-ethylmaleimide (NEM) has been used to selectively block the actions of pertussis toxin-sensitive G-proteins (Nakajima et al., 1991; Shapiro et al., 1994). Unexpectedly, it also increases the M current in sympathetic neurons (Shapiro et al., 2000). Recently, the subunit specificity of NEM action on cloned KCNQ1–3 channels was explored. NEM was found to strongly augment currents from homomeric KCNQ2 but not KCNQ1 or KCNQ3 channels, primarily via a voltage-independent increase in current ampli-

Received Jan. 19, 2004; revised April 7, 2004; accepted April 8, 2004.

This work was supported by an American Heart Association Texas Affiliate research award, a research grant from the Epilepsy Foundation, National Institutes of Health Grant NS43394 (M.S.S.), and an American Heart Association Texas Affiliate postdoctoral fellowship (N.G.). We thank Pamela Martin and Sara Kathryn Boyd for expert technical assistance.

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DOI:10.1523/JNEUROSCI.0882-04.2004

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tudes (Roche et al., 2002). Here we characterize the single-channel properties of cloned KCNQ2–5 channels, extend the subunit specificity of NEM to include KCNQ4 and KCNQ5, and probe the biophysical and molecular mechanism of action by which NEM acts on the sensitive KCNQ channels, at both whole-cell and single-channel levels. We find that NEM does not alter surface expression of the channels but, rather, acts by increasing their maximal open probability by alkylation of a cysteine in the channel C terminus. We suggest that the subunit specificity may arise from very different intrinsic open probabilities of KCNQ2–5 channels.

Materials and Methods

cDNA constructs. Plasmids encoding human KCNQ2, rat KCNQ3, human KCNQ4, and human KCNQ5 (GenBank accession numbers AF110020, AF091247, AF105202, and AF249278, respectively) were kindly given to us by 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). KCNQ3 was subcloned into pcDNA3 (Invitrogen, San Diego, CA) as described previously (Shapiro et al., 2000). KCNQ4 and KCNQ5 were subcloned into pcDNA3.1zeo⁺ and pcDNA3.1zeo[−] (Invitrogen) using *XhoI*–*HindIII* and *XbaI*–*EcoRI*, respectively. Myc-tagged KCNQ2–5 were generated by subcloning each channel in-frame into cytomegalovirus-myc (pCMV) plasmid (Clontech, Palo Alto, CA) behind the myc epitope. The KCNQ3_{N/4C} and KCNQ4_{N/3C} chimeras were generated by restriction cutting wild-type (wt; rat) KCNQ3 and KCNQ4 with *XcmI* and *XbaI*. *XcmI* cuts KCNQ3 at Q385 and cuts KCNQ4 at Q454, and *XbaI* cuts after the stop codon in both cases. Thus, the chimeras contain most but not all of the C terminus downstream of S6. The junction site is just downstream of the highly conserved KCNQ3 “IQ₁” domain tested by Gamper and Shapiro (2003) and downstream of the nonconserved stretch of KCNQ4 just after that domain that is not present in KCNQ3. The cysteine-to-alanine KCNQ4 mutants were made commercially using Quikchange mutagenesis (BioS&T, Montreal, Quebec, Canada) and were sequenced to verify mutagenesis.

Cell culture and transfections. Chinese hamster ovary (CHO) cells were a kind gift of Feng Liu (Department of Pharmacology, University of Texas Health Science Center at San Antonio). Cells were grown in 100 mm tissue culture dishes (Falcon; Becton Dickinson, Mountain View, CA) in DMEM with 10% heat-inactivated fetal bovine serum and 0.1% penicillin and streptomycin in a humidified incubator at 37°C (5% CO₂) and passaged every 3–4 d. Cells were discarded after ~30 passages. For transfection, cells were plated onto poly-L-lysine-coated coverslip chips and transfected 24 hr later with Polyfect reagent (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. For electrophysiological and biochemical experiments, cells were used 48–96 hr after transfection. As a marker for successfully transfected cells, cDNA-encoding green fluorescent protein (GFP) was cotransfected together with the cDNAs of the genes of interest. We found that >95% of green-fluorescing cells expressed KCNQ currents in control experiments.

Whole-cell electrophysiology. Whole-cell patch-clamp experiments were performed at room temperature (22–25°C). Pipettes were pulled from borosilicate glass capillaries (IB150F-4; World Precision Instruments) using a Flaming–Brown P-97 micropipette puller (Sutter Instruments, Novato, CA) and had resistances of 2–3 MΩ when filled with internal solution and measured in Ringer’s solution. Membrane current was measured under voltage clamp with pipette and membrane capacitance cancellation, sampled at 1 kHz, and filtered at 100–200 Hz by an Axopatch 1D (Axon Instruments, Union City, CA) or EPC-9 (HEKA, Lambrecht, Germany) amplifier. Data acquisition and analysis were performed by Pulse software (HEKA) and an ITC-16 interface (Instrutech, Port Washington, NY). The whole-cell access resistance was typically 4–10 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 (VaveLink 8; Automate Scientific). Bath solution exchange was complete

by <30 sec. To observe GFP fluorescence, a mercury lamp was used in combination with an Eclipse TE 300 inverted microscope equipped with a HQ FITC filter cube (Nikon, Melville, NY).

Several voltage protocols were used to study KCNQ currents in CHO cells. To evaluate the augmentation of the current by NEM at saturating voltages, CHO cells were held at 0 mV, and 800 msec hyperpolarizing steps to −60 mV, followed by 1 sec pulses back to 0 mV, were applied every 4 or 6 sec. The amplitude of the current was usually defined as the difference between the holding current at 0 mV and the current at the beginning (after any capacity current as subsided) of the 1 sec pulse back to 0 mV. To evaluate the voltage dependence of activation, cells were held at the potential of −70 mV, and a family 800 msec voltage steps was applied every 3 sec, from −80 mV to 50 mV, with the amplitude taken as the maximal outward current at each potential. Voltage dependence was quantified as the amplitude of the tail current at −60 mV versus test potential. In most experiments, XE991 or linopirdine (50 μM) was used to verify current identity. CHO cells have negligible endogenous macroscopic K⁺ currents under our experimental conditions, and 50 μM XE991 or linopirdine completely blocked the K⁺ current in KCNQ-transfected CHO cells but had no effect on currents in nontransfected cells. Cells exhibited variable “rundown” in the amplitude of KCNQ currents and usually stabilized within several minutes of whole-cell dialysis. Cells in which the rundown exceeded 3%/min were discarded. All results are reported as mean ± SEM. Voltage dependence was evaluated by fitting the individual activation curves to a Boltzmann equation: $I = I_{\max}/\{1 - \exp[(V_{1/2} - V)/k]\}$, where $V_{1/2}$ is the voltage that produces half-maximal activation of the conductance, and k is the slope factor. Dose–response curves were fitted by a Hill equation of the form $I/I_0 = A/[NEM]^n/(EC_{50}^n + [NEM]^n)$, where EC_{50} is the concentration corresponding to half-maximal activity; A is the maximal augmentation by NEM; and n is the Hill coefficient.

Cell-attached patch/single-channel electrophysiology. Channel activity was recorded 48–96 hr after transfection. Pipettes had resistances of 7–15 MΩ when filled with a solution of the following composition (in mM): 150 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4 with NaOH. Cells were bath-perfused with a “high-K⁺” solution in which the KCl concentration was raised from 5 to 40 mM to clamp the resting membrane potential near −30 mV. The methods of recording and analysis were similar to those previously used for studying unitary M-type channels (Selyanko and Brown, 1999; Selyanko et al., 2001). Data were analyzed using PulseFit, IgorPro (Wavemetrics, Lake Oswego, OR), and TAC (Bruxton, Seattle, WA) software. Currents were recorded using an Axopatch 1-D amplifier (Axon Instruments). The data were sampled at 4 kHz and filtered at 500 or 200 Hz. To evaluate any error in our estimate of open probability (P_o) caused by choice of filtering frequency, we compared P_o values for records filtered at 500 Hz and those same records digitally refiltered at 200 Hz. We found that at P_o values between 0.1 and 0.8, such errors were <5%. Only at extremely low P_o , in the range of <0.01, did the lower filtering cause a more appreciable error, which for $P_o = 0.01$ was an underestimation of 14%.

At a given potential, the single-channel amplitude (i) and the P_o were calculated by fitting all-point histograms with single- or multi-Gaussian curves. All estimates of P_o at all potentials were made by analysis of >20 sweeps. The difference between the fitted “closed” and “open” peaks was taken as i , and the ratio of the area under the fitted open Gaussian to the total area under the entire Gaussian was taken as P_o . The presence of only one channel in a patch was assumed if P_o was >0.25 for >1 min without superimposed openings, especially at strongly depolarized potentials at which P_o is highest. In cases in which P_o was low, the absence of superimposed openings is not a reliable indicator of the number of channels in the patch, and in those cases, P_o may have been overestimated. When superimposed openings were observed, the number of channels in the patch was estimated from the maximal number of superimposed openings, and the time spent at each current level was estimated from the area under its associated Gaussian curve. In cases in which channel activity was produced by more than one channel in the patch, current levels were calculated by fitting the histograms with multiple Gaussian curves, and P_o was calculated according to the following equation (Selyanko and Brown, 1999), which assumes that all channels in the patch have the same

P_o and that the gating of each channel occurs independently of each other:

$$NP_o = \left(\sum_{j=1}^N t_{ij} \right) / T,$$

where t_j is the time spent at each current level corresponding to $j = 0, 1, 2, \dots, N$; T is the duration of the recording; and N is the number of current levels (minimum number of active channels). P_o – V_m relationships were fitted by a Boltzmann equation: $P_o/P_{o,\max} = 1/[1 + \exp\{(V_{1/2} - V_m)/k\}]$, where P_o is the channel open probability, $P_{o,\max}$ is the maximum P_o obtained at strongly depolarized membrane potentials, V_m is the assumed membrane potential (estimated as $V_{\text{rest}} - V_{\text{pipette}}$, where $V_{\text{rest}} = -30$ mV), $V_{1/2}$ is the half-activation potential (i.e., the potential at which $P_o = 0.5 P_{o,\max}$), and k is the slope factor. Because many investigators analyze single-channel events using “threshold analysis” rather than the all-points histogram analysis used here, we analyzed a number of patches using the former method as well using the TAC software package (Bruxton) to ensure that our estimates of P_o were not in error. Three patches each in low-, medium-, and high- P_o cases were analyzed using both methods. In all cases, the difference in analyzed P_o between the two methods was <2.5%.

Biotinylation of cell surface protein and immunoblotting. Cells were grown in 100 mm culture dishes and individually transfected with myc-tagged KCNQ2–5 and GFP. After 48 hr, cells were washed three times with PBS at room temperature (22–25°C) and treated with 50 μ M NEM or with only Ringer’s solution (control) for 8–10 min; the cells were washed three times with cold PBS; and cell surface proteins were biotinylated by EZ-link sulfo-NHS-S-S-biotin (0.5 mg/ml; Pierce, Rockford, IL) in PBS. After incubation at 4°C for 1 hr, cells were washed five times with ice-cold PBS to remove any remaining biotinylation reagent. Cells were then harvested with a rubber policeman in gentle lysis buffer (GLB; 75 mM NaCl, 50 mM HCl-Tris, 2 mM EGTA, 1% Nonidet P-40, and 10% glycerol) plus the protease inhibitor phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO), and lysate proteins were quantified with a BCA assay (Pierce). Proteins (150 μ g/reaction) were mixed with 50 μ l of streptavidin-immobilized beads (Pierce); beads were first washed four times with GLB overnight at 4°C. Beads were pelleted, washed thoroughly in GLB, and incubated in Laemmli sample buffer at 50°C for 30 min, and bound proteins were separated using SDS-PAGE, followed by electroblotting onto nitrocellulose membranes. Immunoblots were probed with mouse anti-myc primary antibodies (Clontech) at 1:1000 dilution overnight at 4°C in a blocking solution containing 5% nonfat dry milk (Carnation) in TBS and Tween 20 and subsequently treated with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:25,000 dilution, 45 min, room temperature; Jackson ImmunoResearch, West Grove, PA). Blots were developed with enhanced chemiluminescence (Supersignal; Pierce) and exposed on x-ray film (Biomax).

Total internal reflection fluorescent microscopy. To selectively illuminate the plasma membrane and its associated channel subunits, we used total internal reflection fluorescent (TIRF) microscopy (Axelrod et al., 1984; Steyer and Almers, 2001). Cells used for TIRF microscopy were plated on glass-bottomed 35 mm dishes (MatTek, Ashland, MA). The method was similar to that described previously by Merrifield et al. (2002) and Taraska et al. (2003). In brief, TIRF microscopy was performed using an inverted TE2000 microscope with “through-the-lens” laser illumination. An “evanescent field” (EF) is generated by total internal reflection (TIR) generated when the light beam strikes the dielectric interface between the glass coverslip and cellular plasma membrane and cytoplasm at a glancing angle greater than the critical angle (θ_c), given by $\theta_c = \sin^{-1}(n_2/n_1)$, where n_2 and n_1 are the refractive indices of the two media, in this case, cytoplasm and glass, respectively. Samples were illuminated by epifluorescence with wide-field or EF light and viewed through a Plan Apo TIRF 60 $\times$ oil-immersion high-resolution (1.45 numerical aperture) objective (Nikon). TIR generates an EF that declines exponentially with increasing distance from the interface between the cover glass and plasma membrane, illuminating only a small optical slice of the cell (~ 300 nm) including the plasma membrane. Thus, using EF

illumination, only fluorophores in or very close to the plasma membrane contribute to emission, whereas those deeper in the cytoplasm do not. The selective excitation of membrane fluorophores was verified in control experiments in which CHO cells were cotransfected with the membrane-localized enhanced GFP-F (EGFP-F) construct (Clontech) and the red-fluorescing cytoplasmic protein dsRed2 (Clontech). The cells were illuminated either with a mercury lamp for wide-field illumination or with the 488 nm line of an argon laser (for EGFP-F) and a green helium and neon laser (for dsRed2) for TIRF illumination. Under wide-field illumination, both membrane-localized green fluorescence and cytoplasmic red fluorescence were observed. Under TIRF, however, only a green-fluorescing “footprint” of the cell could be observed, with little red fluorescence at all (Tong et al., 2004).

KCNQ2 and KCNQ5 were yellow fluorescent protein (YFP)-tagged by subcloning into the pEYFP-N1 vector (Clontech), which appends YFP to the C terminus of the channels. The 488 nm line of a 10 mW argon laser was used for TIRF illumination of YFP-tagged channels. This system was also interfaced with a mercury lamp and the appropriate dichroic excitation and emission filter sets for TIRF, as well as wide-field imaging of YFP, EGFP-F, and dsRed2. Images were collected and processed as above with a CCD camera interfaced to a personal computer running MetaMorph software (Universal Imaging, Downingtown PA).

Solutions and materials. For whole-cell recording, the external solution used to record KCNQ currents in CHO cells contained (in mM): 160 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4 with NaOH. The regular pipette solution contained (in mM): 160 KCl, 5 MgCl₂, 5 HEPES, 10 EGTA, 3 K₂ATP, and 0.1 NaGTP, pH 7.4 with KOH. For perforated patch experiments, the pipette solution also contained amphotericin B (12 μ g/ml). Reagents were obtained as follows: DMEM, fetal bovine serum, penicillin, and streptomycin, Invitrogen; and ATP, GTP, NEM, and amphotericin B, Sigma. XE991 and linopirdine were kind gifts from Michael E. Schnee (DuPont, Billerica, MA).

Results

NEM augments currents of KCNQ2, KCNQ4, and KCNQ5 channels but not currents of KCNQ3 channels

It has been shown that the cysteine-alkylating reagent NEM increases the amplitude of the M-current of rat superior cervical ganglion neurons and of homomeric KCNQ2 and heteromeric KCNQ2/3 channels but not of homomeric KCNQ3 or KCNQ1 channels (Shapiro et al., 2000; Roche et al., 2002). We first wished to extend the investigation of the subunit specificity of NEM action to homomeric KCNQ4 and KCNQ5 channels. Throughout, we included tests on KCNQ2 and KCNQ3 in our investigations as positive or negative controls, respectively. For these experiments, we individually expressed KCNQ2–5 channels in CHO cells and tested the effects of NEM application on the whole-cell current. To focus on the voltage-independent augmentation of the currents, we assayed the effect of NEM at the holding potential of 0 mV, at which activation is near maximal. NEM (50 μ M) enhanced the current amplitudes of KCNQ4, KCNQ5, and KCNQ2 channels by threefold to fourfold (Fig. 1A–C). The augmentation of the current did not reverse 10 min after washing out of NEM. Consistent with previous work, NEM did not increase the amplitude of KCNQ3 currents at 0 mV (Roche et al., 2002). These data are summarized in Figure 1E. The augmented current from KCNQ2-expressing cells was nearly all blocked by 10 mM tetraethylammonium (TEA) ions, consistent with it being an increase arising from KCNQ2 channels (Shapiro et al., 2000). The NEM-augmented currents from cells expressing KCNQ4 and KCNQ5 were completely inhibited by linopirdine or XE991 (both 50 μ M) (Fig. 1B), specific inhibitors of KCNQ channels (Zaczek et al., 1998). These results indicate that the current augmentation by NEM is specific to KCNQ channels and is not reversible. We investigated the dose–response relationship of NEM on KCNQ2 channels (Fig. 1F). The augmentation of the

Figure 1. NEM enhances the amplitudes of currents in homomeric KCNQ4, KCNQ5, and KCNQ2 but not KCNQ3 channels. Plotted are current amplitudes at 0 mV during the experiments from CHO cells expressing KCNQ4 (A), KCNQ5 (B), KCNQ2 (C), and KCNQ3 (D), studied under whole-cell clamp. Voltage pulses using a classic M-channel protocol (A, inset) were given every 4 sec. Bath applications of NEM (50 μM), TEA (10 mM), or linopiridine (50 μM) are indicated by the bars. Representative current sweeps at the indicated times during the experiments are shown above each plot. E, Bars are mean augmentations by NEM of the currents at 0 mV. For KCNQ4, KCNQ5, KCNQ2, and KCNQ3, they were 2.9 ± 0.4 ($n = 7$), 3.5 ± 1.0 ($n = 6$), 3.3 ± 0.6 ($n = 5$), and 1.1 ± 0.1 ($n = 4$), respectively. F, Dose–response curve for NEM action on KCNQ2 currents, pooled from five experiments. The data were fitted by a Hill equation with an EC_{50} of 14.4 ± 1.5 μM and a Hill coefficient of 1.1 ± 0.4 ($n = 5$).

current by NEM was quantified after application of each concentration for 5 min. The data were fit by a Hill equation, yielding an EC_{50} of 14.4 ± 1.5 μM ($n = 5$) and a Hill coefficient of 1.1 ± 0.4 .

It was shown that NEM causes an ~ 10 mV hyperpolarizing shift of the activation curves of KCNQ2 and KCNQ3 currents, although this effect does not account for the strong augmentation of KCNQ2 current amplitudes (Roche et al., 2002). We next asked whether NEM similarly shifts the voltage dependence of KCNQ4 and KCNQ5 channels. A voltage protocol more common for studying voltage-gated K⁺ channels was used, as shown in Figure 2A, inset. Activation was assayed by measuring the tail current amplitudes after a family of depolarizing steps and plotting the normalized amplitudes versus test potential. The resulting activation curves were fitted by Boltzmann equations. The results of this analysis for KCNQ2–5 currents are shown in Figure 2. In addition to the large augmentation in current amplitude at all voltages, we found that NEM shifted the half-activation potential ($V_{1/2}$) of the KCNQ2 and KCNQ3 activation curves by -14.3 ± 1.2 mV ($n = 7$) and -11.3 ± 5.7 mV ($n = 3$), similar to that previously reported (Roche et al., 2002). NEM also shifted the voltage dependence of activation for KCNQ4 and KCNQ5, with a shift of $V_{1/2}$ of -7.5 ± 1.5 mV ($n = 5$) and -18.6 ± 1.6 mV ($n = 8$), respectively. Although NEM did shift the voltage dependence of KCNQ3, thus increasing the currents at submaximal voltages, it did not significantly increase the currents at voltages

positive to 0 mV. The effect of NEM on the voltage dependence of activation of KCNQ2–5 is summarized in Figure 2E.

We also saw that NEM accelerated the rate of activation of all KCNQ2–5 channels. We quantified the effect of NEM on the kinetics of activation and deactivation of KCNQ4 and KCNQ5 at 0 and -60 mV, respectively, by fitting activating or deactivating currents by single exponentials. For both channels, NEM decreased the activation time constant (τ_{act}) by threefold to fourfold and slightly decreased the deactivation time constant (τ_{deact}) by ~ 1.3 -fold. For KCNQ4, τ_{act} was 127 ± 11 msec before NEM and 47 ± 7 msec after NEM ($n = 11$); τ_{deact} was 106 ± 12 msec before NEM and 94 ± 14 msec after NEM ($n = 6$). For KCNQ5, τ_{act} was 139 ± 15 msec before NEM and 37 ± 5 msec after NEM ($n = 9$); τ_{deact} was 115 ± 7 msec before NEM and 84 ± 7 msec after NEM ($n = 7$). Because the voltage dependence of activation reflects the balance between activation and deactivation rates, we asked whether the observed shifts in the voltage dependence of activation were of the magnitude expected from the measured changes in τ_{act} and τ_{deact} . We modeled activation curves using a highly simplified two-state gating scheme in which α and β are the rate constants of going from closed to open or open to closed, respectively, and P_o is then given by $\alpha/(\alpha + \beta)$. As a baseline, we used the precise measurements of activation and deactivation as a function of voltage recently presented for KCNQ2 and 3 heteromultimers (Prole et al., 2003) and modeled the predicted effects on $V_{1/2}$ of our observed changes in τ_{act} and τ_{deact} for KCNQ4 and KCNQ5. Assuming that the effect of NEM on gating kinetics is voltage-independent, the predicted shifts in $V_{1/2}$ are -7 and -10 mV for KCNQ4 and KCNQ5, respectively. The predicted shift for KCNQ4 is almost exactly what we observed. Although the predicted shift in $V_{1/2}$ for KCNQ5 is slightly less than observed, a two-state scheme is highly simplified but does indicate that the observed shifts in voltage dependence are of the magnitude expected for the observed acceleration of gating kinetics.

Biotinylation of cell surface proteins

NEM action is not immediate, requiring 3–8 min to reach its maximal level. Two general mechanisms could account for the augmentation by NEM of KCNQ currents: an increase in the number of functional channels in the cell membrane or an increase in the P_o of the channels. We tested the first possibility by performing biotinylation assays and immunoblots to specifically label and quantify the KCNQ channels in the cell membrane. We compared biotinylated (cell surface) KCNQ channel protein from control cells with those after 10 min NEM treatment. KCNQ2–5 subunits were epitope-tagged by introduction of myc epitope to their N terminus and individually expressed in CHO cells. The properties of the KCNQ2–5 channels were not affected by introduction of the myc epitope (data not shown). 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 weights of ~ 100 kDa for KCNQ2, 110 kDa for KCNQ3, 80 kDa for KCNQ4, and 125 kDa for KCNQ5. Lysate from cells expressed with wt KCNQ3 was not labeled, showing the specificity of the myc antibodies, and lysate from nonbiotinylated cells was not retained by the streptavidin beads, showing specific binding to the beads (data not shown).

Shown in Figure 3A are immunoblots of cell surface (top row) and total lysate (bottom row) from cells subjected to NEM treatment or from control cells. The amount of protein in the lanes of the blot was estimated by measuring the pixel intensity of their

Figure 2. Effect of NEM on the voltage dependence of activation of KCNQ2–5. Shown are families of currents from KCNQ4 (A), KCNQ5 (B), KCNQ2 (C), and KCNQ3 (D) channels, elicited by a range of depolarizations from -80 to 40 mV in 10 mV increments (A, inset) before and after application of NEM ($50 \mu\text{M}$). Plotted below the current traces are the fractional activation versus voltage, quantified as the tail current amplitudes following the range of test potentials, normalized to the maximal tail current after application of NEM. The activation curves were fitted by Boltzmann equations (see Materials and Methods). For KCNQ4, KCNQ5, KCNQ2, and KCNQ3, before NEM (open circles), $V_{1/2}$ and k were -23.1 and 10.4 , -31.0 and 13.1 , -27.5 and 9.9 , and -52.3 and 5.7 mV, respectively; after NEM (filled circles), they were -33.4 and 8.4 , -43.7 and 7.5 , -34.5 and 10.3 , and -55.8 and 4.6 mV, respectively. The dotted curves in A–C are the fitted Boltzmann relations of the control data, scaled up so that maximal activation is equal to that of the post-NEM curve. E, Bars are mean $V_{1/2}$ of KCNQ4, KCNQ5, KCNQ2, and KCNQ3 relations before (open) and after NEM (hatched). Student's t test, $p < 0.05$ in each group.

corresponding antibody-labeled bars. The experiment shown in Figure 3A,a, reveals no increase in relative cell surface expression in the NEM-treated cells compared with control cells. These experiments are summarized in Figure 3A,b. For each channel, the ratio of cell surface protein in NEM-treated to control cells was normalized by the ratio of total channel protein in the corresponding lysate. We found that for KCNQ2–4, there was no significant increase in cell surface protein caused by NEM treatment but the suggestion of an increase for KCNQ5. Thus, we turned to an alternate and more powerful method of answering this question.

Total internal reflection fluorescence microscopy

We desired a method that allowed direct observation of channels localized to the plasma membrane so that we could monitor “live” any NEM-induced trafficking of channels to the surface. Thus, we used TIRF microscopy (Axelrod et al., 1984; Axelrod, 2003). TIRF illumination involves directing a laser beam at the interface between two transparent media of differing refractive indices at a glancing angle. By the laws of optics, at an angle greater than the critical angle determined by ratio of the two refractive indices (see Materials and Methods), the light beam is not primarily transmitted to the second media but is instead reflected; however, not all the light energy is reflected back; a component penetrates into the second media as an “evanescent wave” that decays exponentially in intensity over a distance of only several hundred nanometers. Thus, we can selectively excite only fluorophores located within ~ 300 nm of the plasma membrane by directing laser light at such a glancing angle through a

special TIRF objective (Steyer and Almers, 2001). Any molecules located deeper in the cytoplasm will not be illuminated.

We constructed (YFP)-tagged KCNQ2 and KCNQ5 and expressed them in CHO cells. The YFP-tagged channels expressed well, and CHO cells expressing them gave normal-looking currents under whole-cell clamp that were augmented by NEM like untagged channels (data not shown). We looked under TIRF illumination for any change in plasma membrane YFP-tagged KCNQ channels induced by NEM treatment. Figure 3B, insets, shows images from such an experiment. Under normal wide-field epifluorescence, the YFP-tagged KCNQ5 channels are seen to be expressing both at the membrane and throughout the intracellular compartment, with little preferential localization (Fig. 3B, left). The same cell was then imaged under TIRF illumination, both before and several minutes after NEM application (center, right). Under these conditions, only channels in or very near the membrane can be observed and are seen to be localized in discrete puncta. We estimated membrane localization of the channels by measuring the pixel intensity of images acquired every 3 sec. In this experiment, NEM did not increase the intensity coming from YFP-tagged KCNQ5. The results from all such experiments for YFP-tagged KCNQ2 ($n = 7$) and KCNQ5

($n = 4$) were pooled by temporally aligning the images to NEM application (Fig. 3B). There was no increase in KCNQ5 intensity and only a barely detectable transient increase in KCNQ2 intensity induced by NEM. As a positive control, we cotransfected CHO cells with M₁ receptors and the EGFP-phospholipase C δ (PLC δ)-pleckstrin homology domain construct, which binds to phosphoinositide-(4,5)-bisphosphate (PIP₂) and is thus an optical reporter of PIP₂ hydrolysis (Stauffer et al., 1998; Raucher et al., 2000). We easily detected reduction of membrane fluorescence from such cells imaged under TIRF illumination on application of a muscarinic agonist, as expected from the consequent activation of PLC (data not shown). Thus, the TIRF experiments, like the biotinylation experiments, do not indicate that the NEM-induced augmentation is attributable to increased trafficking of channels to the membrane.

Single-channel characteristics of KCNQ2–5

Because NEM did not change the channel number in the cell membrane, we reasoned that its effect was likely to be an increase in the P_o of the channels. We thus investigated this at the single-channel level. Although the single-channel characteristics of KCNQ2 and KCNQ3 have been investigated (Selyanko et al., 2001), those of KCNQ4 and KCNQ5 have not been described. We assayed the single-channel behavior of KCNQ2–5 using cell-attached patch recordings. Single-channel or multichannel currents were recorded from CHO cells individually transfected with KCNQ2–5, with 40 mM K⁺ in the bath to set the resting membrane potential (V_{rest}) near -30 mV. Membrane potentials (V_m) were calculated as $V_m = V_{\text{rest}} - V_{\text{pipette}}$, where V_{rest} was assumed

Figure 3. NEM does not induce trafficking of channels to the plasma membrane. *A*, Surface expression of homomeric myc-tagged KCNQ2–5 channels was evaluated by biotinylation labeling. Shown are the biotinylated proteins (top) and lysates (lower) after or without NEM treatment. *B*, Surface expression of homomeric YFP-tagged KCNQ2 and KCNQ5 evaluated by TIRF microscopy. Images are of two cells expressing YFP-tagged KCNQ5 under wide-field epifluorescence (left) or under TIRF illumination before (middle) or after (right) NEM treatment. Note the punctate membrane expression of the channels seen in the TIRF images. Plotted are the pooled data from all such experiments for YFP-tagged KCNQ2 (open circles; $n = 7$) and YFP-tagged KCNQ5 (filled circles; $n = 4$), by temporally aligning all experiments to NEM application. Images were acquired every 3 sec under constant bath perfusion, and surface expression was estimated by quantification of pixel intensity. Care was taken to not saturate the camera at any point in the image. NEM was bath-applied at the time shown by the arrow.

to be -30 mV, and V_{pipette} was the voltage applied in the pipette. We identified expressed KCNQ2–5 channels by their (1) reversal potential near E_K , (2) voltage dependence expected from their macroscopic behavior, and (3) sensitivity to linopiridine.

Multichannel current sweeps recorded in cell-attached mode using a typical M-channel protocol were averaged, and the resulting ensemble currents for KCNQ4 and KCNQ5 were similar to their whole-cell currents (Fig. 4*A,a,C,a*). We found both to have an unexpectedly small unitary current amplitude. At $V_m = 0$ mV, those of KCNQ4 and KCNQ5 were 0.21 ± 0.01 pA ($n = 15$) and 0.19 ± 0.02 pA ($n = 11$) (Fig. 4*A,b,C,b*). Measured as the chord conductance over a range of voltages, their single-channel conductances were correspondingly low. For KCNQ4 and KCNQ5, they were 2.1 ± 0.1 pS ($n = 12$) and 2.2 ± 0.1 pS ($n = 7$), respectively (Fig. 4*B,b,D,b*). We estimated their P_o by fitting all-points histograms of the current records by Gaussian curves (Fig. 4*B,a,D,a*) and found them to be quite low. For KCNQ4 and KCNQ5 at $V_m = 0$ mV, which should be near maximal P_o (judged

from macroscopic activation curves), they were 0.07 ± 0.02 ($n = 6$) and 0.17 ± 0.03 ($n = 7$), respectively. The extrapolated reversal potentials were in the range of -90 to -100 mV, near that expected for a K⁺-selective channel with 5 mM K⁺ in the pipette and 120–160 mM K⁺ assumed in the cytoplasm. The identified KCNQ4 and KCNQ5 channels had steady-state voltage-dependent activation at V_m potentials greater than -60 mV. Mean P_o – V_m relationships for KCNQ4 and KCNQ5 (Fig. 4*B,c,D,c*) were fitted by Boltzmann equations, yielding half-activation potentials and slope factors close to those of activation curves generated by whole-cell currents (Fig. 2). In nontransfected CHO cells, no such single-channel currents were observed (data not shown). Thus, KCNQ4 and KCNQ5 channels have low single-channel conductance and open probability but have a gating behavior expected from their macroscopic characteristics.

Compared with KCNQ4 and KCNQ5, KCNQ2 and KCNQ3 channels had much larger single-channel amplitudes (Fig. 5*A,C*). At 0 mV, they were 0.55 ± 0.04 pA ($n = 6$) and 0.64 ± 0.07 pA ($n = 6$), respectively. Their conductances were correspondingly higher. For KCNQ2 and KCNQ3, they were 6.2 ± 0.3 pS ($n = 6$) and 8.5 ± 0.3 pS ($n = 5$), respectively, which is threefold to fourfold larger than that of KCNQ4 or KCNQ5. These parameters of KCNQ2 and KCNQ3 are very similar to those previously reported for these channels in CHO cells (Selyanko et al., 2001). We found that KCNQ3 exhibited a strikingly higher P_o at 0 mV than did the other channels. That of KCNQ2 (0.17 ± 0.06 ; $n = 6$) was similar to that of KCNQ5, but the P_o of KCNQ3 was much higher (0.89 ± 0.05 ; $n = 5$) (Fig. 5*A,B,c,D,c*). For many patches containing a single KCNQ3 channel, the channel remained nearly continually open at 0 mV, with only brief closures (some very brief closures may have been missed due to filtering). Mean P_o was calculated over a range of voltages, and the resulting P_o – V_m relationships were fitted by Boltzmann equations (Fig. 5*B,c,D,c*) with the half-activation potentials and slope factors close to those previously reported in both cell-attached (Selyanko et al., 2001) and whole-cell recording (Roche et al., 2002; Gamper et al., 2003; this study).

In whole-cell recording, the amplitudes of currents from cells expressing KCNQ4 or KCNQ5 are large, whereas those of cells expressing KCNQ2 are small, and those of cells expressing KCNQ3 are very small. Thus, the rank order of whole-cell current amplitudes at 0 mV is: KCNQ4 $\sim$ KCNQ5 $>$ KCNQ2 $>$ KCNQ3. The rank orders of maximal P_o and unitary conductances, however, were quite different. For maximal P_o , it was KCNQ3 $\gg$ KCNQ2 $\sim$ KCNQ5 $>$ KCNQ4, and for unitary conductance, it was KCNQ3 $>$ KCNQ2 $>$ KCNQ4 $\sim$ KCNQ5. Thus, the tonic amplitude of whole-cell currents must be primarily determined by the number of functional channels in the cell membrane. This conclusion was corroborated by our cell-attached patch experiments. Using similarly sized pipettes, most patches from cells expressing KCNQ4 or KCNQ5 contained more than one channel, but from those expressing KCNQ3, there was never more than one channel in the patch, and most patches contained none.

Analysis of NEM augmentation at the single-channel level

To determine whether the increase in macroscopic current amplitude of KCNQ4, KCNQ5, and KCNQ2 caused by NEM can be attributed to an increase in single-channel conductance, P_o , or both, we tested its action on cell-attached patches (Table 1). We found that channel activities of KCNQ4, KCNQ5, and KCNQ2 steadily increased during bath application of NEM, reaching their maximal level within 3–6 min. Representative experiments from patches containing a single channel are shown in Figure 6, left. At

Figure 4. Single-channel properties of KCNQ4 and KCNQ5 channels. *A*, KCNQ4. *a*, Single (left) and ensemble ($n = 52$; right) records from a multichannel KCNQ4 cell-attached patch (left), using a typical M-current voltage protocol, as in Figure 1. *b*, Single sweeps at the holding potential of $V_m = 0$ mV applied to a cell-attached patch containing a single KCNQ4 channel before or after bath application of linopirdine ($50 \mu\text{M}$; bottom). Also shown is a representative segment of one sweep (as indicated) on a faster time scale. *B*, *a*, All-point amplitude histograms for the sweeps shown in *A*, fitted by a double-Gaussian curve (smooth lines). The closed-state peak has been set to 0 pA. The fitted single-channel amplitude (i) was 0.18 pA, and the P_o was 0.07. *b*, Single-channel amplitudes (mean $\pm$ SEM) against a range of membrane potentials. The mean slope conductance obtained by fitting the points by a line was 2.1 ± 0.1 pS ($n = 12$). *c*, Mean P_o against a range of V_m , fitted by a Boltzmann equation. The maximal P_o , $V_{1/2}$, and k were 0.07 ± 0.01 , -18.8 ± 2.5 mV, and 9.4 ± 2.2 mV ($n = 6$), respectively. *P_o* was estimated as described in Materials and Methods. *C*, *D*, Similar analyses for cell-attached patches containing several (*C*, *a*) or a single (*C*, *b*) KCNQ5 channels. *D*, *a*, The fitted single-channel amplitude and P_o at 0 mV were 0.19 pA and 0.22, respectively. *b*, The fitted chord conductance was 2.2 ± 0.1 pS ($n = 7$). *c*, The P_o - V_m curve yielded values for maximal P_o , $V_{1/2}$, and k of 0.19 ± 0.04 , -19.6 ± 5.6 mV, and 11.1 ± 5.4 mV ($n = 5$), respectively. 0 and C represent the open-channel and closed-channel current level, respectively.

0 mV, the P_o of KCNQ4, KCNQ5, and KCNQ2 increased from 0.07 ± 0.02 ($n = 6$) to 0.18 ± 0.02 ($n = 6$), from 0.17 ± 0.03 ($n = 6$) to 0.58 ± 0.1 ($n = 4$), and from 0.17 ± 0.06 ($n = 6$) to 0.60 ± 0.03 ($n = 7$), respectively. On the other hand, their unitary conductances were not significantly altered by NEM (Fig. 6, right). From such experiments over a range of voltages, we calculated P_o - V_m relationships before or after NEM treatment (Fig. 6, middle). The data were fitted by Boltzmann equations, indicating a strong increase in P_o at all voltages. In addition, there was a hyperpolarizing shift in activation. For KCNQ5, NEM shifted the $V_{1/2}$ from -20 to -44 mV, similar to its -19 mV shift on macroscopic activation. For KCNQ4, NEM shifted the $V_{1/2}$ from -19 to -24 mV, also close to its -7 mV shift in macroscopic activation. The $V_{1/2}$ from one good KCNQ2 single-channel patch (Fig. 6C) also displayed a -20 mV shift by NEM application. For KCNQ3, although NEM cannot increase its P_o at saturating voltages because of its tonic value near unity, it did increase it at threshold voltages because of the voltage shift of activation like that seen in whole cell (data not shown).

The C termini of KCNQ3 and KCNQ4 determine NEM action and current density

What is the origin of the strong augmentation of the currents by NEM on KCNQ2, KCNQ4, and KCNQ5 but not on KCNQ3? Because NEM acts by alkylating cysteine residues, one possibility is that there are structural aspects that confer sensitivity, such as

cysteines, that are different between the channels. Because it easily and rapidly crosses the membrane, our patch-clamp experiments cannot localize the site of NEM action to one side of the membrane or the other. Expression of wild-type KCNQ4 produces large saturating currents that are strongly augmented by NEM; expression of KCNQ3 produces small saturating currents that are nearly NEM-insensitive (Roche et al., 2002; this study). To examine the regions of the channel important for NEM action, we constructed two chimeric proteins that swapped nearly the entire C terminus of KCNQ3 and KCNQ4: KCNQ3_N/Q4_C and KCNQ4_N/Q3_C (Fig. 7A,B, diagrams). The junction site (see Materials and Methods) is just after the post-S6 IQ₁ domain tested for calmodulin binding by Gamper and Shapiro (2003) but well upstream of the "SID" and "A/B/Cd" domains implicated by two other laboratories for determining subunit assembly and expression (Maljevic et al., 2003; Schwake et al., 2003). We examined both the sensitivity of the currents from these chimeras to NEM as well as their tonic current densities in transfected cells. At 0 mV, which is near saturating activation for both chimeras, NEM increased the current amplitude of KCNQ3_N/Q4_C by more than eightfold (Fig. 7A,D), which is even greater than the threefold augmentation of currents of KCNQ4. On the other hand, NEM had no action on the currents of KCNQ4_N/Q3_C (Fig. 6B,D), similar to its little action at

saturating voltages on KCNQ3. Thus, the sensitivity to NEM augmentation seems to correlate with the C terminus. Unlike the case for KCNQ4 channels, the voltage dependence of the KCNQ3_N/Q4_C chimera was not shifted by NEM (Fig. 7A, bottom, right), perhaps indicating the distinct mechanism responsible for the voltage shift. Current densities also appeared to correlate with the identity of the C terminus. Thus, cells expressing the KCNQ3_N/Q4_C chimera produced large currents similar in amplitude to those expressing KCNQ4, and those expressing the KCNQ4_N/Q3_C chimera produced small currents, like for KCNQ3. These results are consistent with previous studies implicating domains in the channel C terminus important for channel assembly and expression (Maljevic et al., 2003; Schwake et al., 2003).

We then asked whether the striking difference in NEM action on currents of the two chimeras were attributable to their different open probabilities. Single-channel recordings from a patch containing a single KCNQ4_N/Q3_C channel showed that maximal P_o of this chimera was near unity at -10 mV (0.90 ± 0.05 ; $n = 5$), like that of KCNQ3 (Fig. 8A). Thus, as for KCNQ3, NEM cannot augment the current of this chimera at saturating voltages because P_o is already so high. In striking contrast, recordings from a patch containing two KCNQ3_N/Q4_C channels (Fig. 8B) indicated that the maximal P_o for this channel was >40 -fold less (0.02 ± 0.01 ; $n = 4$) much more like that of KCNQ4 ($P_o = 0.07$). Thus, NEM can augment its activity, doing so by nearly sixfold in the patch shown here, increasing P_o from 0.014 to 0.09 (Fig. 8B). We

conclude that the intracellular C terminus of KCNQ3 or KCNQ4 determines the extent of NEM action because of its strong influence on channel gating. Additionally, the combined observations of much lower $P_{o,max}$ of KCNQ3_N/4_C channels but their much larger whole-cell currents, compared with KCNQ4_N/3_C channels, strongly suggest that there are many more functional channels in the plasma membrane if they have the C terminus of KCNQ4, rather than that of KCNQ3.

NEM action on KCNQ4 localizes to a specific cysteine

Because NEM acts by alkylating cysteines, we sought to determine its locus of action by making several specific cysteine mutations, focusing on the intracellular C terminal of KCNQ4. We choose to investigate KCNQ4 because of its robust expression as homomultimers and its shorter C terminus, relative to KCNQ2 or KCNQ5. For instance, the distal domain of KCNQ2 truncated at G653 by Roche et al. (2002), eliminating the six most distal KCNQ2 cysteines (which did not affect NEM augmentation), does not exist in KCNQ4. Because NEM has strong actions on KCNQ2, 4, and 5, we examined cysteines that are present in all three channels in analogous positions in the C tail. Three cysteine-to-alanine KCNQ4 mutants were tested for NEM augmentation, and in only one, C519A, most (but not all) of the NEM effect was abolished (Fig. 9A). For that mutant, NEM augmentation was only 1.4 ± 0.1 ($n = 14$), compared with 2.9 ± 0.4 ($n = 7$) for wild-type KCNQ4. In addition, there was no voltage shift caused by NEM of the activation of C519A (Fig. 9B). Two other cysteine mutants, C427A and C418A, responded to NEM with an augmentation of the currents that were near that of wild-type KCNQ4. These data are summarized in Figure 9D.

Because the lack of KCNQ3 current augmentation by NEM was found to be attributable to the very high tonic P_o of KCNQ3 channels, we asked whether the reduced NEM action on KCNQ4 C519A was likewise attributable to a high P_o of this channel. However, single-channel analysis of C519A in cell-attached patches showed that this was not the case (Fig. 9C). Although there was some variability, the maximal P_o was indeed still low. At 0 mV, the P_o of C519A was only 0.041 ± 0.024 ($n = 4$), similar to that of wild-type KCNQ4. NEM application increased it to 0.062 ± 0.034 ($n = 4$). The mean increase in P_o of the C519A mutant was 1.4 ± 0.4 , congruent with the weak effect of NEM on this mutant in whole cell. Thus, we conclude that NEM primarily acts on KCNQ4 by alkylation of C519 in the C terminus.

Comparing the macroscopic and microscopic data, NEM augmentations of KCNQ2–5 on whole-cell current amplitudes and on the P_o of single-channel currents at 0 mV were very consistent. For KCNQ3, NEM cannot increase the whole-cell current or

Figure 5. Single-channel properties of KCNQ2 and KCNQ3 channels. *A, B*, Similar analysis as in Figure 4 for a cell-attached patch containing a single KCNQ2 channel. *B, a*, The fitted unitary current amplitude and P_o at 0 mV were 0.52 pA and 0.11, respectively. *b*, The fitted chord conductance was 6.5 ± 0.2 pS ($n = 6$). *c*, The P_o – V_m curve for this one patch yielded values for $V_{1/2}$ and k of -33.9 and 13.2 mV, respectively. *C, D*, Similar analysis for a cell-attached patch containing a single KCNQ3 channel. Linopiridine ($50 \mu\text{M}$; bottom) blocked but did not completely inhibit channel activity (as might be expected because it cannot directly access the channel as in whole-cell experiments). *D, a*, The fitted unitary current amplitude and P_o at 0 mV were 0.63 pA and 0.90, respectively. *b*, The fitted chord conductance was 8.5 ± 0.3 pS ($n = 5$). *c*, The P_o – V_m curve yielded values for maximal P_o , $V_{1/2}$, and k of 0.89 ± 0.08 , -44.1 ± 3.9 mV, and 8.6 ± 3.5 mV ($n = 6$), respectively. O and C represent the open-channel and closed-channel current levels, respectively.

Table 1. Single-channel properties of KCNQ2–5 channels

	P_o	i (pA)	γ (pS)
KCNQ4	0.07 ± 0.02 ($n = 6$)	0.21 ± 0.01 ($n = 15$)	2.1 ± 0.1 ($n = 12$)
KCNQ4 + NEM	0.18 ± 0.02 ($n = 6$)	0.195 ± 0.01 ($n = 9$)	2.0 ± 0.1 ($n = 10$)
KCNQ5	0.17 ± 0.03 ($n = 6$)	0.19 ± 0.02 ($n = 11$)	2.2 ± 0.1 ($n = 7$)
KCNQ5 + NEM	0.58 ± 0.1 ($n = 4$)	0.17 ± 0.02 ($n = 10$)	2.0 ± 0.2 ($n = 9$)
KCNQ2	0.17 ± 0.06 ($n = 6$)	0.55 ± 0.04 ($n = 6$)	6.2 ± 0.3 ($n = 6$)
KCNQ2 + NEM	0.60 ± 0.03 ($n = 7$)	0.56 ± 0.04 ($n = 7$)	6.3 ± 0.4 ($n = 4$)
KCNQ3	0.89 ± 0.05 ($n = 5$)	0.64 ± 0.07 ($n = 6$)	8.5 ± 0.3 ($n = 5$)

P_o and i were fitted by Gaussian curves from the single-channel currents at 0 mV. P_o was measured at 0 mV, which is very near maximal P_o in all cases. Values are mean $\pm$ SEM; n is the number of cells.

single-channel openings at saturating voltages because it has a P_o there near unity. For KCNQ4, KCNQ5, and KCNQ2, the threefold to fourfold augmentation by NEM on whole-cell currents at saturating voltages is attributable to the threefold to fourfold increase of the P_o of the channels at the single-channel level. Our data strongly suggest that the enhancement of macroscopic KCNQ2, KCNQ4, and KCNQ5 by NEM can be nearly all attributed to a voltage-independent increase in P_o of the channels rather than an increase in their unitary conductance or the number of functional channels in the cell membrane. Most of the action is at a cysteine in the C-terminal domain that seems to be critical for modulating M-type K⁺ channels.

Discussion

The existence of five members of the KCNQ family of K⁺ channels with distinct properties has provided us the opportunity to examine their function by making structural and physiological comparisons. The earlier observations by Roche et al. (2002) of

Figure 6. Single-channel analysis of NEM action. *A*, Left, Current records at 0 mV from a cell-attached patch containing two KCNQ4 channels before and after bath application of 50 μ M NEM. NEM increased the P_o from 0.03 to 0.17. Linopiridine (50 μ M) inhibited the openings in the patch. Middle, Mean P_o against a range of V_m for KCNQ4 channels in the presence or absence of NEM, fitted by Boltzmann equations. Before NEM (open circles), the maximal P_o , $V_{1/2}$, and k were 0.07 ± 0.02 , -18.8 ± 2.5 mV, and 9.4 ± 2.2 mV, respectively; after NEM (filled circles), they were 0.18 ± 0.02 , -24.1 ± 4.8 mV, and 13.2 ± 4.0 mV, respectively. Right, Linear fits of current–voltage relationships of single KCNQ4 channels plotted as unitary current amplitudes against a range of V_m . The unitary conductance was not significantly changed by NEM, which was 2.1 ± 0.1 pS before NEM (open circles) and 2.0 ± 0.1 pS after NEM (filled circles) ($n = 10$). *B*, Similar analysis of NEM action on single KCNQ5 channels. Left, Current records of a single KCNQ5 channel at -10 mV in a cell-attached patch. P_o increased from 0.1 (Control) to 0.5 (NEM). Middle, Mean P_o versus V_m yielding values for maximal P_o , $V_{1/2}$, and k . Before NEM (open circles), they were 0.17 ± 0.03 , -19.6 ± 5.6 mV, and 11.1 ± 5.4 mV, respectively; after NEM (filled circles), they were 0.58 ± 0.10 , -44.1 ± 3.9 mV, and 8.6 ± 3.5 mV ($n = 4$), respectively. Right, Fitted chord conductances for KCNQ5 channels of 2.2 ± 0.1 pS before NEM (open circles) and 2.0 ± 0.2 pS after NEM (filled circles) ($n = 7$). *C*, Left, Similar analysis of NEM effect for a cell-attached patch containing a single KCNQ2 channel. NEM increased the P_o from 0.12 to 0.38, and linopiridine (50 μ M) reduced it to near zero. Middle, P_o – V_m curves from the same patch yielded values for maximal P_o , $V_{1/2}$, and k of 0.36, -33.9 mV, and 13.2 mV, respectively, before NEM (open circles); after NEM (filled circles), they were 0.71, -54.9 mV, and 10.3 mV, respectively. Right, Fitted chord conductances of 6.2 ± 0.3 pS before NEM (open circles) and 6.3 ± 0.4 pS after NEM (filled circles) ($n = 4$). O and C represent the open-channel and closed-channel current levels, respectively.

large NEM action on KCNQ2 but little on KCNQ1 or KCNQ3 suggested that there might be important properties very different between the channels. Thus, we extended the analysis of NEM action to include KCNQ4 and KCNQ5 as a further comparative probe. We find that, as for KCNQ2, NEM augments currents of KCNQ4 and KCNQ5. For all three NEM-sensitive channels, the dramatic action is a voltage-independent increase in macroscopic current amplitudes. We investigated possible mechanistic explanations to account for these observations. Two possibilities presented themselves: NEM could increase macroscopic currents by rapidly promoting trafficking of channels to the plasma membrane (e.g., the exocytotic machinery protein first identified as the “NEM-sensitive factor”), or it could act by altering the gating or permeation properties of channels already present. After ruling out the former mechanism, we then turned to single-channel analysis of KCNQ2–5 channels. Although such analysis of KCNQ2 and KCNQ3 has been reported previously (Selyanko et al., 2001), there had been no such reports for KCNQ4 and KCNQ5.

Patch analysis of KCNQ4 and KCNQ5 shows both to have an unexpectedly small unitary conductance and low maximal P_o . Our analysis of the single-channel properties of KCNQ2 and KCNQ3 is mostly similar to that described previously (Selyanko

et al., 2001), including the observation that the maximal open probability of KCNQ3 is higher than that of KCNQ2. The action of NEM at the single-channel level is to increase P_o , with no change of unitary conductance. We had expected that the differential action of NEM between KCNQ3 and KCNQ2, KCNQ4, and KCNQ5 to be attributable to a cysteine present in the NEM-sensitive channels but not in KCNQ3. However, the differential effect of NEM at the whole-cell level can be explained by the very different tonic P_o of the different channels. KCNQ2, KCNQ4, and KCNQ5 all have rather low maximal P_o , and NEM alkylation increases this several-fold in all three cases, with the maximum effect possible being the inverse of maximal P_o in control. KCNQ3, however, already has a maximal P_o near unity; little further increase is possible; therefore, little augmentation of whole-cell currents is seen at saturating voltages. Thus, NEM reveals some important feature of KCNQ channels, different among the subtypes, that influences gating. The weak effect of NEM on whole-cell KCNQ3 currents is wholly consistent with this channel having a very high tonic P_o , and the increases in macroscopic current amplitudes and in unitary P_o observed among KCNQ2–5 channels are qualitatively congruent.

What structural component of KCNQ3 underlies this very high open probability? Our initial hypothesis was that it would localize to the C terminus. This supposition came from the C-terminal loci of putative binding sites on the channels of Ca^{2+} /calmodulin (Wen and Levitan, 2002; Yus-Najera et al., 2002; Gamper and Shapiro, 2003) and of domains important for subunit assembly (Schwake et al., 2003). The analysis of our chimeras at the macroscopic and single-channel levels indicates that our hypothesis is correct. The KCNQ4_N/Q3_C chimera that contains the C terminus of KCNQ3 has a very high maximal P_o like that of KCNQ3, and NEM has little action on its whole-cell currents. Conversely, the KCNQ3_N/Q4_C chimera that contains the C terminus of KCNQ4 has a very low maximal P_o like that of KCNQ4, and NEM strongly augments its whole-cell currents. These chimeras suggest that the C terminus of KCNQ3 must contain some unique structural features that confer high maximal P_o to the channel. Interestingly, our chimera junction site is downstream of the domain suggested to be a site of binding to PIP_2 (Zhang et al., 2003), indicating that differential affinity for PIP_2 is unlikely to be involved. Last, we confirm recent work implicating C-terminal domains in assembly and expression of KCNQ channels at the plasma membrane (Maljevic et al., 2003; Schwake et al., 2003).

Further supporting the role of the C terminus as a “channel-modulatory domain” is the localization of NEM action, on at least KCNQ4, to a cysteine just before the IQ₂ domain analogous to what we have implicated in calmodulin binding to KCNQ2 and KCNQ3 (Gamper and Shapiro, 2003). The strong reduction of NEM action on KCNQ4 by the C519A mutation localizes the

major site of action of NEM to the channel itself rather than on one of the many modulatory proteins that act on M-type channels. However, the secondary effects of NEM such as the shift in voltage dependence and the speeding of activation are equally possible because of alkylation of a modulatory protein as to alkylation of another channel cysteine. For example, strong actions on both of these parameters of KCNQ3–5 are caused by Src-tyrosine kinase (Gamper et al., 2003).

We considered whether we could link the augmentation of the currents and P_o at saturating voltages and the shift in voltage dependence to a common mechanism. Such a link would be possible if NEM alkylation caused a stabilization of the open state in a linear-type state gating scheme. Without going into undue detail here, such a scheme would predict NEM action to manifest itself in strong increases in current at saturating voltages for the channels with low maximal P_o (KCNQ2, KCNQ4, KCNQ5, and the KCNQ3_N/4_C chimera) but little shift in voltage dependence and little current augmentation for a channel with high maximal P_o (KCNQ3) but a large shift in voltage dependence. However, we see little correlation between channel maximal P_o and the NEM-induced shift and conclude that the two phenomena have different mechanisms. Similar conclusions have been reached for the dual actions of the compound retigabine on KCNQ channels, for which voltage-dependent and -independent modifications on channel P_o were ascribed to distinct mechanisms (Tatullian et al., 2001; Tatullian and Brown, 2003). It will be interesting to see whether the molecular determinants of retigabine action on the channels correspond to those found for NEM action and for the subunit-specific $P_{o,max}$ reported here.

Because KCNQ3 does not have a cysteine in the position analogous to 519 in KCNQ4, the near removal of NEM action in the C519A mutant could have been attributable simply to that mutation increasing its $P_{o,max}$ to near unity like KCNQ3, but that was not the case because KCNQ4-C519A had no higher maximal P_o than wt KCNQ4. Thus, the difference in $P_{o,max}$ conferred by the C terminus of KCNQ3 versus that of KCNQ4 is not simply attributable to the presence of a cysteine in that position. We posit that alkylation of KCNQ4 (and probably KCNQ2 and KCNQ5) at the 519 position mimics in some way the high- P_o action of the KCNQ3 C terminus and conclude that the localization of NEM action to a cysteine

Figure 7. NEM augmentation is conferred by the C terminus of KCNQ3 or KCNQ4. *A*, Diagram of the KCNQ3_N/4_C chimera (top left), consisting of most of the KCNQ4 C terminus grafted onto the rest of KCNQ3. The thick bars in the N and C termini indicate regions strongly conserved among KCNQ1–5 channels. Plotted are families of currents and activation curves generated from their normalized tail current amplitudes before and after NEM application (top right, bottom left). The dotted line is the control curve scaled up to match the post-NEM curve, and the bars (bottom right) are mean $V_{1/2}$ before and after NEM. The mean fitted $V_{1/2}$ was -26.9 ± 2.8 mV ($n = 5$) in control, not significantly different from the $V_{1/2}$ after NEM (-30.8 ± 2.8 mV; $n = 5$). *B*, Inverse KCNQ4_N/3_C chimera (left), consisting of the C terminus of KCNQ3 grafted onto the rest of KCNQ4. Right, Currents before and after NEM treatment from this chimera using a classical M-current protocol. The currents from this chimera were too small to generate meaningful activation curves. *C*, Bars are mean current densities of the two chimeras and of wt KCNQ3 and KCNQ4 for comparison. Those of KCNQ3_N/4_C and KCNQ4_N/3_C were 13.0 ± 3.5 and 4.6 ± 1.0 pA/pF ($n = 6$; $p < 0.05$), respectively. Those of wt KCNQ4 and KCNQ3 were 17.1 ± 5.1 and 3.0 ± 0.7 pA/pF ($n = 7$; $p < 0.05$), respectively. *D*, Bars are mean NEM-induced augmentations of current amplitudes of two chimeras ($p < 0.001$).

Figure 8. Single-channel analysis of NEM action on the KCNQ3/4 chimeras. *A*, Current records of a cell-attached patch containing a single KCNQ4_N/3_C channel at $V_m = -10$ mV before and after bath application of $50 \mu\text{M}$ NEM. The fitted P_o values from Gaussian curves were 0.92 in control and 0.94 after NEM. *B*, Current records of a cell-attached patch containing two KCNQ3_N/4_C channels at $V_m = -10$ mV before and after bath application of $50 \mu\text{M}$ NEM. The fitted P_o values from Gaussian curves were 0.014 in control and 0.09 after NEM. O and C represent the open-channel and closed-channel current levels, respectively.

Figure 9. The C519A mutation abolishes most of NEM augmentation of KCNQ4 currents. *A*, Whole-cell currents from a cell transfected with KCNQ4 C519A, elicited by a family of voltage steps in 10 mV increments from -80 to 50 mV before and after bath application of NEM. *B*, Activation curves generated by tail current analysis of currents like those in *A*, fitted by Boltzmann equations. The fitted values of $V_{1/2}$ and k were -21.0 ± 1.2 and 10.0 ± 0.7 mV ($n = 6$) in control (open circles) and -19.3 ± 1.1 and 9.8 ± 0.8 mV ($n = 6$) after application of $50 \mu\text{M}$ NEM (filled circles). *C*, Sweeps at $V_m = 0$ mV from a cell-attached patch containing at least two KCNQ4 C519A channels before and after application of $50 \mu\text{M}$ NEM. The mean P_o values of 22 similar sweeps were 0.11 in control and 0.14 after NEM application. *D*, Bars are mean augmentations by NEM of currents from wt KCNQ4 and three cysteine-to-alanine mutants. For wild-type KCNQ4, C519A, C427A, and C418A, they were 2.9 ± 0.4 ($n = 7$), 1.4 ± 0.1 ($n = 14$), 2.2 ± 0.2 ($n = 4$), and 2.5 ± 1.0 ($n = 4$), respectively. $**p < 0.01$.

that KCNQ3 does not have is, most probably, coincidence. Our earlier study (Roche et al., 2002) suggested that a cysteine in the S5 transmembrane domain could be a crucial site of NEM action on KCNQ2, but we do not have single-channel data to estimate the P_o of the C242T mutant used in that study. Indeed, the reported reduction of NEM augmentation from 5- to 2.5-fold by that mutation might have been the result of a twofold increase in maximal P_o from, say, 0.2 – 0.4 caused by the C242T mutation alone. However, because NEM still augments KCNQ4-C519A currents by 1.4-fold, alkylation of the analogous S5 cysteine may make some contribution.

The site of action at the C terminus of two inhibitory signaling pathways involving PIP_2 or intracellular $[\text{Ca}^{2+}]$ has suggested that domains here influence gating only in an inhibitory way. Our results in this work suggest that the C terminus may have an excitatory or stabilizing role as well. Two general mechanisms could be at work here. Gating of the channel could be maximally stabilized in the absence of an interaction between the C terminus and the rest of the channel, and inhibitory stimuli, such as PIP_2 depletion (Suh and Hille, 2002) or Ca^{2+} /calmodulin binding (Gamper and Shapiro, 2003), would be to promote such an interaction that would in some manner destabilize opening. In the context of the results presented here, such a scheme would envision the C terminus of KCNQ3 channels tonically most lacking this interaction, compared with the others, and NEM alkylation reducing it for KCNQ2, KCNQ4, and KCNQ5. The other scheme would involve the C terminus acting as an “autoexcitatory” domain, as is the role of the N terminus shown for cyclic nucleotide-gated channels (Varnum and Zagotta, 1997). In this scenario, an interaction between the C terminus and the rest of the channel stabilizes opening. In the context of this paper, the autoexcitatory model would predict that the C terminus of KCNQ3 in particular is able to maximally fulfill this stabilizing role, and NEM alkylation

enhances it for KCNQ2, KCNQ4, and KCNQ5. We look forward to further analysis of the structural mechanism by which the C termini of M-type channels regulate gating.

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