KCNQ1/KCNE1 assembly, co-translation not required

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Key words: potassium channel, accessory subunit, channel assembly, protein purification, trafficking

Abbreviations: KV, voltage-gated potassium channel; prKCNE1, purified human KCNE1 protein; IKS, slow delayed rectifier current; ER, endoplasmic reticulum; CHX, cycloheximide; BFA, brefeldin A

Introduction

Voltage-gated potassium (KV) channels are often assembled with accessory proteins that increase their functional diversity. KCNE proteins are small accessory proteins that modulate voltage-gated potassium (Kv) channels. Although the functional effects of various KCNE proteins have been described, many questions remain regarding their assembly with the pore-forming subunits. For example, while previous experiments with some Kv channels suggest that the association of the pore-subunit with the accessory subunits occurs co-translationally in the endoplasmic reticulum, it is not known whether KCNQ1 assembly with KCNE1 occurs in a similar manner to generate the medically important cardiac slow delayed rectifier current (IKs). In this study we used a novel approach to demonstrate that purified recombinant human KCNE1 protein (prKCNE1) modulates KCNQ1 channels heterologously expressed in Xenopus oocytes resulting in generation of IKs. Incubation of KCNQ1-expressing oocytes with cycloheximide did not prevent IKs expression following prKCNE1 injection. By contrast, incubation with brefeldin A prevented KCNQ1 modulation by prKCNE1. Moreover, injection of the trafficking-deficient KCNE1-L51H reduced KCNQ1 currents. Together, these observations indicate that while assembly of KCNE1 with KCNQ1 does not require co-translation, functional KCNQ1-prKCNE1 channels assemble early in the secretory pathway and reach the plasma membrane via vesicular trafficking.

Voltage-gated potassium channels are often assembled as heteromultimeric complexes consisting of pore-forming (α) subunits and accessory proteins. Members of the KCNE family are small accessory proteins with a single-transmembrane domain that function to control or modulate Kv channels in tissues such as the heart, cochlea and small intestine.1,5 Heterologous expression experiments have demonstrated that KCNE proteins alter the properties of several Kv channels,1,5 and that all KCNE proteins (KCNE1-KCNE5) interact with KCNQ1 (Kv7.1), each yielding a distinct phenotype.4,5 Thus, the observed functional properties of KCNQ1, and other Kv channels, vary depending on the associated KCNE subunit. KCNE1 affects several biophysical properties of KCNQ1 channels (i.e., increased whole-cell current density, slower activation and activation at depolarized potentials) to generate the slow delayed rectifier current, IKs.6,7

Although many studies describe the effects of KCNEs on Kv channel function, many questions remain regarding the structural biology of KCNE proteins and their assembly with α subunits. The structural determinants of KCNE1 modulation of KCNQ1 have been inferred by mutagenesis, chimera and chemical modification experiments,8,20 and recently the structure of full-length human KCNE1 has been obtained by solution NMR analysis.21 Nevertheless, questions remain regarding the timing and subcellular location for the assembly of KCNE proteins with the pore-forming subunits. Some studies suggest that KCNE1 and KCNQ1 can associate at the plasma membrane,22,23 but others propose that assembly occurs early in the secretory pathway.24,25 Previous experiments with other Kv channels suggest that the association of α-subunit with the accessory subunits Kvβ and KChAP occurs co-translationally.26,27 Both KChAP27 and Kvβ29 are soluble cytosolic proteins. However, KCNEs are integral membrane proteins29 and their assembly with Kv channels may differ from that of cytoplasmic accessory proteins.

In this study we investigated KCNE1 and KCNQ1 assembly using a novel approach in which Xenopus oocytes expressing human KCNQ1 were injected with purified recombinant KCNE1 protein (prKCNE1). The injected protein modulated KCNQ1 function rapidly after protein injection (t½ ~ 3 h) as determined by the appearance of IKs at the plasma membrane. Modulation of KCNQ1 by injected prKCNE1 was not prevented by inhibiting new KCNQ1 synthesis with cycloheximide. However, modulation of KCNQ1 channels by prKCNE1 was inhibited by the...
secretory pathway blocker brefeldin A. Together these observations indicated that assembly of KCNE1 with KCNQ1 can occur independent of protein translation, and that functional KCNQ1–prKCNE1 channels assemble early in the secretory pathway then reach the plasma membrane through vesicular trafficking.

Results and Discussion

Time-course of prKCNE1 modulation of KCNQ1 channels. Our previous results demonstrated that purified recombinant human KCNE1 protein (prKCNE1) injected into Xenopus oocytes associates with KCNQ1 channels translated in vivo to generate Ikᵣ.10 In this study we have determined the time-course for the functional interaction of prKCNE1 with KCNQ1 following protein injection by monitoring the appearance of Ikᵣ currents. Figure 1B illustrates current traces recorded from KCNQ1-expressing oocytes at 2, 4, 6 or 18 h following prKCNE1 injection. Addition of prKCNE1 slowed channel activation and increased the current magnitude in a time-dependent manner. We were unable to measure current prior to 2 h post protein-injection because the oocytes had excessive leak. Figure 1C depicts the apparent half-maximal activation voltage (V½) determined for the whole-cell currents at each time point (solid bars). At the early time points (e.g., 2–4 h post prKCNE1 injection) isochronal activation curves from individual cells have variable shifts in V½ (note larger standard error for these time points in Fig. 1C) while whole-cell current recordings exhibit complex gating suggesting that these currents are carried by two channel populations: KCNQ1-alone and KCNQ1–KCNE1 channels (Fig. S1). The time-course for prKCNE1 functional interaction with KCNQ1 was determined by plotting the change in V½ (∆V½) as compared with KCNQ1-only channels (Fig. 1D). The data were fit with a monoexponential function yielding an apparent t½ of ∼3 h (Fig. 1D). These results demonstrated that prKCNE1 solubilized in detergent micelles readily associates with endogenously processed KCNQ1 channels, and that most of the KCNQ1 channels at the plasma membrane appear fully modulated by prKCNE1 10–12 h after protein injection. Moreover, these data also indicated that KCNQ1-KCNE1 assembly does not require the simultaneous translation of both proteins.

IKᵣ channel assembly does not require new KCNQ1 synthesis. Previous experiments with other Kᵥ channels indicated that the association of α-subunit with the accessory subunits Kᵥβ and KChAP occurs cotranslationally while the nascent α-subunit is still attached to the ribosome complex.26,27 In order to test whether prKCNE1 associates with KCNQ1 during α-subunit translation, we inhibited protein synthesis with cycloheximide [CHX (50 µg/ml), Suppl. Results (Fig. S2) show that CHX prevented functional expression of KCNQ1 channels]. Block of KCNQ1 synthesis was inferred by the decrease in functional KCNQ1 expression following exposure to CHX. In these experiments, we delayed the application of CHX for 48 h after KCNQ1 cRNA injection in order to obtain a measurable number of functional KCNQ1 channels at the plasma membrane. Current decrease is assumed to be caused by the removal of KCNQ1 channels from the plasma membrane by constitutive endocytosis coupled with disruption of channel synthesis. As illustrated in Figure 2A, KCNQ1-expressing oocytes exposed to ethanol (EtOH, vehicle) exhibited steadily increasing current levels throughout the experiment phase (KCNQ1 expression has not peaked after 48 h). In contrast, current amplitude recorded from KCNQ1-expressing oocytes treated with CHX initially rose but then progressively declined after ∼6 h incubation (Fig. 2A).

We then tested the effect of blocking KCNQ1 synthesis on co-assembly with prKCNE1 by first treating KCNQ1-expressing
Channels Volume 4 Issue 2

110

KCNQ1-expressing oocytes (48 h post cRNA injection) were first exposed to BFA or EtOH (vehicle) for 3 h, sufficient time to detect the block of vesicle delivery to the plasma membrane (Fig. 3A). Both treatment groups were then injected with prKCNE1 and further incubated with CHX or EtOH for 8 h. This period allows for consistently observing KCNQ1 modulation following prKCNE1 injection (Fig. 1). Figure 2B illustrates representative whole-cell currents recorded from KCNQ1-expressing oocytes treated with CHX or EtOH and injected with prKCNE1. Whole-cell currents measured at +50 mV and V_½ values were similar for both treatments [Fig. 2C, CHX (solid bars), EtOH (empty bars)]. Longer incubations with CHX or EtOH yielded similar results (24 h post protein injection, data not shown). These results show that incubation of KCNQ1-expressing oocytes with the protein-synthesis inhibitor CHX did not prevent expression of I_Ks following prKCNE1 injection. This observation strongly suggests that correct assembly of functional KCNQ1-KCNE1 channel complexes does not require co-translation of the two proteins in this system. The post-translational assembly of KCNE1 with KCNQ1 suggests that these proteins could associate outside the ER.

This is a distinct mechanism than the co-translational assembly of the voltage-gated K^+ channel K_\text{ca,1.2} with the accessory subunits K_\text{ca,β} and KChAP.26,27 This difference may be due to the accessory protein. Both KChAP26 and K_\text{ca,β} are soluble cytosolic proteins, while KCNE1 is a trans-membrane protein.28 Interestingly, the assembly of the α-subunit of the voltage-gated rat brain Na^+ channel with its accessory subunit β, another trans-membrane accessory protein,29 also occurs post-translationally.30 Whether co-translation-independent assembly of KCNQ1 with KCNE1 is subunit specific or a property shared by all KCNE proteins is unresolved.

KCNQ1 modulation by prKCNE1 requires vesicular trafficking. Previous studies demonstrated that proteins injected into Xenopus oocytes reach many compartments including the plasma membrane.33-35 We envisioned two possible pathways for prKCNE1 to reach the plasma membrane: simple diffusion from the cytoplasm to the plasma membrane, or vesicular trafficking following the insertion of prKCNE1 into the endoplasmic reticulum (ER) or Golgi membranes. Brefeldin A (BFA) is a fungal metabolite that blocks anterograde trafficking of secretory vesicles from the ER and causes the movement of Golgi membranes into the ER.36 BFA was demonstrated to inhibit the insertion of newly synthesized channel proteins [ENaC37 and ROMK38] into the plasma membrane of Xenopus oocytes. BFA (5 μM) also prevents KCNQ1/KCNE1 complexes from reaching the plasma membrane (Suppl. Results, Fig. S3). Figure 3A shows that BFA exposure decreased whole-cell currents recorded from oocytes injected with KCNQ1 and KCNE1 cRNAs. Current decrease occurred as early as ∼3 h after BFA exposure, and did not occur with EtOH (vehicle) treatment alone. These results also indicated that KCNQ1-KCNE1 channels were removed from the plasma membrane with a t_{1/2} ∼ 9 h (Fig. 3A, solid line). Interestingly, a turnover rate of ∼11 h was reported for KCNQ1-KCNE1 channels in COS-7 cells.39

To test whether prKCNE1 reaches the plasma membrane by vesicular trafficking through the secretory pathway, KCNQ1-expressing oocytes (48 h post cRNA injection) were first exposed to BFA or EtOH (vehicle) for 3 h, sufficient time to detect the block of vesicle delivery to the plasma membrane (Fig. 3A). The BFA- or EtOH-treated oocytes were then injected with prKCNE1 and further incubated in either BFA or EtOH for 6 or 15 h. These time intervals were selected because KCNQ1 modulation by prKCNE1 was evident at 6 h post protein injection (Fig. 1) and 15 h should be sufficient time for prKCNE1 to diffuse to the plasma membrane (typical oocyte radius = 0.5 mm). Figure 3B depicts representative whole-cell currents recorded from KCNQ1-expressing oocytes exposed to either EtOH (top traces)
incubations and currents recorded ~18 h later, but only non-modulated KCNQ1 currents were detected in the prKCNE1-injected oocytes. In summary, exposure to BFA prevented the appearance of $I_{Ks}$ following prKCNE1 injection.

Although the results indicate that vesicular trafficking was required for prKCNE1 and KCNQ1 to form functional $I_{Ks}$ channels at the plasma membrane, these results do not determine whether prKCNE1 associates with KCNQ1 in an intracellular compartment (i.e., ER or Golgi) and the assembled channel complex reaches the plasma membrane by vesicular trafficking, or whether prKCNE1 is transported by vesicular trafficking to the plasma membrane where it associates with KCNQ1 channels that are already present. KCNE1 and KCNQ1 assembly has been proposed to occur at either the plasma membrane or the ER. Previous studies demonstrated that proteins injected into Xenopus oocytes reach many compartments including the plasma membrane. If the injected KCNE1 protein could form functional $I_{Ks}$ channels by associating with KCNQ1 in a post-ER/Golgi compartment (i.e., plasma membrane), we would have observed $I_{Ks}$-like currents following injection of prKCNE1 into KCNQ1-expressing oocytes treated with BFA. However, only KCNQ1 currents were recorded following BFA treatment suggesting that functional assembly of prKCNE1 with KCNQ1 at the plasma membrane does not occur.

BFA block of KCNQ1 modulation by prKCNE1 demonstrates that vesicular traffic, not direct fusion to the plasma membrane, is required for the appearance of functional prKCNE1-KCNQ1 channels. This observation suggests that functional assembly of prKCNE1 with KCNQ1 occurs early in the secretory pathway (i.e., ER or Golgi). This is a similar pathway to that reported for the Shaker $K^+$ channel and accessory subunits. Experiments by Nagaya and Papazian demonstrated that Shaker $K^+$ channel $\alpha$ subunits assemble with $K_V\beta$ accessory subunits in the ER, and together are transported to the Golgi apparatus in route to the plasma membrane.

In order to further localize the site of prKCNE1-KCNQ1 assembly we injected KCNQ1-expressing oocytes with the non-dominant, trafficking-deficient KCNE1-L51H mutant protein. This mutant KCNE1 does not reach the plasma membrane, it associates with KCNQ1 in the ER and prevents the channel from reaching the plasma membrane, thus reducing KCNQ1 currents without altering the kinetics. Figure 4A illustrates whole-cell currents and current-voltage relationships obtained from KCNQ1-expressing oocytes before and ~18 h after injecting either prKCNE1 (upper trace) or prKCNE1-L51H (lower trace). Injection of prKCNE1 into KCNQ1-expressing oocytes yielded $I_{Ks}$ currents, while introduction of prKCNE1-L51H reduced current amplitude without altering the kinetics.

The results with the trafficking-deficient KCNE1-L51H suggest that KCNQ1-prKCNE1 assembly may occur in the ER. Experiments carried out with KCNQ1 and KCNE1 in HEK-293, CHO and COS-7 cells support the notion that KCNQ1-KCNQ1 assembly occurs early in the secretory pathway, most likely in the ER. KCNE1 has a tetrapeptide motif (KKLE) positioned in a cytosolic juxtamembrane domain that resembles known ER retention signals for type I membrane proteins. This raises the intriguing
An alternative explanation is that only prKCNE1 that enters the early secretory pathway and associates with KCNQ1 is properly glycosylated,25 and this protein represents a very small fraction of the total amount injected. This would be reasonable as our calculations indicate that ∼0.025% of the injected prKCNE1 forms functional complexes with KCNQ1 channels at the plasma membrane. These calculations are based on the whole-cell current amplitude measured after prKCNE1 injection (2 µA at +50 mV), the single-channel conductance for I_Ks channels (8pS), the estimated open probability (0.5), a subunit stoichiometry of two prKCNE1 molecules per IKs channel, and ∼8 ng prKCNE1 injected per oocyte.

Methods

Functional analysis. Complementary DNAs encoding KCNQ1 and KCNE1 were constructed in plasmid vectors pSP64T and pRc/CMV, respectively, as previously described.12 Briefly, Xenopus laevis oocytes (stage V-VI) were microinjected with either sterile...
water (control) or cRNA that was transcribed in vitro from EcoRI (pSP64T-KCNQ1) or XbaI (pRC/CMV-KCNE1) digested linear DNA templates using Sp6 or T7 RNA polymerase and the mMessage mMachine transcription system (Ambion Inc., Austin, USA). Injected oocytes were incubated at 18°C for 48–96 h in Leibovitz’s media (Invitrogen, Carlsbad, USA) diluted 1:1 with water and supplemented with penicillin (150 units/ml) and streptomycin (150 µg/ml).

Whole-cell currents were recorded at room temperature (RT) 1–4 d after injections with an OC-725B amplifier (Warner Instruments Corp., Hamden, USA). Oocytes were bathed at RT (22–25°C) in a modified ND96 solution (in mM: 96 NaCl, 4 KCl, 2 MgCl₂, 0.1 CaCl₂, 5 HEPES, pH 7.6, ~200 mosmol/kg). Cycloheximide (CHX) and brefeldin A (BFA) stocks were dissolved in ethanol (EtOH). Currents were recorded using Clampex 7 (Molecular Devices Corp., Sunnyvale, USA), filtered at 500 Hz and digitized at 2 kHz. Data were analyzed and plotted using Clampex, SigmaPlot 2000 (SPSS Science, Chicago, USA), SigmaStat (SPSS Science) and Origin 7.0 (OriginLab, Northampton, USA) softwares. Current-voltage and normalized isochronal voltage-activation relationships were obtained by measuring current at 2 s during depolarizing pulses between -50 and +60 mV from a holding potential of -80 mV. The normalized isochronal data were fit with a Boltzmann function of the form: 1/[1 + exp((V - V1/2)/k)], where V1/2 is the apparent half-maximal activation voltage and k is the slope factor. At early time points (e.g., 2, 4 hours) after prKCNE1 injection when there may be mixed channel populations, single Boltzmann fits serve to approximate an average V1/2. Oocytes with base-line currents larger than those measured for water-injected oocytes (typically ~0.10 µA at -80 mV) were considered leaky and not used.

Overexpression, purification and incorporation of prKCNE1 and prKCNE1-L51H into Xenopus oocytes. The expression, purification and reconstitution of recombinant human KCNE1 protein have been previously described.9 Briefly, the cDNA for recombinant human His₆-tagged KCNE1 was expressed in bacteria, solubilized from inclusion bodies, purified on nickel-NTA agarose resin and eluted as detergent micelles in 0.2% 1-myristyryl-2-hydroxy-sn-glycero-3-[phosphor-rac-(1-glycerol)] (LMPG, Avanti Polar Lipids Inc., Alabaster, USA).

Oocytes were first injected with KCNQ1 cRNA (6 ng) and incubated for 24 or 48 h. Following incubation, KCNQ1-expressing oocytes were injected with 10 nl of protein-containing (0.8 mg/ml) or protein-free detergent micelles.

Biochemical analysis. KCNQ1-expressing oocytes (48 h post cRNA injections) were injected with prKCNE1 and harvested ~18 h later. KCNQ1 and KCNQ1-cRNA-injected oocytes were harvested 64–70 h post cRNA injections. Oocyte membranes were prepared using a previously published method and western blotting was performed as described previously.45–49 Immunoblot for KCNE1 proteins were performed overnight at 4°C with anti-KCNE1 (1:200; Alomone Labs, Jerusalem, Israel), and for KCNQ1 proteins for 1 h at room temperature with a goat anti-KCNQ1 (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, USA). PNGase-F treatment was performed according to the supplier’s instructions (New England Biolabs, Ipswich, USA).

Chemicals were from Sigma (St. Louis, USA) unless otherwise stated. All experimental conditions were tested in oocytes from at least three frogs. Data are represented as means ± SEM, and in some figures error bars are smaller than the symbols. The numbers of oocytes used in experiments (N) are provided in the figure legends. Statistical significance was determined using unpaired Student’s t-test or one-way ANOVA, (pairwise comparisons analyzed with the Tukey Test).

Conclusion

In summary, our results strongly suggest that purified recombinant KCNE1 protein associates with KCNQ1 channels early in the secretory pathway, possibly in the ER, and that the complex is then transported to the plasma membrane by vesicular trafficking. Our results do not exclude that prKCNE1 reaches other compartments including the plasma membrane, or that prKCNE1 and KCNQ1 may associate at the plasma membrane. However, our results indicate that these proteins do not assemble into functional Iᵥ channels at this location. Our results also demonstrated that KCNE1 and KCNQ1 do not require cotranslation for proper assembly. This is a different mechanism than that observed for assembly of Kᵥ channels with cytoplasmic accessory subunits. The observation that these channel proteins can assemble post-translationally raises interesting questions about how specific Kᵥ–KCNE complexes are formed in tissues known to express more than one type of KCNE and Kᵥ channel protein.44–46 The use of recombinant KCNE proteins and Kᵥ channels expressed in Xenopus oocytes may assist in answering these important questions.

Acknowledgements

This work was supported by grants from the Vanderbilt Discovery Grant Program (Carlos G. Vanoye) and DK061359 (Carlos G. Vanoye), HL077188 (Alfred L. George Jr) and DC00716 (Charles R. Sanders) from the National Institutes of Health.

Note

Supplementary materials can be found at:

www.landesbioscience.com/supplement/VanoyeCHAN4-2-Sup.pdf

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