Phosphorylation-dependent Functional Coupling of hSlo Calcium-dependent Potassium Channel and Its hβ4 Subunit*

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The auxiliary β4 subunit of the brain slowpoke calcium-dependent potassium (slo) channel is expressed predominantly in the brain. Co-expression of β4 subunit with the slo channel α subunit in HEK293 and Chinese hamster ovary cells slows channel activation and deactivation and also shifts the voltage dependence of the channel to more depolarized potentials. We show here that the functional interaction between the hβ4 subunit and the slo channel is influenced by the phosphorylation state of hβ4. Treatment of cells with okadaic acid (OA) reduces the effect of hβ4 on slo channel activation kinetics and voltage dependence but not on slo channel deactivation kinetics. The effect of OA can be blocked by mutating three putative serine/threonine phosphorylation sites in hβ4 (Thr-11/Ser-17/Ser-210) to alanines, suggesting that OA potentiates phosphorylation of hβ4 and thereby suppresses its functional coupling to the slo channel. Mutation of Ser-17 alone to a negatively charged residue (S17E) can mimic the effect of OA. Mutating all three phosphorylation sites in hβ4 to negatively charged residues (T11D/S17E/S210E) not only suppresses the effect of hβ4 on slo channel activation kinetics and voltage dependence, it also suppresses its effect on slo channel deactivation kinetics. Co-immunoprecipitation/Western blot experiments indicate that all of these hβ4 mutants, as well as the wild-type hβ4, bind to the slo channel. Taken together, these data suggest that phosphorylation of the β4 subunit dynamically regulates the functional coupling between the β4 subunit and the pore-forming α subunit of the slo channel. In addition, phosphorylation of different residues in hβ4 differentially influences its effects on slo channel activation kinetics, deactivation kinetics, and voltage dependence.

Large conductance Ca2+-dependent potassium (KCa) or maxi K) channels are ubiquitously expressed in neurons and many other tissues. They contribute to action potential repolarization and influence neurotransmitter release (1–4). Although the native KCa channels exhibit diverse phenotypic properties, there is only a single gene encoding this potassium channel. The slowpoke gene that encodes the KCa channel has been cloned in many species (5–8). Alternative mRNA splicing of this gene can potentially generate a large number of distinct channel isoforms (9). Yet another mechanism of generating KCa channel diversity is to associate the pore-forming α subunit with a group of auxiliary β subunits (10–15). The β subunits influence such diverse aspects of KCa channel function as kinetic behavior, voltage dependence, and sensitivity to toxins and modulators (11, 13, 16–19).

Like other types of potassium channels, as well as sodium and calcium channels, native and recombinant KCa channels are modulated by post-translation modification such as phosphorylation and oxidation/reduction (20–25). Furthermore, KCa channels from brain have been shown to be intimately associated with protein kinase and phosphoprotein phosphatase activity (4, 20, 26–28). There is considerable variation in the sensitivity of different types of KCa channel to protein kinase modulation (24, 29). This variation could be due to different subunit composition of native KCa channels, providing different substrates for post-translational modification (11, 14, 19, 30, 31). In the case of voltage-activated potassium (Kv) channels and L-type calcium channels, it is known that the β subunits of these channels are substrates for protein kinases (32–35). It has been shown previously that the human β4 subunit influences slo channel activation kinetics and voltage dependence (11, 13, 15). In the present study we investigated the effect of phosphorylation of the β4 subunit on its functional interaction with the slowpoke α subunit.

EXPERIMENTAL PROCEDURES

Cloning and Transient Expression in CHO Cells—hβ4 was cloned into the pRRES2-EGFP vector (CLONTECH), a bicistronic vector that allows co-expression of hβ4 and green fluorescent protein in the same cell. We used the Quick-change protocol (Stratagene) to make point mutations in the β4 subunit. Briefly, a PCR was performed, using the wild-type β4 as the template and a pair of complementary mutagenesis primers. The PCR mixture was then cut with the enzyme DpnI to digest the template wild-type β4. After DpnI digestion, the PCR product was used to transform competent bacterial cells, and the mutant plasmid of β4 was then amplified. All mutant constructs were verified by sequencing (University of Pennsylvania Sequencing Facility, Philadelphia).

CHO cells were maintained in Ham’s F-12 nutrient mixture supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were seeded on 35-mm culture dishes and transfected 2 days later with the appropriate slo and β4 DNA using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals).

Electrophysiology—CHO cells were used for recording 1–3 days after transfection. The slo channel was cloned into the pcDNA3 vector. The β4 subunit was cloned into the pRRES2-EGFP vector (CLONTECH). Cells were transfected with both constructs, and transfected cells were identified by their green fluorescence; all such cells were found to express slo current. Recording electrodes were pulled to have resistances of 1.5–2.0 megohms when filled with regular pipette solution. slo current was recorded in the whole-cell recording mode. Solutions for the

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1 The abbreviations used are: CHO, Chinese hamster ovary; OA, okadaic acid; PKA, cAMP-dependent protein kinase; h, human.
recording as follows: bath solution, 130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, pH 7.2; and pipette solution, 150 mM KCl, 0.5 mM MgCl₂, 3.4 mM CaCl₂, 5 mM EGTA, 10 mM HEPES. Recording was performed using an Axopatch 200A amplifier and pClamp8 software (Axon Instruments, Foster City, CA). Data were analyzed off line using pClamp8 software. For measuring the time course of slo channel activation and deactivation, the cells were held at −80 mV, and the membrane potential was stepped to +40 mV for 200 ms and then back to −80 mV. For measuring the current-voltage relationship of the slo channel, the cells were held at −80 mV, and depolarizations in steps of 20 mV were then applied for 200 ms. The maximum instantaneous tail current after stepping the membrane potential back to −80 mV was measured. To quantify the activation kinetics, the activation of the slo current was fitted with a two-exponential function, giving rise to a fast and a slow time constant (τ). Similarly, the deactivation current was fitted with a two-exponential function. The half-activation voltage (V_{1/2}) was derived by fitting the current-voltage relationship curve with a Boltzmann function. All results were expressed as mean ± S.E. Statistical significance was assessed using the Student's t test in SigmaPlot software.

**RESULTS**

**Time-dependent Change of Slo Channel Activation Kinetics in the Whole-cell Recording Configuration**—We showed previously that β4 slows slo channel activation in the inside-out recording configuration (11). Similar modulation of slo channel activation by β4 was observed in the whole-cell recording configuration (Fig. 1, A and B). However, this effect of β4 on slo channel activation kinetics gradually decreased with time after going into whole-cell mode. In cells expressing both slo and β4, the time constant of slo channel activation decreased over the 3-min recording period, from an initial value of 3.4 ± 0.6 ms to a plateau level of about 0.8 ms (Fig. 1E). In contrast, in cells expressing slo alone, the time constant of slo channel activation stayed unchanged over the same recording period (Fig. 1, C, D, and E). We have found a two-exponential function was necessary to fit accurately the time course of slo channel activation and deactivation. Because both the fast and the slow time constants were modulated similarly in the following experiments, as summarized in Table I, we have presented only the fast time constants in the figures.

Co-expression with β4 also slowed slo channel deactivation in the whole-cell recording mode. Unlike the time-dependent change of slo channel activation in the presence of β4, the time constant of slo channel deactivation did not change with time (Fig. 1, A, B, and F). These data suggest that, in the presence of β4, there was a time-dependent change of slo channel activation kinetics attributable to the whole-cell recording configuration. This change with time in the presence of β4 was unlikely to be due to the time required for Ca²⁺ equilibration, which is much faster. Note that the time-dependent change in activation time constant was seen only in cells expressing both slo and β4 and not in cells expressing slo alone (Fig. 1E). In addition, the time constant of slo channel deactivation did not change with time in the absence or presence of β4 (Fig. 1F). All of these data suggest that the concentration of intracellular free Ca²⁺ was equilibrated in less than 30 s. The same time-dependent change in slo channel activation was also observed when the slo channel was co-expressed with mutant h4β with three serine/threonine residues mutated to alanines (T11A, S17A, S210A). The mechanism of this time-dependent change in slo channel activation has yet to be investigated, but it is unlikely that phosphorylation of h4β is responsible for this phenomenon.

**Inhibition of Phosphoprotein Phosphatase with Okadaic Acid Suppresses the Effect of β4 on slo Channel Activation**—Sequence analysis using ScanProsite revealed three potential

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2 H. Wen and I. B. Levitan, unpublished observations.
Phosphorylation-dependent Coupling of hβ4 to hSlo Channel

TABLE I

| Time constant of hSlo channel (mean ± S.E.) |
|-------------------------------------------|
| hSlo                                      | hSlo + hβ4                                      | hSlo + hβ4 + OA
dehphosphorylated (B) | hSlo + hβ4 AAA (C) | hSlo + hβ4 DEE (D) | hSlo + hβ4 S17E |
| τ₁ (ms) | 0.59 ± 0.05 | 2.34 ± 0.24 | 1.47 ± 0.22 | 3.25 ± 0.46 | 3.72 ± 0.84 | 0.99 ± 0.12 | 1.56 ± 0.17 | 9.40 ± 0.72 |
| τ₂ (ms) | 4.38 ± 0.49 | 13.51 ± 1.22 | 10.31 ± 1.44 | 17.81 ± 1.61 | 23.53 ± 3.65 | 6.94 ± 0.74 |

OA, okadaic acid 0.5 μM.
AAAA, T11A/S17A/S210A.
DEE, T11D/S17E/S210E.

serine/threonine phosphorylation residues in hβ4 (S11EE, S15IR, and RKF420). The serine at position 11 is a consensus casein kinase II phosphorylation site; the serine at position 17 is a protein kinase C site; and the serine at position 210 is a PKA site. We were interested in whether phosphorylation of β4 could modulate its functional interaction with the slo channel. Preincubating cells expressing both slo and β4 in 0.5 μM okadaic acid (OA) for 10–30 min largely blocked the effect of β4 on channel activation (Fig. 2A). This difference in channel activation was better revealed in the normalized current (normalized to the maximum current amplitude) as shown in Fig. 2B. Treatment of cells expressing both β4 and slo with OA decreased the time constant of slo channel activation, although the time constant of activation is still greater than that in cells expressing slo alone. These differences in slo channel activation are quantified in Fig. 2C. In cells expressing both slo and β4, OA treatment reduced the time constant of slo channel activation from 2.3 ± 0.2 to 1.5 ± 0.2 ms (p < 0.001) (Fig. 2C). The same OA treatment in cells expressing slo alone did not significantly change channel activation (p > 0.5) (Fig. 2C).

The effect of OA treatment on slo channel activation kinetics could be due to a change in the phosphorylation state of either the slo channel or the β4 subunit. We tried to distinguish these two possibilities by mutating all three putative phosphorylation residues in hβ4 to alanines (the β4 AAA mutation). The β4 AAA mutant increased the activation time constant to the same extent as the wild-type β4 (p > 0.05) (Fig. 2D). Interestingly, in cells expressing both slo and β4AAA, OA treatment did not alter the effect of β4 AAA on slo channel activation (Fig. 2D).

Mutation of the hβ4 Serine/Threonine Phosphorylation Sites to Negatively Charged Residues Suppresses the Effect of β4 on slo Channel Activation—The results with okadaic acid treatment and β4 AAA mutation suggest that phosphorylation of β4 could dynamically modulate the functional interaction between β4 and the slo channel. To test this hypothesis further, and to determine the critical phosphorylation site in β4, we mutated the three potential serine/threonine phosphorylation sites individually and in combination. Mutation to alanine removes any possibility of that residue being phosphorylated, and mutation to a negatively charged amino acid is assumed to mimic phosphorylation. Cells were transfected with slo and wild-type β4 or various mutant β4s, and the time constant of slo channel activation was measured. As shown in Fig. 3A, mutating all three phosphorylation sites to negatively charged residues (β4 DEE) largely suppressed the effect of β4 on slo channel activation. The single mutation β4 S17E was sufficient to suppress the effect of β4 on slo channel activation. Neither the single mutation T11D nor the single mutation S210E had any effect on the functional interaction between β4 and the slo channel (data not shown). These data are quantified in Fig. 3B. The triple mutation β4 DEE decreased the effect of β4 on the time constant of slo channel activation; the time constant of activation was 2.3 ± 0.2 ms for the wild-type β4 and 1.0 ± 0.1 ms for the β4 DEE mutation (p < 0.001) (Fig. 3B). The time constant of activation for the triple mutation β4 DEE was still greater than that for slo alone (p < 0.002). The single mutation β4 S17E suppressed the effect of β4 on channel activation; in the presence of β4 S17E the activation time constant was 1.6 ± 0.2 ms (p < 0.01) (Fig. 3B). This effect of the single mutation β4 S17E is similar to that of OA treatment (p > 0.7) (compare Figs. 2B and 3B). Mutating Ser-17 to alanine had no effect on the action of hβ4 on channel activation (Fig. 3B).

The Triple Mutation hβ4 DEE Suppresses the Effect of β4 on slo Channel Deactivation—Co-expressing β4 with the slo channel slowed slo channel deactivation (Fig. 1F). We tested whether the effect of β4 on the deactivation kinetics of the slo channel is also modulated by phosphorylation of the β4 subunit. Preincubating cells expressing both slo and β4 in 0.5 μM OA for 10–30 min did not alter the effect of β4 on the time constant of slo channel deactivation (p > 0.2) (Fig. 4, A and B). OA treatment also did not change the time constant of slo channel deactivation in cells expressing slo alone (Fig. 4B).
expressing slo alone (p > 0.9) (n = 7).

However, when we mutated all three putative serine/threonine phosphorylation sites in β4 to negatively charged residues (β4 DEE mutation), the effect of β4 on the deactivation kinetics of the slo channel was eliminated (Fig. 5, A and B). In cells expressing both slo and the triple mutation β4 DEE, the time constant of slo channel deactivation was 1.3 ± 0.3 ms, which was significantly different from that in cells expressing slo alone and wild-type β4 (p < 0.001) (Fig. 5B). The single mutation β4 S17E was not sufficient to suppress the effect of β4 on slo channel deactivation kinetics (p > 0.2) (Fig. 5B). β4 S17A was as effective as wild-type β4 in increasing the deactivation time constant (Fig. 5B).

**Phosphorylation-dependent Modulation of slo Channel Voltage Dependence by β4**—We showed previously in the inside-out recording configuration that β4 modulates the voltage dependence of the slo channel (11). Under the conditions we used in the present study (i.e., the whole-cell recording configuration and overexpression in CHO cells), the current amplitude of the slo channel was so large (up to 20 nA) that an accurate measurement of the current-voltage relationship of the slo channel was impossible. In addition, we had limited our recording to the initial 30 s in order to see the modulation of the slo channel by β4, and this does not allow sufficient time to make the necessary input resistance compensation. Nevertheless, we made an estimate of the voltage dependence of the slo channel in the presence of either wild-type β4 or various mutant β4s. Because the current amplitude of the slo channel was similar under all these conditions, all the I-V measurements were subject to the same magnitude of error. Similar to what we showed with inside-out recordings, β4 shifted the current-voltage relationship curve to more depolarized membrane potentials (Fig. 6A). The V1/2 of slo alone was 14 ± 3.2 mV, which was increased to 40 ± 4.9 mV in the presence of wild-type β4 (p < 0.001) (Fig. 6A). The same okadaic acid treatment, which suppressed the effect of β4 on slo channel activation kinetics, also significantly suppressed the shift of the I-V curve caused by β4 (p < 0.01). The V1/2 of the slo channel in the presence of β4 and OA was 19 ± 5.7 mV. As shown in Fig. 6, the single mutation β4 S17E also significantly suppressed the effect of β4 on slo channel deactivation (data not shown).

**Phosphorylation-independent Binding of β4 to the slo Channel**—The lack of effect of β4 DEE and β4 S17E on the functional properties of the slo channel could be due to two possibilities. The mutant β4 subunits may not bind to the slo channel; alternatively, they may bind to the slo channel but not interact with it functionally. To distinguish between these two possibilities, we tested the binding between the mutant β4 subunits and the slo channel by co-immunoprecipitation. HEK293 cells were transfected with hSlo channel together with wild-type β4 subunit or mutant β4 subunits. The hSlo channel was immunoprecipitated with a specific antibody recognizing the hSlo channel. As shown in Fig. 7, we
could detect both the wild-type β4 subunit and all the mutant forms of β4 subunit in the immunoprecipitates. As shown previously (11), there were two bands on the Western blot corresponding to the epitope-tagged β4 subunit and a higher molecular weight form of the β4 subunit. This higher molecular weight band was seen for both the wild-type β4 subunit and the β4 DEE and β4 S17E mutants, although it was largely reduced in the β4 AAA mutant (Fig. 7). The identity of this second band is unknown, but we currently are investigating it. In any event, it is clear that the β4 mutants we used in the present study still bind to the slo channel, and thus a lack of binding cannot account for the functional differences we observe.

**hβ4 Is a Phosphoprotein**—We also examined the phosphorylation of β4 directly using radioactive 32P labeling of transfected cells. When wild-type β4 was co-expressed with hSlo in HEK293 cells and the hSlo protein was immunoprecipitated, we could detect a 32P-labeled band in the immunoprecipitate corresponding to the molecular weight of β4 (Fig. 8). In contrast, there was no 32P-labeled band of this molecular weight when hSlo was expressed alone in HEK293 cells (Fig. 8). This demonstrates that β4 is indeed a phosphoprotein. There are multiple potential intracellular phosphorylation sites in the β4 sequence, including the three serine/threonine residues on which we have focused and an additional threonine and two tyrosine residues. The triple alanine mutation (β4 AAA) reduced the 32P label in β4, confirming that one or more of these mutated residues is phosphorylated (Fig. 8). However there is still substantial 32P label associated with β4 AAA (Fig. 8), suggesting that some or all of the other potential residues are also phosphorylated and contribute to the total 32P signal we observe.

**DISCUSSION**

The function of an ion channel can be modulated by its associated auxiliary subunits. We show here that the functional interaction between the α subunit of the slo channel and the β4 subunit is itself subject to modulation. Phosphorylation of the β4 subunit dynamically modulates its functional coupling to the α subunit. This provides strong evidence that the protein complex associated with this ion channel is subject to dynamic modulation.

We showed previously that the slo channel α subunit binds tightly to its β4 subunit (11). This physical interaction is stable, because it is maintained during co-immunoprecipitation in detergent solution. When co-expressed in HEK293 or CHO cells, β4 dramatically modulates the kinetic behavior and toxin sensitivity of the slo channel. There are three putative serine/threonine phosphorylation sites in the β4 subunit, and we tested whether β4 could be phosphorylated and the α—β interaction could be modulated. For this purpose, we employed the whole-cell recording configuration in the present study. Similar to what we saw previously with recording from inside-out patches, the activation and deactivation kinetics of whole-cell slo channel current-voltage relationship are modulated by β4. Interestingly, the modulation of slo channel activation kinetics is only observed within the 1st min of recording. This suggests that after going into the whole-cell mode, either the slo channel α subunit and/or the β4 subunit is modified, and the functional interaction between them disappears. This time-dependent phenomenon is currently being investigated.

Native KCa channels in neurons, smooth muscles, and endocrine tissues have all been shown to be modulated by phosphorylation (4, 20, 24, 25, 37). In their recording of KCa channels
**Phosphorylation-dependent Coupling of hβ4 to hSlo Channel**

A Blot: anti-β4

![Western blots of cell lysates or hSlo immunoprecipitates](image)

**FIG. 7. Mutations of serine/threonine phosphorylation sites in β4 do not eliminate the binding of β4 to the slo channel.** Shown are Western blots of cell lysates or hSlo immunoprecipitates (IP), probed with an antibody against the V5 epitope of the V5-epitope tagged β4 subunits (A), or an antibody recognizing the hSlo channel (B). Both the wild-type β4 subunit and the mutant β4 subunits were detected in the hSlo immunoprecipitates (A).

B Blot: anti-hSlo

![Western blots of cell lysates or hSlo immunoprecipitates](image)

**FIG. 8. β4 is a phosphoprotein.** Shown here is an autoradiograph of 32P-labeled proteins expressed in HEK293 cells. 1st lane, hSlo alone expressed in HEK293 cells; there was no 32P-labeled band corresponding to the molecular weight of β4. 2nd lane, wild-type β4 co-expressed with hSlo; a 32P-labeled band of the molecular weight of β4 was detected in the hSlo immunoprecipitate. 3rd lane, triple alanine mutation (β4 AAA) co-expressed with hSlo; the 32P label in β4 was reduced.

reconstituted in lipid bilayers, Reinhart et al. (23) classified the neuronal large conductance K_{Ca} channels into two types according to channel kinetics. These two types of channel also respond differently to PKA treatment. The open probability of type 1 K_{Ca} channels is increased by PKA, whereas the open probability of type 2 K_{Ca} channels is decreased by PKA (24). The pore-forming α subunits of K_{Ca} channels have numerous phosphorylation sites. For example, serine 942 is a consensus site for PKA phosphorylation in Drosophila slo (27), although mutating this residue does not block the PKA modulation of slo function. It is also conceivable that part of the diverse response of K_{Ca} channels to protein kinase modulation is due to their association with different β subunits. The data presented here strongly support this hypothesis.

When cells expressing slo channel α subunit alone were treated with okadaic acid, an inhibitor of protein phosphatase 1 and 2A (38), there was no significant change in channel activity. On the other hand, when we treated cells expressing both slo α subunit and β4 subunit with okadaic acid, a dramatic suppression of the effect of β4 on slo current activation kinetics and voltage dependence was observed. This suppression effect is likely to be due to changes in phosphorylation of the β4 subunit. First, mutating all three putative serine/threonine phosphorylation sites in β4 to alanines abolishes this effect of okadaic acid treatment. Second, mutating all three sites to negatively charged residues, or even the single mutation S17E, mimics the effect of okadaic acid treatment. The phosphorylation of β4 thus seems sufficient for uncoupling this functional interaction with the α subunit. The loss of functional interaction between the β4 subunit and the slo channel α subunit in the present study is not likely due to dissociation of the two proteins. This is supported by the co-immunoprecipitation experiments that show that the mutant β4 subunits bind to the slo α subunit as well as does the wild-type β4. The results of these biochemical experiments also suggest that mutations of the putative phosphorylation residues did not grossly alter the structure of the β4 protein. The effect of these β4 mutations on the modulation of the slo channel thus must be due to alteration of functional interactions between these two channel subunits.

Our data suggest that the activation kinetics, deactivation kinetics, and voltage dependence of the slo channel are differentially modulated. For example, the time constant of slo channel activation decreases with time after going into whole-cell recording mode, whereas the time constant of slo channel deactivation does not change with time. It was also noted that the triple mutation β4 DEE influenced not only the effect of β4 on slo channel activation kinetics and voltage dependence but also the effect of β4 on slo channel deactivation (Figs. 3 and 5). In contrast, the single mutation β4 S17E or OA treatment altered the effect of β4 only on the activation kinetics and voltage dependence of the slo channel. These differential effects on activation kinetics, deactivation kinetics, and voltage dependence are summarized in Table II. One possible explanation is that uncoupling the effect of β4 subunit on slo channel deactivation kinetics may require phosphorylation of β4 on multiple residues, and this is not mimicked in the single mutation β4 S17E or achieved by OA treatment. These results suggest that different kinetic parameters of the slo channel can be modulated by fine-tuning of the phosphorylation state of the β4 subunit.

We found by 32P labeling that hβ4 is indeed a phosphoprotein, and the β subunits of some Kv channels and of L-type Ca^{2+} channels have been shown to be modulated by phosphorylation (32, 34). It was also shown that phosphorylation of Kv1.1 α subunit at a specific residue is necessary for the channel to be sensitive to Kvβ1.1-induced fast inactivation (39). Similarly, the fast inactivation of Kv1.5 conferred by Kvβ1.3 is modulated by PKA activation, but in this case the effect of PKA is mediated by phosphorylation of the Kvβ1.3 subunit (34). PKA activation increases L-type Ca^{2+} channel activity, and this modulation is at least partially mediated by the phosphorylation of the Ca^{2+} channel β2 subunit, because mutating two serine residues in the β2 subunit blocked this up-regulation of the L-type Ca^{2+} channel by PKA (32). These data, together with the present results implicating phosphorylation in the functional interaction between the slo channel and its β4 sub-

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3 Y. Zhou and I. B. Levitan, manuscript in preparation.
unit, provide strong evidence that the interaction between pore-forming α subunits and auxiliary subunits can be subject to dynamic regulation. Such dynamic regulation of subunit interactions thereby provides another mechanism for generating diversity of ion channel function. It will be interesting to determine how this type of modulation is utilized under physiological conditions.

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