Phosphorylation Is Required for Alteration of Kv1.5 K⁺ Channel Function by the Kvβ1.3 Subunit*

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The Kv1.5 K⁺ channel is functionally altered by coassembly with the Kvβ1.3 subunit, which induces fast inactivation and a hyperpolarizing shift in the activation curve. Here we examine kinase regulation of Kv1.5/Kvβ1.3 interaction after coexpression in human embryonic kidney 293 cells. The protein kinase C inhibitor calphostin C (3 μM) removed the fast inactivation (66 ± 1.9 versus 11 ± 0.25%, steady state/peak current) and the β-induced hyperpolarizing voltage shift in the activation midpoint (V_{1/2}) (−21.9 ± 1.4 versus −4.3 ± 2.0 mV). Calphostin C had no effect on Kv1.5 alone with respect to inactivation kinetics and V_{1/2}. Okadaic acid, but not the inactive derivative, blunted both calphostin C effects (V_{1/2} = −17.6 ± 2.2 mV, 38 ± 1.8% inactivation), consistent with dephosphorylation being required for calphostin C action. Calphostin C also removed the fast inactivation (57 ± 2.6 versus 16 ± 0.6%) and the shift in V_{1/2} (−22.1 ± 1.4 versus −2.1 ± 2.0 mV) conferred onto Kv1.5 by the Kvβ1.2 subunit, which shares only C terminal sequence identity with Kvβ1.3. In contrast, modulation of Kv1.5 by the Kvβ2.1 subunit was unaffected by calphostin C. These data suggest that Kvβ1.2 and Kvβ1.3 subunit modification of Kv1.5 inactivation and voltage sensitivity require phosphorylation by protein kinase C or a related kinase.

Voltage-gated K⁺ channels represent a structurally and functionally diverse group of membrane proteins. These channels establish the resting membrane potential and modulate the frequency and duration of action potentials in nerve and muscle (1, 2). Multiple Shaker-like K⁺ channel α and β subunit genes have been cloned from mammalian brain, heart, skeletal muscle, pancreas, and smooth muscle and functionally expressed in heterologous systems (2). The Kvβ1.1, 1.2, 1.3, and 3.1 subunits confer varying degrees of rapid inactivation onto members of the Kv1 family of delayed rectifiers (3–6). In addition, Kvβ1.2, 1.3, and 2.1 modify the voltage dependence of Kv1.5 channel opening by shifting the midpoint of activation 8–20 mV in the hyperpolarizing direction (4, 5, 7). Each Kvβ subfamily is derived from a separate gene (Kvβ1, Kvβ2, and Kvβ3), whereas additional variability in the Kvβ1 subfamily results from alternative splicing in the N-terminal region, thus yielding the Kvβ1.1, 1.2, and 1.3 subunits (4). The variable N-terminal domains are responsible for the functional differences, whereas the conserved C-terminal domain most likely governs assembly with the α subunit (8, 9).

Voltage-gated K⁺ channels are regulated via both serine/threonine and tyrosine phosphorylation. Kv1.1 and Kv1.2 currents are down-regulated by PKC activation (10, 11). Kv1.2 is down-regulated in part by a G-protein/PKC-dependent phosphorylation of tyrosine 332, whereas PKA phosphorylation of threonine 46 increases current (12, 13). Canine, but not human, Kv1.5 channel activity is decreased also by PKC activation in Xenopus oocytes (11), whereas the human Kv1.5 is down-regulated by tyrosine phosphorylation in HEK cells (14). Kv1.3 is down-regulated by both PKA and PKC in T-lymphocytes (15). K⁺ channel α/β interactions are also influenced by phosphorylation. Lotan and co-workers (16) showed that PKA phosphorylation of serine 446 in the C terminus of Kv1.1 is necessary for the channel to be fully sensitive to fast inactivation conferred by the Kvβ1.1 subunit. Functional interactions between Kv1.5 and Kvβ1.3 are also regulated by PKA phosphorylation, with phosphorylation of serine 24 in the Kvβ1.3 subunit decreasing the β-induced fast inactivation (17).

The Kvβ1.3 subunit converts Kv1.5 from a delayed rectifier with a modest degree of slow inactivation to a channel with both fast and slow components of inactivation (9). In addition, the activation curve is shifted in the hyperpolarizing direction. The present study was performed to determine whether PKC pathways modulate this particular α/β interaction. We present data indicating that both the rapid inactivation and voltage sensitivity conferred onto Kv1.5 by Kvβ1.3 require PKC phosphorylation after heterologous expression in HEK 293 cells. Thus, the kinase systems active in heterologous systems must be taken into account when comparing currents in native cells to those generated from cloned channels. It remains to be determined whether this PKC requirement involves direct phosphorylation of either the α or β subunits and/or other kinase systems.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased from Sigma unless indicated otherwise. Calphostin C, bisindolylmaleimide, phorbol 12-myristate 13-acetate, and okadaic acid were from Calbiochem. Tissue culture media and reagents, including LipofectAMINE, were obtained from Life Technologies, Inc. Calphostin C was activated by a 5-min exposure to light.

DNA Constructs—Human Kv1.5 (1,284 nucleotides) and Kvβ1.3 (53–1,500 nucleotides) were inserted in tandem into the same vector with the Kv1.5 subunit placed 3' to the Kvβ1.3 subunit and behind an internal ribosomal entry sequence (IRES), thus generating a dual cistronic mRNA. A modified pBluescript vector (pATG-deleted pBluescript) that had

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‡ The abbreviations used are: PKC, protein kinase C; PKA, protein kinase A; HEK 293, human embryonic kidney cells; IRES, internal ribosome entry sequence.

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the β-galactosidase ATG at position 1415 removed (mutated to an NheI site) was used. The Kv1.3/RES/Kv1.5 pbK vector was constructed as follows. A 590-base pair IRES (18) was subcloned into EcoRI/EcoRV-prepared pbS5K+. The above-mentioned K+ channel subunit fragments were blunted and subcloned into blunted XbaI and Clal sites of the fastest linker that flanked the IRES. This construct was then digested with SnaI, blunted, and digested with NotI to release the Kv1.3/RES/Kv1.5 sequence. This fragment was then inserted into the NotI/SmaI-digested ATG-deleted pbKCMV.

For a limited number of experiments, Kv1.3 was co-expressed with a C-terminal deletion mutant of Kv1.5 (ΔC57hKv1.5), a 68-amino acid N-terminal deletion mutant of Kv1.3 (ΔN68Kv1.3) and wild type Kv1.5 (Kv1.3) as 1:2:1 into Xenopus oocytes. The wild type Kv1.5 vector was made by removing an upstream NheI/SnaI fragment containing the β-galactosidase ATG from the previously described Kv1.5/pbK construct (7). The wild type Kv1.21 in wild type pbK has been previously described (7).

Construction of the Kv1.3-Kv1.5 tandem was as follows. The single SphI site at nucleotide 316 of the Kv1.3 sequence was removed, and the SphI site was changed to an SphI site using the QuickChange site-directed mutagenesis kit from Stratagene. This fragment was then ligated in-frame to the SphI site just 5’ to the Kv1.5 start codon as described previously (9).

Transfection of HEK 293 Cells—Recently thawed HEK 293 cells (ATCC#1573-CRL) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% horse serum. Cells were transiently transfected by the lipofectAMINE method according to supplier’s directions. Soluble GFP was coexpressed with the channel subunits to identify cells for voltage clamp analysis as described previously (7). The transient transfections used 2.5 ng of hKv1.5/pbK, 4 µg of Kv1.3/RES/hKv1.5 pbK and Kvb1.3pbK, 1.5 µg of Kvb1.2pbK, 4.0 µg of Kvb1.2pbK, 4.0 µg of ΔN68Kv1.3pbK, 4.0 µg of Kv1.3/IRES/ΔC57hKv1.5, 0.4 µg of Kv1.3-Kv1.5 tandem, and 0.5 µg of GFP/pCI (7) mixed with 25 µL of lipofectAMINE reagent. The lipofection mixture was applied overnight, after which the type Kv1.5 in the cell medium was restored. After 24–48 h the cells were removed from the dish using brief trypsinization, washed twice with maintenance medium, and stored at room temperature for recording within the next 12 h. Three µL calphostin C was added to cells and incubated for 0.5–2 h at room temperature before seal formation and voltage clamp. Five µL MgCl2 and was adjusted to pH 7.2 with KOH, yielding a final intracellular K+ concentration of ~145 mEq. The bath solution contained 130 mM NaCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 10 mM glucose and was adjusted to pH 7.35 with NaOH.

Pulse Protocols and Analysis—The holding potential was ~80 mV, and the cycle time for the protocols was 20 s. The standard protocol to obtain current-voltage relationships and activation curves consisted of 250-ms pulses that were imposed in 10-mV increments between ~80 and ~60 mV. The steady state currents were obtained at the end of the 250-ms depolarizations to ~60 mV. Percent inactivation was calculated as 1 − (steady state current/peak current amplitude). Deactivating tail currents were recorded at ~30 or ~50 mV. The activation curve was obtained from the ratio of tail current amplitudes measured immediately after decay of the capacitive transients. The voltage dependence of channel opening (activation curve) was fitted with a Boltzmann equation, y = Y1/(1 + exp(-(E - Eh)/k)), in which k represents the slope factor, and Eh represents the voltage at which 50% of the channels are open. The results are expressed as the mean ± S.E. The Student’s t test was used to calculate the statistical significance of the differences between two populations. Values of p < 0.05 were considered to indicate statistical significance.

RESULTS

The Kv1.3 subunit alters Kv1.5 function by 1) inducing a rapid, but incomplete, voltage-dependent inactivation, 2) a hyperpolarizing shift in the midpoint of activation, 3) a slowing of deactivation, and 4) enhanced slow inactivation (4, 9). Our previous work has shown that the fast inactivation conferred by Kv1.3 onto Kv1.5 is reduced specifically by PKA phosphorylation of serine 24 in the β subunit (17). The present study was performed to determine whether PKC pathways also regulated this α/β interaction. Since PKC inhibitors such as calphostin C directly inhibit L-type Ca2+ channels (20), we first incubated Kv1.5-transfected HEK 293 cells with a variety of PKC inhibitors to test for direct effects on the Kv1.5 channel. We found that the PKC inhibitor chelerythrine was a potent open channel blocker with a Kd value in the low micromolar range when applied to the bath solution (data not shown). This block was apparent within minutes of application to the bath. Given the charge and hydrophobicity of this compound, such open channel block was not surprising because it is structurally similar to well characterized open channel blockers such as nifedipine (21). However, 3 µM calphostin C, which is structurally distinct from chelerythrine, showed no open channel block of the Kv1.5 current (compare panels A and B, Fig. 1), although this agent did slow the rate of both activation and deactivation as well as decrease the slope of the activation curve (panels B, C, and D, respectively). Since direct addition of calphostin C to the bath solution produced these minor effects within 5 min,
Okadaic acid alone had no effect on the Kv1.5/Kv voltage shift, whereas okadaic acid prevented this removal. The data presented in 30 min after calphostin C addition, was loss of inactivation formation or once, when the seal was maintained for more than cells were preincubated with calphostin C for 1–2 h before seal observed when the agent was added directly to the bath after phosphorylation is required to remove the attenuation the calphostin C effect, as predicted if protein de-

Panel D shows currents from Kv1.5 plus Kv1.3-transfected cells that were incubated with 3 μM okadaic acid for 30 min before a 2-h incubation with both 3 μM okadaic acid and 3 μM calphostin C. The activation curves, as obtained from peak tail current amplitude, for either Kv1.5 alone, Kv1.5 plus Kv1.3, Kv1.5 plus Kv1.3 with phorbol 12-myristate 13-acetate (PMA), Kv1.5 plus Kv1.3 with calphostin C, and Kv1.5 plus Kv1.3 with calphostin C and okadaic acid are shown in panel E. Panel F shows outward currents recorded from cells transfected with Kv1.5 plus Kv1.3 after overnight treatment with 5 μM bisindolylmaleimide.

They most likely represent a direct effect on the channel as opposed to involving kinase pathways.

The effects of calphostin C on the Kvβ1.3-induced modification of Kv1.5 current were examined next. As shown in Fig. 2, panels A and B, the Kvβ1.3 subunit induced its characteristic fast inactivation upon Kv1.5. Preincubation with calphostin C (3 μM) for 2 h essentially removed the β-induced fast inactivation as shown in panel C. This effect of calphostin C was not observed when the agent was added directly to the bath after seal formation and incubated for 30 min or less. Only when the cells were preincubated with calphostin C for 1–2 h before seal formation or once, when the seal was maintained for more than 30 min after calphostin C addition, was loss of inactivation observed. The data presented in panel D show that 3 μM okadaic acid, an inhibitor of protein phosphatases 1 and 2A (22), attenuated the calphostin C effect, as predicted if protein dephosphorylation is required to remove the β-induced inactivation. The data summarized in panel E illustrate that calphostin C also affected the β-induced hyperpolarizing shift in the activation curve. Calphostin C completely removed the β-induced voltage shift, whereas okadaic acid prevented this removal. Okadaic acid alone had no effect on the Kv1.5/Kvβ1.3 current or the current induced by Kv1.5 alone (data not shown). Although calphostin C removed the β-induced inactivation and the hyperpolarizing shift in the activation curve, it did not negate the β-induced slowing of deactivation but rather slowed it further (τ = 34.6 versus 41.5 ms, without and with calphostin C treatment, respectively). This calphostin C-induced slowing of deactivation agrees with the data of Fig. 1 indicating calphostin C directly slows Kv1.5 deactivation. The PKC inhibitor bisindolylmaleimide also inhibited β-induced inactivation as shown in panel E. Phorbol 12-myristate 13-acetate (10 μM, added directly to the bath or preincubated with the cells for either 2 or 12 h) had no effect on the Kv1.5/Kvβ1.3 current (data not shown).

Deletion of the N-terminal 68 amino acids of Kvβ1.3 removes the fast inactivation but not the β-induced shift in activation midpoint (9). To determine whether these N-terminal amino acids, including a consensus sites for PKC phosphorylation at serine 34, are required for the calphostin C effects, we examined the effect of calphostin C on this truncated β subunit coexpressed with Kv1.5. As shown in Fig. 3, panels A and B, no fast inactivation was observed both in the absence and presence of calphostin C. However, the hyperpolarizing shift in the activation was still present, and calphostin C treatment returned the activation midpoint to that observed in the absence of a β subunit (panel C). These data indicate that the first 68 amino acids of Kvβ1.3, although being essential for the β-induced fast inactivation, are not required for the β subunit to render αβ-derived current PKC-sensitive.

The fast inactivation conferred onto Kv1.1 by the Kvβ1.1 subunit requires phosphorylation at a PKA site (serine 446) in the α subunit C terminus (23). To determine whether the two PKA and two PKC sites present in the C terminus of Kv1.5 were involved in the PKC regulation of Kv1.5/Kvβ1.3 interaction, we studied a C-terminal truncation of Kv1.5 (ΔC57Kv1.5) that is missing these potential phosphorylation sites. As illustrated in Fig. 3D, the Kvβ1.3 subunit induced its characteristic fast inactivation upon this truncated channel, and calphostin C continued to negate the β-induced inactivation and voltage shift (panels E and F, respectively). Thus, the phosphorylation sites in the Kv1.5 C terminus play no role in either the β-in-

FIG. 2 Effect of calphostin C on Kv1.5/Kvβ1.3 current. Panels A–C show outward currents recorded from HEK 293 cells transfected with Kv1.5 alone, Kv1.5 plus Kvβ1.3, and Kv1.5 plus Kvβ1.3 followed by treatment with 3 μM calphostin C for 2 h at room temperature before seal formation and voltage clamp, respectively. Panel D shows currents from Kv1.5 plus Kvβ1.3-transfected cells that were incubated with 3 μM okadaic acid for 30 min before a 2-h incubation with both 3 μM okadaic acid and 3 μM calphostin C. The activation curves, as obtained from peak tail current amplitude, for either Kv1.5 alone, Kv1.5 plus Kvβ1.3, Kv1.5 plus Kvβ1.3 with phorbol 12-myristate 13-acetate (PMA), Kv1.5 plus Kvβ1.3 with calphostin C, and Kv1.5 plus Kvβ1.3 with calphostin C and okadaic acid are shown in panel E. Panel F shows outward currents recorded from cells transfected with Kv1.5 plus Kvβ1.3 after overnight treatment with 5 μM bisindolylmaleimide.
The activation curves generated under these two conditions are shown in
panel F to the position observed in the absence of any
currents produced by expression of Kv1.5 with an N-terminal deletion mutant of Kv
induces a hyperpolarizing shift in the activation curve.

rapid inactivation onto Kv1.5 and, as with Kv
acids are identical (4). Fig. 4, completely between isoforms, whereas the C-terminal 329 amino
shows that calphostin C reduces the effect of calphostin C on the currents produced by expression of Kv1.3 with a C-terminal truncated mutant of Kv1.5 that lacked the final 57 amino acids. Again, control currents and those recorded after a 2-h incubation with 3 μM calphostin C are shown in panels A and B, respectively. The activation curves generated under these two conditions are shown in panel C as is the activation curve for Kv1.5 in the absence of any β subunit. The bottom row, panels D–F, show the effect of calphostin C on the currents produced by expression of Kv1.3 with a C-terminal truncated mutant of Kv1.5 that lacked the final 57 amino acids. Again, control currents and those recorded after a 2-h incubation with 3 μM calphostin C are shown in panels D and E, respectively. The activation curves generated under these two conditions are shown in panel F.

duced inactivation or voltage shift or the PKC regulation of these processes.

The Kvβ isoforms are produced by alternative splicing of the N terminus. Thus, the N-terminal amino acids differ completely between isoforms, whereas the C-terminal 329 amino acids are identical (4). Fig. 4, panels A–C, examine the effect of calphostin C treatment on the current generated by Kv1.5 and Kvβ1.2 co-expression. As shown in panel A, Kvβ1.2 confers a rapid inactivation onto Kv1.5 and, as with Kvβ1.3, Kvβ1.2 induces a hyperpolarizing shift in the activation curve. Panel B shows that calphostin C reduces the β-induced inactivation, and panel C shows that calphostin C returned the activation curve to the position observed in the absence of any β subunit. Therefore, the mechanism of calphostin C action is shared between Kvβ1.3 and β1.2.

The Kvβ2.1 protein is derived from a different gene and has a unique N terminus and only 85% identity in the C-terminal region as compared with the Kvβ1 family (4). This subunit does not confer fast inactivation onto the Kv1.5 channel, but it does produce a hyperpolarizing shift in the activation curve (7). Panels D and E in Fig. 4 indicate that calphostin C had no effect on Kvβ2.1 action during the 250-ms depolarizing stimulus to +60 mV. Importantly, panel F shows that calphostin C failed to shift the activation curve toward that observed in the absence of any β subunit (V1/2β = 2.7 ± 1.0 mV). Thus, Kvβ2.1 subunit action, which is primarily a 10–12-mV hyperpolarizing shift in the activation curve, is resistant to calphostin C treatment.

One possible interpretation of the results presented above is that calphostin C either inhibited α/β assembly or induced dissociation of the α/β complexes already on the cell surface. However, it is unlikely that calphostin C was affecting subunit assembly during de novo synthesis, since as little as 30 min of calphostin C treatment was required to remove the inactivation and voltage shift. This amount of time is insufficient to replace all cell surface protein with newly synthesized material, especially at room temperature, because the turnover rate for the Kv1.5 channel is approximately 4 h at 37 °C (24). However, since subunit dissociation remained a possibility, we constructed a tandem α/β complex in which the C terminus of Kvβ1.3 was fused with the N terminus of Kv1.5. As shown in Fig. 5, this tandem construct retained wild type α/β activity in terms of fast inactivation and the activation midpoint (69.48 ± 2.15% inactivation at +60 mV and 250 ms, V1/2 = 21.47 ± 4.19 mV, n = 12) (panels A and B, respectively). Fig. 5, C and D show that this tandem also had the typical response to calphostin C in that both the fast inactivation and hyperpolarizing voltage shift were removed (22.93 ± 3.07% inactivation at +60 mV and 250 ms, activation midpoint = -9.82 ± 8.64 mV, n = 6). Thus, PKC activity is unlikely to be modulating subunit assembly in this expression system.

**DISCUSSION**

The Kvβ1.3 subunit alters the function of Kv1.5 by mainly conferring a rapid but incomplete inactivation and a hyperpolarizing shift in the activation curve. Although the PKC inhibitor calphostin C had only minor effects on the current produced by Kv1.5 alone, this agent dramatically altered the current observed in the presence of the Kvβ1.3 subunit. The
fast inactivation was removed by calphostin C treatment as was the hyperpolarizing shift in the activation curve (Table I). There was also a change in the slope of the activation curve in the presence of calphostin C. However, this slope change is likely to be related to removal of the β-induced inactivation. It is inherently difficult to compare activation curves between Kv1.5 alone and Kv1.5 in the presence of an inactivation-inducing β subunit, for the β-induced inactivation probably affects the peak tail currents as previously discussed (9) and, thus, artificially alters any activation curve generated from peak tail currents. Still, Kvβ1.3 does enhance the voltage sensitivity independent of the induced inactivation, since a hyperpolarizing voltage shift occurs with the Kvβ1.3 N-terminal truncation, and calphostin C treatment returns this activation curve to that observed in the absence of any β subunit (Fig. 3C). Also arguing against α/β dissociation is the fact that forced assembly via use of the α/β tandem shows the expected calphostin C response. However, it remains possible that calphostin C does induce dissociation with separate proteins, but dissociation is not observed when the two subunits are physically linked, since reassociation is highly favored under these conditions.

Care must be exercised to avoid studying a direct action of calphostin C on ion channel function as opposed to modulating kinase pathways. However, calphostin C had little effect on the current produced by Kv1.5 alone. If calphostin C was having a direct effect on the Kv1.5/Kvβ1.3 complex, there would be no preincubation requirement, and okadaic acid would not be expected to antagonize its action. Furthermore, an inactive analog of okadaic acid, okadaic acid tetraacetate (25), did not antagonize calphostin C action (data not shown). Bisindolylmaleimide, another PKC inhibitor, also prevented the Kvβ1.3-induced inactivation. However, an effect of this inhibitor was observed in only 5 of 10 experiments, and the activation curve was not shifted (data not shown). These data may suggest that complete PKC block is required to shift the activation curve, whereas only a partial block is needed to inhibit inactivation. Alternatively, different PKC isoforms could be involved that show differential inhibitor sensitivity, as has been previously described by other investigators in other systems (23, 26, 27). Inhibitor peptides that block Ca2+-dependent PKC isoforms did not alter Kv1.5/Kvβ1.3 interaction (data not shown). This finding agrees with the fact that the BAPTA present in the intracellular solution does not alter Kv1.5/Kvβ1.3 current, which is expected if Ca2+-insensitive PKC isoforms are involved in regulating subunit interaction.

Two major questions raised by the data presented here are, 1) if direct PKC phosphorylation of the Kv1.5/Kvβ1.3 complex occurring, and 2) if direct phosphorylation is occurring, which sites are phosphorylated? Our working hypothesis is that PKC phosphorylation of either the α or β subunit is required to render the Kv1.5 channel sensitive to the β-induced inactivation and voltage shift. Multiple PKC isoforms have been reported in HEK 293 cells (28) as well as PKC activity in the absence of PKC activators (29, 30). Therefore, it is not surprising that aspects of this signaling system are constitutively active and that removal of this phosphorylation requires the use of PKC inhibitors. The time required to observe the calphostin C effects, 1 h on average, is consistent with this proposed mechanism; for time is required for the calphostin C to enter the cell and inhibit the required PKC isoform. Additional time is required for dephosphorylation via protein phosphatases. Also supporting this scenario is the finding that okadaic acid, an inhibitor of protein phosphatases 1 and 2A (22) that...
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FIG. 5. Effects of calphostin C on the Kvβ1.3-Kv1.5 tandem. This tandem was constructed as described under "Experimental Procedures." Control currents and those obtained after a 30-min incubation with 1 μM calphostin C are shown in panels A and C, respectively. Currents were recorded in response to 10-mV depolarizations from a holding potential of −80 mV. Panels B and D contain activation curves derived from the peak tail currents in panels A and C, respectively.

TABLE I
Summary of the calphostin C-induced effects on the channel kinetics
Parameters are determined as described in text. PMA, phorbol 12-myristate 13-acetate.

|                   | Activation midpoint | % Inactivation at +60 mV | Number of experiments |
|-------------------|---------------------|--------------------------|-----------------------|
| Kv1.5             |                     |                          |                       |
| Control           | −2.7 ± 1.0          | 8 ± 2.2                  | 9                     |
| Calphostin C (3 μM) | 0.2 ± 1.9          | 6 ± 0.06                 | 8                     |
| Kvβ1.3/Kv1.5      | −21.9 ± 1.4         | 66 ± 1.9                 | 7                     |
| PMA (10 μM)       | −22.6 ± 0.9         | 65 ± 3.7                 | 7                     |
| Calphostin C (3 μM) | −4.3 ± 2.0*        | 11 ± 0.25*               | 10                    |
| Okadaic acid (3 μM) + calphostin C | −17.6 ± 2.2*     | 38 ± 1.8*                | 7                     |
| DN66Kvβ1.3/Kv1.5  | −15.6 ± 1.6         | 7 ± 0.08                 | 6                     |
| Control           | −4.4 ± 2.3*         | 4 ± 0.04                 | 4                     |
| DC57Kvβ1.5/Kvβ1.3 | −22.1 ± 2.0         | 67 ± 2                   | 5                     |
| Control           | −1.3 ± 2.3*         | 9 ± 0.4*                 | 6                     |
| Kvβ1.2/Kv1.5      | −22.2 ± 1.4         | 57 ± 2.6                 | 6                     |
| Control           | −2.1 ± 0.0          | 16 ± 0.6*                | 6                     |
| Kvβ1.2/Kv1.5      | −18.7 ± 1.3         | 7 ± 0.15                 | 5                     |
| Calphostin C (3 μM) | −16.5 ± 2.1       | 6 ± 0.06                 | 5                     |
| Kvβ1.3/Kv1.5 tandem | −21.47 ± 4.19    | 69.48 ± 2.15*            | 10                    |
| Calphostin C (1 μM) | −9.82 ± 8.64*     | 22.93 ± 3.07*            | 4                     |

* Denotes statistically significant differences.

alone has no effect on Kv1.5/Kvβ1.3 current (data not shown), inhibits the calphostin C effects on both the inactivation and hyperpolarizing voltage shift. Kv channel α and β subunits are basally phosphorylated, and these phosphorylation events probably occur early in biosynthesis (31–33). Hence, it is likely that the channel complex is stably phosphorylated on PKC sites and that this phosphorylation is required for complete α/β functional interaction. Where such a single phosphorylation site may exist is at present unknown. Many potential PKC consensus sequences exist in both the Kv1.5 and Kvβ1.3 proteins (34). Since the Kvβ1.2 subunit, but not the Kvβ2.1 subunit, is sensitive to calphostin C (Fig. 4), we examined these C-terminal sequences for differences in possible PKC phosphorylation sites. Serine 314 in Kvβ1.3 could be phosphorylated by PKC, and the corresponding site is absent in Kvβ2.1. However, mutation of this serine in Kvβ1.3 to alanine had no effect on the control currents or the calphostin C response (data not shown). Either other sites are involved or there is no direct PKC phosphorylation of the α/β complex. For example, PKC can modulate tyrosine kinase phosphorylation of Kv1.5 expressed without a β subunit (12).

In summary, there is no reason at present to believe we are dealing with only a single PKC site on either the Kv1.5 or Kvβ1.3 proteins that must be phosphorylated to allow the β-induced inactivation and voltage shift. Additional experiments are required to define the mechanism by which calphostin C removes the β-induced inactivation and hyperpolarizing voltage shift in the Kv1.5/Kvβ1.3 complex. Since the bisindoylmaleimide does not fully mimic the calphostin C and all known PKC isoforms that are sensitive to both agents, it is possible that a kinase other than PKC is involved. Whatever the exact mechanism, the data presented here introduce additional complexity to the topic of β subunit modulation of Kv channel function.

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