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Biochemical and Biophysical Research Communications 342 (2006) 1–8
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A specific N-terminal residue in Kv1.5 is required for upregulation of the channel by SAP97

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Received 19 January 2006
Available online 31 January 2006

Abstract

We have previously reported that SAP97 enhancement of hKv1.5 currents requires an intact Kv1.5 N-terminus and is independent of the PDZ-binding motif at the C-terminus of the channel [J. Eldstrom, W.S. Choi, D.F. Steele, D. Fedida, SAP97 increases Kv1.5 currents through an indirect N-terminal mechanism, FEBS Lett. 547 (2003) 205–211]. Here, we report that an interaction between the two proteins can be detected under certain conditions but their interaction is irrelevant to the enhancement of channel expression. Instead, a threonine residue at position 15 in the hKv1.5 N-terminus is critically important. Mutation of this residue, which lies within a consensus site for phosphorylation by protein kinase C, to an alanine, completely abrogated the effect of SAP97 on channel expression. Although we were unable to detect phosphorylation of this residue, specific inhibition of kinase C by Calphostin C eliminated the increase in wild-type hKv1.5 currents associated with SAP97 overexpression suggesting a role for this kinase in the response.

Keywords: SAP97; PDZ; Potassium channel; Kv1.5

Precise targeting and anchoring of ion-channels and receptor proteins to specific micro-domains of cellular membranes are important for efficient intercellular communications and signal transduction in all excitable cells. The underlying mechanisms which govern post-translational sorting, trafficking, clustering, anchoring, and organization of these molecules have only recently begun to be unraveled [2–5]. Voltage-gated K^+ channels are the most diverse class of ion-channels that regulate the electrical properties of excitable cells. Different classes of Kv channels are targeted differentially in their cellular environments. For example, Kv1.1 and Kv1.2 localize to the juxtaparanodal regions of myelinated axons, while Kv1.4 is present on axonal shafts of certain neurons [6,7]. Similarly, a restricted regional distribution of Kv channels contributes to variations in the shape and durations of the action potentials in cardiac myocytes [8–10].

A family of PDZ-domain containing modular proteins termed MAGUKs (membrane-associated guanylate kinases), which includes PSD95 and SAP97, has recently emerged as important players in the trafficking and clustering of various Kv1 channels [11,12]. Both PSD95 and SAP97 bind to the same extreme C-terminal sequence (xS/TxV) in many Kv1 subunits via their PDZ-domains. PSD95 clusters with Kv1 channels on the cell surface but SAP97 interacts with most Kv1 channels in the cell interior, preventing the forward trafficking of those channels [12,13].

We have recently uncovered an additional PSD95-interacting domain near the T1 domain in the N-terminal segment of hKv1.5 and found that its co-localization with PSD95 at the surface of HEK293 cells was independent of the canonical PDZ-binding motif [14].

We and others have previously shown that SAP97 co-expression with Kv1.5 significantly enhanced the currents through the channel [1,15–17]. We found also that the

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doi:10.1016/j.bbrc.2006.01.110

increase in currents depends on the N-terminal region of Kv1.5 rather than on the PDZ-binding site at the C-terminus [1]. In this study, we have further investigated this phenomenon in order to narrow down the important residues in the N-terminal region and to gain insight into the possible mechanisms behind this phenomenon.

Materials and methods

Generation of N-terminal hKv1.5 deletion constructs. All of the constructs described in this study were in the pcDNA3 vector (Invitrogen) and were derived from the hKv1.5 cDNA. hKv1.5Δ92 and hKv1.5Δ19 were cloned into pcDNA3 from pET42 clones previously constructed [18]. Other deletion mutants were made by standard PCR-based methods. T7-tagged constructs were made by ligation into T7-a(+)pCDNA3 constructed by us as described earlier [1]. For tetracycline-induced expression of T7-hKv1.5, the tagged channel was sub-cloned into pCDNA4/TO (T-Rex, Invitrogen).

Cell lines, transfections, and preparation of cell lysates. A clonal HEK293 cell line, expressing Kv1.5Δ92 was obtained by transfecting (Lipofactamine 2000), selecting the G418 resistant colonies (500 μg/ml) and serially diluting them. Cell lysates were prepared as previously described [19]. Fifty millimolar NaF was routinely included to inhibit phosphatases. Protein concentrations were determined by Lowry's method (TP0300, Sigma).

Co-immunoprecipitations and electrophoresis. All steps were carried out at 4 °C. Lysates were pre-cleared with 17 μl of 1:1 slurry of Protein G-Agarose beads (Roche) for 4 h, centrifuged at 16,000*g* for 4 min and the supernatants saved. Anti-PSD95 family-specific antibody (K28/86.2, Upstate) was added (4 μg) to the supernatants (or without it in the controls) and gently mixed overnight. The immune-complexes were collected by adding 30 μl of Protein G-Agarose beads for 4 h, centrifuging 4 min at 16,000*g*, washing with 3×30 min with 600 μl of the lysis buffer and spinning as above. The beads were suspended in 45 μl of SDS-containing buffer, heated at 95 °C for 5 min, centrifuged, and electrophoresed in 10% SDS–PAGE. The blots (Hybond, Amersham) were probed with an anti-T7 monoclonal antibody (Novagen) (1:6667, 1 h at room temperature) and HRP conjugated, goat anti-mouse secondary antibody at 1:10,000 (Zymed). Protein bands were visualized using an enhanced chemiluminiscent reagent (Perkin-Elmer).

Phosphothreonine detection. T7-tagged hKv1.5 was immunoprecipitated using anti-T7 monoclonal antibody (Novagen) from cell lysates, which included 100 nM Calyculin as a serine/threonine phosphatase inhibitor, followed by Western blot analysis. The blots were probed with three different anti-phosphothreonine antibodies from a Phosphothreonine Detection Kit (Calbiochem) and the protein bands were visualized by chemiluminiscence as before.

Electrophysiological procedures. Procedures were as previously described [14]. Statistically significant differences were determined using a two-tailed Student's *t* test.

Results

T7-hKv1.5 co-immunoprecipitates with SAP97 after transient co-transfection but not from stable lines

We have previously established that SAP97 enhances Kv1.5 currents by about twofold in an HEK 293 cell line stably expressing hKv1.5 [1]. Unlike others [15,17,20], we could find no evidence of a direct interaction between the two proteins. They could not be co-immunoprecipitated from rat cardiac myocytes or HEK cells and immunocytochemistry/confocal microscopy of rat ventricular myocytes showed that while Kv1.5 was enriched at the intercalated disks, SAP97 failed to colocalize and was instead largely distributed along *Z* lines [1]. We therefore concluded that the currents were enhanced by an indirect mechanism. In an attempt to understand the differences between our results and those of others, we have repeated our experiments in a double transient system, wherein both SAP97 and hKv1.5 were co-transfected into HEK293 cells, in parallel with experiments in which SAP97 was transfected into an HEK293 cell line stably expressing hKv1.5. As in the stable lines, co-transfections consistently yielded significant increases in Kv1.5 current densities, analyzed 12–16 h after transfection (Figs. 1A and B). Mean current densities were about twofold higher in the presence of SAP97 compared to control (control 0.78 ± 0.13 nA/pF; +SAP97 1.5 ± 0.032 nA/pF at +40 mV). Also as in hKv1.5 stable lines [1], channel activation and inactivation kinetics were unaffected by SAP97. These results are in agreement with previous results from a stable HEK 293 cell line, as well as from transiently transfected CHO cells, LM6 cells, and from *Xenopus* oocytes [1,15,17].

Fig. 1C (top panel) shows the expression of T7-tagged hKv1.5 protein in transiently transfected HEK cells (lanes 1 and 2) and from a stable line (lanes 3 and 4), when transfected alone or with SAP97. Two bands straddling ~83 kDa were observed, representing differentially processed forms of Kv1.5 as we have shown previously [14]. A widely used PDZ-domain-specific monoclonal antibody (anti-PSD95 family, K28/86.2) that recognizes SAP97 [15,20] was used in co-immunoprecipitation experiments from cell lysates of both transiently transfected cells with T7-hKv1.5 and stable T7-hKv1.5 cell lines. Fig. 1C (middle and lower panels) shows that specific co-immunoprecipitation of T7-hKv1.5 with SAP97 occurred in the transiently transfected cells but not in the stable line. Interestingly, only the smaller of the two bands (~75 kDa), which likely represents an unglycosylated form of T7-hKv1.5 [14], was present in the co-immunoprecipitates.

Co-immunoprecipitation of T7-hKv1.5 with SAP97 in a tetracycline-regulated expression system

That only transiently expressed hKv1.5 co-immunoprecipitates with SAP97 is surprising. Perhaps transiently transfected cells express extraordinary amounts of the channel and thus allow detection of a very weak or artifactual interaction. To test whether the co-immunoprecipitation of T7-hKv1.5 with SAP97 was related to the expression level of the channel protein, we expressed hKv1.5 in a tetracycline-regulated expression system (T-Rex, Invitrogen). T7-hKv1.5 expression in freshly transfected HEK cells was modulated by incubation with different concentrations of tetracycline for 48 h and cell lysates were then prepared and analyzed for T7-hKv1.5 expression (Fig. 2, top panel). In the absence of tetracycline (lane 1), very little T7-hKv1.5 expression was observed. T7-hKv1.5 protein expression increased with increasing

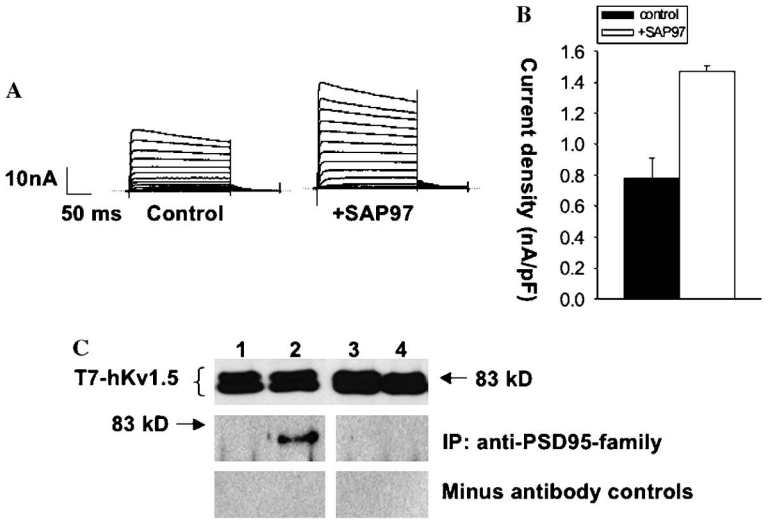

Fig. 1. SAP97 enhances the hKv1.5 currents in transiently transfected HEK293 cells. hKv1.5+pGFP (control) or hKv1.5+SAP97-GFP were co-transfected (1.5 μg each DNA + 3 μl of Lipofactamine 2000) and the whole cell currents were measured after 12–16 h, using a pulse protocol described before [1]. Current traces from a representative example are given (A). Current densities (nA/pF) at +40 mV from control cells and in the presence of SAP97 are shown as mean ± SEM (B). Expression of T7-hKv1.5 in transiently transfected cells (lanes 1 and 2) and in a stable HEK293 line, which was expressing T7-hKv1.5 (lanes 3 and 4) (C). Three micrograms of protein from lysates in the absence (lanes 1 and 3) or in the presence of co-transfected SAP97 (lanes 2 and 4) were subjected to 10% SDS–PAGE and probed with anti-T7 monoclonal antibody. Double bands of T7-hKv1.5 protein were observed ~83 kDa (top panel). Co-immunoprecipitations of T7-hKv1.5 with SAP97 in these lysates (200 μg protein) were carried out by anti-PSD95 family-specific antibody and the blots probed as above.

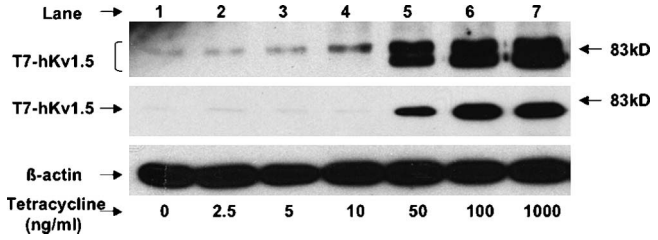

Fig. 2. Tetracycline-regulated expression of T7-hKv1.5 and its co-immunoprecipitation with SAP97. T7-hKv1.5-pCDNA4/TO was co-transfected with tetracyclin repressor (pCDNA6/TR) and SAP97 (1:6:1). Twenty four hours later, tetracycline–HCl was added in the medium at different final concentrations (0–1000 ng/ml) and analyzed 42–48 h later. Expression of T7-Kv1.5 by Western blotting (3 μg protein) (top panel) and its co-immunoprecipitation with SAP97, using the anti-PSD95 family antibody at different doses of tetracycline (lanes 1–7) (middle panel) were carried out as described in Fig. 1. The blot in the top panel was re-probed with anti-β-actin monoclonal antibody (Abcam, 1:20,000) as an internal control protein (lower panel).

tetracycline, approaching saturation at about 1000 ng/ml (lanes 2–7). Both the immature and fully processed forms of T7-hKv 1.5 proteins were evident at higher doses (50–1000 ng/ml), while at lower doses (2.5–10 ng/ml), only the mature protein band was observed.

We tested whether the T7-hKv1.5 protein at the various expression levels could co-immunoprecipitate with the SAP97. Once again, only the smaller of the two T7-hKv1.5 double-bands (~75 kDa) was co-immunoprecipitated (Fig. 2, middle panel). The blots were re-probed with anti-β-actin monoclonal antibody to confirm equal protein loading in lane (lower panel). Thus, the interaction between T7-hKv1.5 and SAP97 in doubly transfected HEK cells is a phenomenon specific to cells transiently expressing hKv1.5 at relatively high levels. The significance of this difference in behavior between stably- and transiently expressed hKv1.5 is unclear. It does account for the differences between the findings of our previous work [1] and those of others [15,17,20], however.

In any case, the binding of SAP97 to hKv1.5 is not involved in the upregulation of the channel by the MAGUK protein. While deletion of the C-terminal −ETDL, the PDZ-binding sequence, was sufficient to eliminate co-immunoprecipitation with SAP97 (Fig. 3B), all of which expressed well in the HEK cells (Fig. 3A), it had no effect

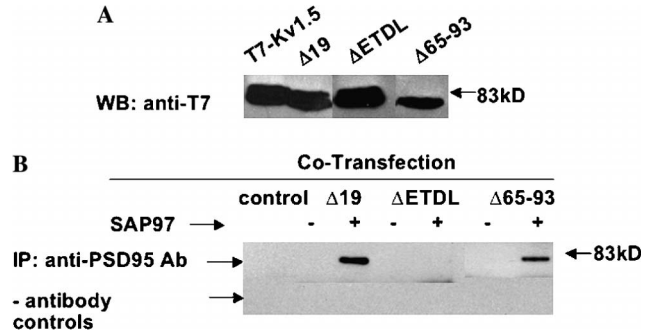

Fig. 3. Expression and co-immunoprecipitation of the deletion mutants with SAP97. Cell-lysates from cells transiently expressing the deletion mutants (3.5 μg protein) were subjected to Western blotting by 10% SDS–PAGE and probed with anti-T7 antibody (A). Transient transfection, with or without SAP97 was carried out (indicated above the lanes). Lysates, including the untransfected control (200 μg protein), were co-immunoprecipitated with anti-PSD95 antibody (4 μg) and analyzed as above (B).

on the responsiveness of the channel to SAP97 overexpression. As we have previously demonstrated [1], SAP97 overexpression increased hKv1.5ΔETDL-encoded currents to the same extent as it did that of the wild-type channel (data not shown). Interestingly, both the hKv1.5 N-terminal deletion mutant T7-hKv1.5Δ19, which prevented the increase in the currents, and the T7-hKv1.5Δ65–93, which did not prevent the increase in currents in response to SAP97 co-expression, could efficiently co-immunoprecipitate with SAP97 (Fig. 3B).

Identification of a specific residue in the hKv1.5 N-terminus that is essential for SAP97-mediated increases in current amplitude

We previously reported that the first 209 amino acids of hKv1.5 and not the C-terminal −ETDL residues (the canonical PDZ-domain recognition site) were required for the enhancement of currents by SAP97 [1]. To further define the necessary region within the Kv1.5 N-terminus, we tested the sensitivity of smaller hKv1.5 deletion mutants to SAP97 overexpression. Because we found that the C-terminal interaction with SAP97 is irrelevant to upregulation of hKv1.5 and we can find no evidence of interaction between the two proteins in cardiac myocytes [1], we generally employed HEK293 cells stably expressing the relevant Kv1.5 mutants for these investigations. To start, we transfected a clonal HEK 293 line stably expressing a smaller hKv1.5 N-terminal deletion mutant, hKv1.5Δ92, which lacks the N-terminal most 92 amino acids, with SAP97. As shown in Fig. 4B, SAP97 failed to enhance the hKv1.5Δ92 peak current densities, demonstrating that residues within the first 92 amino acids of hKv1.5 are necessary for SAP97 to exert its effect. Similar results were obtained from transiently transfected HEK cells transfected with both this mutant and SAP97 (data not shown). The deleted 92-residue segment contains two copies of a proline-rich RPLPPLP sequence (spanning aa 65–82; Fig. 4A), known to interact with src tyrosine kinase SH3 domains [21] and that we have implicated in the regulation of hKv1.5 endocytosis [22]. Because of its known association with src tyrosine kinase in myocytes and regulation in other cell types [21,23,24], we speculated that this region in hKv1.5 might be involved in the SAP97-effect via a phosphorylation-dependent mechanism. Some isoforms of SAP97, which itself contains an SH3 domain, have been shown to interact intra-molecularly with a proline-rich insert and thus influence Kv1.5 currents [17]. Further downstream, a P-X-X-P sequence (aa 88–92) is present which could also potentially bind src kinase or other proteins.

To test whether this proline-rich region was important to responsiveness to SAP97 overexpression, we deleted the entire proline-rich sequence (residues 65–93) (Fig. 4A). The resultant hKv1.5Δ65–93 mutant cell line was transiently transfected with SAP97 and whole-cell currents were measured. As shown in Fig. 4C, SAP97 enhanced the current densities by about twofold, just as in wild-type (control $n = 7$; +SAP97, $n = 11$, shown at +40 mV). Thus, it is unlikely that this proline-rich domain is involved in modulation of hKv1.5 expression by SAP97. Consistent with this interpretation, we failed to detect evidence for tyrosine phosphorylation of hKv1.5 protein when blots of hKv1.5 co-immunoprecipitated with SAP97 were probed with a broad-range, anti-phosphotyrosine monoclonal antibody (clone 4G10, Upstate) (data not shown).

To further narrow down the site of modulation, we deleted the first 30 amino acid residues from hKv1.5 (hKv1.5Δ30) and transfected it with or without SAP97 and again measured whole-cell potassium currents. As shown in Fig. 4C, the SAP97-dependent increase in the mean current densities was also eliminated in this mutant

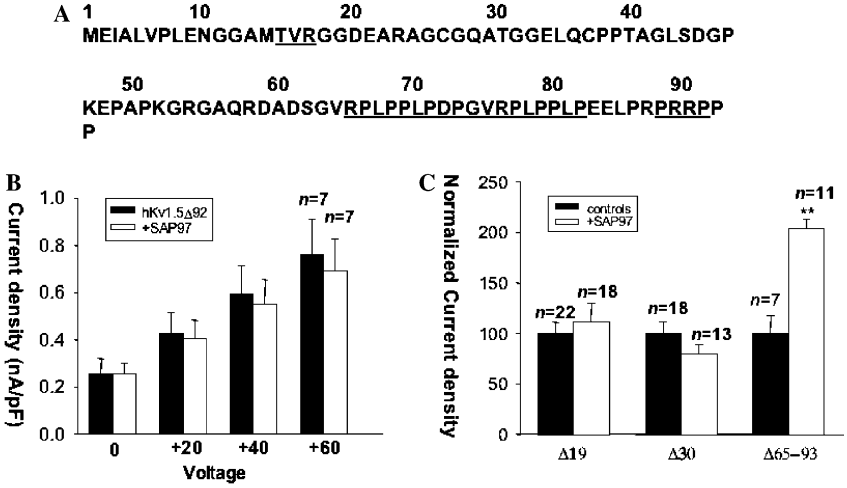

Fig. 4. Amino acid sequence of the N-terminal 92 amino acid residues of hKv1.5 and effects of SAP97 on the currents from different mutants in this region. The P-rich region (aa 65–93) and a possible PKC site (aa 15–17) are underlined to highlight their possible relevance in SAP97-mediated effects (A). A clonal line, expressing the hKv1.5Δ92 was transfected with SAP97 and the whole-cell currents were measured as in Fig. 1. Current densities (nA/pF) at different voltage are shown as means ± SEM (B). Effect of co-transfected SAP97 on the current densities of N-terminal mutants was tested. Normalized current densities (nA/pF) at +40 mV are expressed as means ± SEM and shown here as percent of control (C). Note that a significant increase occurred only in hKv1.5Δ65–93 mutant ($p < 0.05$).

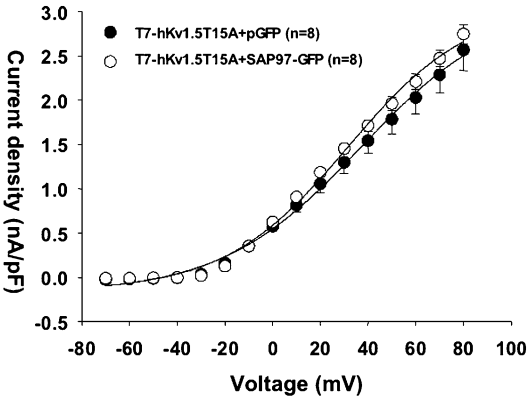

Fig. 5. Threonine 15 is required for SAP97-mediated increases in hKv1.5 currents. Peak current densities from HEK293 cells stably expressing hKv1.5T15A transfected with either the empty vector (pGFP) or SAP97-GFP. From −80 mV, cells were depolarized to between −70 and +80 mV in 10 mV steps followed by repolarization to −40 mV. Peak current amplitudes from controls (filled symbols) and SAP97-GFP co-expressing cells (open symbols) were normalized to cell capacitance.

(values shown at +40 mV), indicating that the region necessary for the response was missing in this mutant, too. We next deleted the first 19 amino acids (hKv1.5Δ19) and tested its effect on the currents by SAP97 exactly as above. With this mutant also, there was no increase in the mean current densities in the presence of SAP97 (Fig. 4C, control $n = 22$; +SAP97, $n = 18$, data shown at +40 mV, not significant), suggesting that this segment contained the site necessary for SAP97-mediated modulation.

The N-terminal most 19 amino acids of hKv1.5 harbor a TVR sequence (residues 15–17) which might be recognized by protein kinase C (S/T-X-R). We hypothesized that the potentially phosphorylated threonine residue in this sequence might be important for upregulation of hKv1.5 by SAP97. To test this possibility, we converted the threonine to an alanine by site-directed mutagenesis, generating hKv1.5T15A. As shown in Fig. 5, when co-expressed in HEK293 cells, this mutation, like the N-terminal deletions, eliminated the SAP97-dependent increases in current densities. Mean current densities were equivalent in the presence or absence of SAP97 (2.74 ± 0.11 and 2.57 ± 0.23 nA/pF at +80 mV, respectively).

A specific inhibitor of protein kinase C activity prevents the increase in hKv1.5 currents associated with SAP97 overexpression

Because T15 is part of a consensus protein kinase C phosphorylation site, we tested whether inhibition of kinase C would influence hKv1.5 responsiveness to SAP97 overexpression. HEK293 cells stably expressing hKv1.5 ± transient transfection with SAP97 were incubated with 100 nM Calphostin C, a specific, cell-permeant inhibitor of protein kinase C (Calbiochem), for 6 h or overnight. As shown in Fig. 6A, cells treated with Calphostin C exhibited no increase in hKv1.5 functional expression in response to SAP97 co-expression. Whereas SAP97 co-expression increased peak current density from 0.43 ± 0.06 to 0.88 ± 0.11 nA/pF at +80 mV in untreated cells, currents in SAP97-coexpressing Calphostin C-treated cells remained indistinguishable from control at 0.44 ± 0.05 or 0.37 ± 0.04 nA/pF, respectively. Calphostin C had no significant effect on T7-hKv1.5T15A current densities. Peak current density at +80 mV in control cells, stably expressing T7-hKv1.5T15A, was 5.2 ± 1.5 nA/pF and in the Calphostin C treated cells it was 3.3 ± 1.3 nA/pF ($p = 0.171$; not significant, Fig. 6B).

Threonine 15 does not appear to be phosphorylated in control or SAP97-co-expressing cells

To test whether the phosphorylation state of T15 in hKv1.5 is affected by SAP97 co-expression, cell lysates were prepared from an HEK293 cell line stably expressing T7-hKv1.5. Control and SAP97 co-expressing cells were tested with or without 100 nM Calphostin C treatment (5 h incubation). Following immunoprecipitation with anti-T7 antibody (Novagen), the samples were electrophoresed, blotted, and probed for the presence of phosphothreonine in T7-hKv1.5, with 3 different anti-phosphothreonine antibodies using the Calbiochem's phosphothreonine detection kit. To ensure that hKv1.5 had indeed been immunoprecipitated, the blots were probed also for hKv1.5 using the anti-T7 antibody. As shown in Fig. 7, although T7-hKv1.5 was efficiently immunoprecipitated, no signal was detected with any of the anti-phosphothreonine antibodies in the region of the blots where T7-hKv1.5 was present (left panel).

Discussion

The interactions of PDZ-domain-containing proteins with the *Shaker*-like Kv1 channels are of considerable interest because of the PDZ-proteins' abilities to regulate the intracellular trafficking, organization, distribution, and expression of the channels on the cell surface. The present work deals with the question of how SAP97 enhances Kv1.5 currents in heterologous cells, a well established phenomenon, the mechanism of which remains uncertain [1,15,17]. The results presented here exclude a role of SAP97 binding to Kv1.5 in effecting the current enhancement and show that a specific N-terminal threonine residue (T15) is required in this phenomenon.

T7-hKv1.5 co-immunoprecipitated with SAP97 only when both proteins were transiently transfected, but not in the hKv1.5-stable lines (Fig. 1C). Several high-expression stable lines were tested during the course of this study, some expressing hKv1.5 even more abundantly than in the transient, but in no case did hKv1.5 co-immunoprecipitate with SAP97 in these lines. Interestingly, antibody to SAP97 co-immunoprecipitated only the faster migrating, relatively unglycosylated [14] form of the channel from the transiently transfected cells. This was true also of hKv1.5 expressed using a tetracycline-induced expression system (Fig. 2).

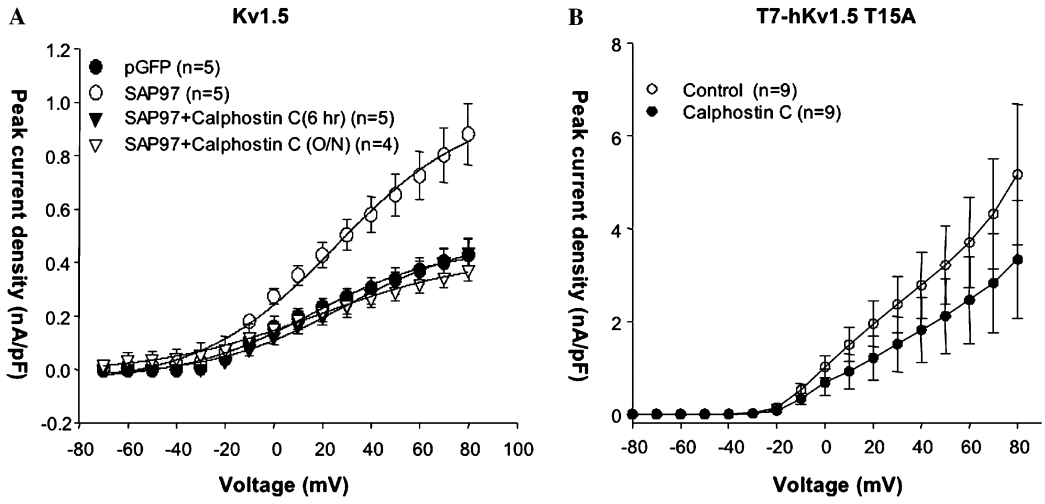

Fig. 6. Calphostin C blocks SAP97-mediated increase in hKv1.5 currents but does not affect Kv1.5T15A expression. (A) Peak currents from HEK293 cells stably expressing hKv1.5 transfected with empty vector (pGFP) or SAP97-GFP ± 100 nM Calphostin C. From −80 mV, cells were depolarized to between −70 and +80 mV in 10 mV steps followed by repolarization to −40 mV. Peak current densities from control cells (filled circles); with co-expressing SAP97 (open circles) and after Calphostin C-treatment of the co-expressing cells for different durations (triangles). (B) Effect of Calphostin C treatment on the peak current densities from HEK293 cells, stably expressing hKv1.5T15A. Control (pGFP; open circles) and Calphostin C-treated cells (filled circles).

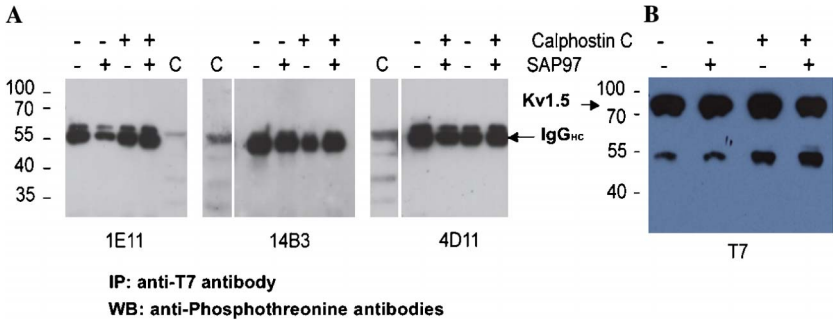

Fig. 7. hKv1.5 is not detected by 3 different anti-phosphothreonine antibodies. T7-hKv1.5 was immunoprecipitated from cell lysates from a stably expressing cell line with (+) or without (−) transfection with SAP97. To test the effect of Calphostin C, the cells were exposed to the drug (100 nM) for 5 h. Equal amounts of proteins were applied in each lane. (A) Three different anti-threonine antibodies were used to probe the blots to detect hKv1.5 (left panel). Lane C indicates the positive control supplied with the antibody. (B) The blot on the right shows the same immunoprecipitation reactions probed with anti-T7 antibody to detect the T7-hKv1.5 (indicated). IgG_{HC} of the antibody used is indicated.

While evidently not relevant to upregulation of hKv1.5, these differences between stable and transient expression are intriguing nevertheless. There are several recent reports of differing properties of proteins expressed stably versus by transient transfection. TRPC7, for example, has been shown to function as a receptor-operated channel if transiently expressed, but as a both a receptor- and a store-operated channel if stably expressed [25]. The authors speculated that cellular components necessary to couple TRPC7 to store depletion are slowly up-regulated in the stable cell lines. Another study in mouse fibroblasts demonstrated that the cells were much more susceptible to apoptosis if Ras was expressed transiently as opposed to stably [26]. Others, too, have found significant differences in target-protein expression between stable and transient lines [27–29]. It would be very interesting to elucidate the mechanisms of intracellular trafficking and sorting or regulation of other endogenous components that cells may utilize depending upon the expression environment.

We previously established that deletion of the bulk of the hKv1.5 N-terminus (hKv1.5ΔN209) eliminates the responsiveness of the channel to SAP97 co-expression [1]. Initially, we speculated that the N-terminal, proline-rich sequence in hKv1.5 might be involved in the SAP97-effect via a phosphorylation-dependent mechanism. We have recently shown this sequence to be important for the endocytosis of hKv1.5 [22] and this sequence is known to associate with src tyrosine kinase in myocytes and other cell types where the kinase has profound effects on channel expression [21,24,30]. Also, some isoforms of SAP97, which itself contains an SH3 domain, have been shown to interact intra-molecularly with a proline-rich region and thus influence hKv1.5 currents [17]. We were surprised, therefore, to find that deletion of the proline-rich segment in hKv1.5 had no effect on the SAP97-induced increase of hKv1.5 currents (Fig. 4). Clearly, the regulation of Kv1.5 surface expression is complex.

hKv1.5Δ19 proved to be the most informative deletion mutant. It was insensitive to SAP97 overexpression yet it fully retained its ability to co-immunoprecipitate with it in the transient lines (Figs. 3 and 4). Thus, it was clear that the necessary and sufficient information for the increase in currents in response to SAP97 overexpression resides in this region. The finding that alanine substitution of a threonine residue (T15) in a potential protein kinase C recognition sequence, TVR, in this region both eliminated the response of hKv1.5 to SAP97 co-expression and dramatically increased its baseline functional expression (Fig. 5) suggested an obvious potential means of modulation by SAP97. That is, SAP97 may act by modulating the phosphorylation state of this threonine residue. Consistent with this possibility, Calphostin C, a specific inhibitor of protein kinase C, completely blocked the enhancement of currents by SAP97 (Figs. 6A and B). However, we were unable to detect phosphothreonine in the T7-hKv1.5 channel with any of the three different anti-threonine antibodies that we used (Fig. 7). While it is possible that the phosphothreonine antibodies were failing to detect phosphorylation that was in fact present, it seems unlikely that all three antibodies would fail. Thus, while it remains formally possible that T15 phosphorylation is important to the modulation of hKv1.5 the more likely possibility is that SAP97 exerts its effect by some other mechanism that requires the presence of this residue.

Collectively, our results show that while there is indeed a specific and stable physical interaction between hKv1.5 and SAP97 under some conditions (by virtue of the canonical −ETDL motif located at the C-terminal end), this interaction is not relevant to the increase in hKv1.5 currents associated with SAP97 overexpression. That hKv1.5 currents can be up-regulated in stable lines, without any demonstrable interaction with SAP97 (Fig. 1), and that the two proteins failed to co-localize in rat myocytes or to co-immunoprecipitate from heart lysates in our previous studies [1], are consistent with this interpretation. In a recent study, deletion of the canonical PDZ-binding site at the C-terminus of a cardiac sodium channel did not alter the currents and other motifs were shown to be involved in regulating the currents [31]. Our results in this study provide evidence that the modulation of hKv1.5 by SAP97 occurs by a novel mechanism that requires the presence of a T15 residue at the Kv1.5 N-terminus.

Acknowledgments

We are grateful to Dr. Ed Moore for helpful discussion throughout the course of this study. We thank Grace Lu for excellent technical assistance.

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