Alternative splice isoforms of small conductance calcium-activated SK2 channels differ in molecular interactions and surface levels

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Abbreviations: nAChR: nicotinic acetylcholine receptor; IHCs, inner hair cells; OHC, outer hair cells; CaM, calmodulin; CaMBD, CaM-binding domain; HC, hippocampus; Ctx, cortex; CICR, Ca2+-induced Ca2+ release

Small conductance Ca2+-sensitive potassium (SK2) channels are voltage-independent, Ca2+-activated ion channels that conduct potassium cations and thereby modulate the intrinsic excitability and synaptic transmission of neurons and sensory hair cells. In the cochlea, SK2 channels are functionally coupled to the highly Ca2+-permeant α9/10-nicotinic acetylcholine receptors (nAChRs) at olivocochlear postsynaptic sites. SK2 activation leads to outer hair cell hyperpolarization and frequency-selective suppression of afferent sound transmission. These inhibitory responses are essential for normal regulation of sound sensitivity, frequency selectivity, and suppression of background noise. However, little is known about the molecular interactions of these key functional channels. Here we show that SK2 channels co-precipitate with α9/10-nAChRs and with the actin-binding protein α-actinin-1. SK2 alternative splicing, resulting in a 3 amino acid insertion in the intracellular 3' terminus, modulates these interactions. Further, relative abundance of the SK2 splice variants changes during developmental stages of synapse maturation in both the avian cochlea and the mammalian forebrain. Using heterologous cell expression to separately study the 2 distinct isoforms, we show that the variants differ in protein interactions and surface expression levels, and that Ca2+ and Ca2+-bound calmodulin differentially regulate their protein interactions. Our findings suggest that the SK2 isoforms may be distinctly modulated by activity-induced Ca2+ influx. Alternative splicing of SK2 may serve as a novel mechanism to differentially regulate the maturation and function of olivocochlear and neuronal synapses.

Introduction

SK2 channels are widely expressed in electrically excitable cells of the nervous system, including sensory hair cells, neurons, and muscle.1-5 They are voltage-independent, Ca2+-activated ion channels that flux potassium cations along their concentration gradient into the extracellular space, thereby hyperpolarizing the membrane potential. They are activated by Ca2+ signaling, and affect both intrinsic excitability and synaptic transmission.6,7 Studies of the cochlea in SK2 knockout mice demonstrate that SK2 is essential in prehearing inner hair cells (IHCs) for proper differentiation and function of afferent synapses onto primary auditory neurons that signal sound reception to the brain.6,9 SK2 is also required in outer hair cells (OHCs) for the differentiation and maintenance of efferent olivocochlear synapses; olivocochlear activation leads to OHC hyperpolarization that suppresses the active mechanical processes by which OHCs amplify auditory signals detected by IHCs.10-12 Efferent inhibition is critical to normal hearing sensitivity and frequency selectivity13,14 and may protect the cochlea from noise-induced damage.15-18

SK2 tetrameric channels are activated by increases in intracellular Ca2+ (sub-micromolar range). The Ca2+-binding protein calmodulin (CaM), which is constitutively associated with the SK2 intracellular C-termini, regulates Ca2+ sensing and channel gating.19 Binding of Ca2+ to CaM induces a conformational change in the SK2-CaM complex, which leads to the opening of the channel.19 SK2 channels are functionally coupled to local Ca2+ sources in micro- or nanodomains in different cell types, allowing rapid channel activation in response to Ca2+ influx.21 SK2 channels are coupled to voltage-gated Ca2+ channels in cardiac muscle, NMDA receptors in neurons, and α9/10-nAChRs in cochlear hair cells.1,23,24 The molecular mechanisms underlying the close coupling of SK2 channels to their Ca2+ source are poorly defined.
Accumulating evidence suggests that SK2 channels are essential organizers of efferent olivocochlear synapses on OHCs. On normal developing hair cells, the first appearance of SK2 clusters coincides with that of α9/10-nAChR clusters, and synaptic responses to efferent stimulation appear contemporaneously with ACh-evoked SK currents, suggesting that SK2 channels may be required at the onset of synaptic function. Importantly, SK2-null mice exhibit a complete absence of ACh-evoked currents from OHCs, despite normal levels of nAChR subunit mRNAs. Further, efferent presynaptic terminals do form on the SK2 null hair cells during early postnatal development, but subsequently degenerate, demonstrating that SK2 channels are required for their maintenance. In comparison, hair cells of α9- and α10-nAChR subunit knockout mice lack cholinergic sensitivity, but retain efferent innervation, albeit with abnormal, hypertrophied bouton morphology. Moreover, α9-knockout mice display normal SK2 localization at postsynaptic sites. Taken together, these studies suggest that SK2 channels localize at postsynaptic sites independent of α9/10-nAChRs, whereas SK2 is essential for α9/10-nAChR surface membrane expression and/or function. These findings lead to the suggestion that SK2 may be physically linked to α9/10-nAChRs in a preassembled complex and SK2 thereby directs the intracellular trafficking and surface membrane expression/ stable retention of the receptors. Alternatively, SK2 may direct the assembly of a postsynaptic protein complex that is required for surface α9/10-nAChRs to be functional. These data highlight the importance of identifying molecular interactions of SK2 channels.

Several protein binding partners that modulate SK2 surface expression have been identified. Constitutive, Ca2+-independent CaM interactions are required for SK2 surface membrane expression in heterologous cells. In cardiac myocytes, SK2 interacts with α-actinin-2, a member of the α-actinin family of actin cross-linking proteins. This interaction links SK2 to its native Ca2+-source, Ca1.3 channels, and is required for SK2 surface membrane expression in cardiac myocytes and in heterologous cells, suggesting that α-actinin-2-mediated linkage to the actin cytoskeleton may promote SK2 surface membrane insertion and/or stable retention. SK2 channels are also constitutively bound to the protein kinase CK2 and the PP2A protein phosphatase, which modulate channel Ca2+ sensitivity through regulation of CaM phosphorylation.

Our studies sought to identify molecular interactions of SK2 channels that may function in cochlear hair cells because of the key role of these channels in the organization and function of olivocochlear synapses. We focus on chicken SK2 because alternative splice isoforms have been recently identified but little is known about their specific properties and developmental expression levels. Here, we identify α-actinin-1 as a novel binding partner of SK2 in chicken hair cells. We provide the first demonstration of a physical association between SK2 and α9/10-nAChRs, based on their co-precipitation from heterologous cells. We show developmental regulation of the relative levels of transcripts encoding the SK2 alternative splice variants during stages of synaptic maturation in the embryonic chicken cochlea. Similarly, we demonstrate that mouse hippocampal and cortical neurons also express the SK2 splice variants, and developmentally regulate their levels. Intriguingly, our heterologous expression studies show that the SK2 isoforms exhibit differences in their molecular interactions, surface levels, and regulation by Ca2+ and CaM. The differential properties of SK2 splice variants suggest a molecular complexity of SK2 channels at synapses.

Results

SK2 interacts with α-actinin-1 in cochlear hair cells

To begin to define mechanisms of SK2 channel localization and functional coupling to its local Ca2+ source in cochlear sensory hair cells, we sought to identify proteins that interact with SK2.
In cardiac muscle, SK2 channels interact with α-actinin-2, which is necessary for surface membrane expression. To determine whether α-actinins may interact with SK2 channels in a distinct cell type, inner ear hair cells, we first identified α-actinin isoforms expressed in these cells. We performed reverse-transcription (RT)-PCR with specific primers to amplify α-actinin from total RNA isolated from E19 chicken basilar papillae (equivalent of the mammalian cochlea). The PCR primers were designed against conserved regions flanking the EF-hand region of α-actinin, which differs in each isoform. Sequencing indicated that the α-actinin isoform expressed in the basilar papilla is not α-actinin-2, a muscle-specific, non-Ca$^{2+}$-sensitive isoform, but a Ca$^{2+}$-sensitive non-muscle isoform. We tested whether α-actinin localizes at olivocochlear synapses in sensory hair cells in vivo, using a pan-specific α-actinin monoclonal antibody. SK2 is concentrated at these postsynaptic sites, as indicated by the juxtaposition of SK2 immunolabeled surface clusters to the large calyx-type olivocochlear presynaptic terminal, marked by SV2 synaptic vesicle staining (Fig. 1A). We were unable to directly demonstrate co-localization of SK2 with α-actinin because of the poor match between optimal fixation conditions for their immunostaining. Instead, we showed increases in developmental expression of SK2-ark splice variant in chicken cochlear hair cells in vivo. Sequence alignments of the C-terminus of chicken (ch) splice variants, SK2 and SK2-ark (24), mouse SK2 (GenBank accession number P58390) and SK2-ARK, trout SK2 (NP_001117783) and mouse SK1 (Q9EQK3), Numbers indicate amino acids of chicken SK2. Source: http://multalin.toulouse.inra.fr/multalin/ (B) ARK insertion (lower sequence) creates a unique restriction endonuclease site (Hpy1888) that is not present in chicken SK2 (upper sequence) that lacks the insert. (C) Quantification of relative abundance of SK2-ARK mRNA, compared with SK2, during chicken cochlear development, ranging from embryonic day (E)12–14 to E20. Identification of cDNA clones of SK2-ARK (lanes 1,3) and SK2 (lanes 2,4) by using Hpy1888 restriction digestion and size separation of the products by agarose gel electrophoresis. The cDNAs were generated by RT-PCR amplification from cochlear total RNA with primers to conserved sequences that flank the ARK insertion site and subcloning (~150 clones analyzed in total per age). Bottom: quantification of the relative abundance of SK2-ARK transcript. n = 3 separate RT-PCR experiments with independent RNA extractions, 50–60 clones per age per experiment. (D) Graph of developmental increases in the relative abundance of SK2-ARK transcripts, detected by q-PCR, in the mammalian hippocampus (HC) and cortex (Ctx) from postnatal day 8 to 3 mo of age (adult). n = 6 animals per age, 3 separate q-PCR experiments with independent RNA extractions. Bars and data points in (C) and (D) represent mean ± SEM; * P < 0.01 Student t test compared with levels at E12–14.
did not co-precipitate with SK2-C-MBP, and MBP alone did not co-precipitate α-actinin-1-GST. Taken together, our results show that α-actinin-1 interacts directly with SK2 channels and both localize at postsynaptic sites in chicken cochlear hair cells in vivo.

**SK2 interacts with α9/10-nAChRs**

Proper function of olivocochlear synapses on hair cells requires close physical proximity (co-localization) and functional coupling of SK2 channels with α9/10-nAChRs, but a physical association has not been demonstrated to date. To test for their interaction, we utilized heterologous expression in *Xenopus laevis* oocytes, rather than the native proteins in hair cells, because of the lack of reliable antibodies that recognize α9- and α10-nAChR subunits. We epitope tagged the chicken α10-nAChR subunit C-terminus end with hemagglutinin (HA). Oocytes were microinjected with cRNA encoding α9, α10-HA, SK2, and α-actinin-1. SK2 channels were immunoprecipitated from membrane fractions isolated from oocytes three days after injection, the time determined experimentally to provide optimal expression levels. As a positive control, exogenously expressed SK2 co-precipitated with α-actinin-1 from oocyte membrane fractions (Fig. 2A), consistent with the co-precipitation of these endogenous proteins from cochlear lysates (Fig. 1D). Importantly, SK2 channels co-precipitated with HA-tagged α9/10-nAChRs (Fig. 2B). The interaction is specific, as SK2 did not co-precipitate with other membrane proteins, such as the endogenous sodium potassium ATPase (Fig. 2B). As an additional negative control, SK2 antibody did not pull down HA-tagged α9/10-nAChRs from oocytes not co-expressing exogenous SK2 (see Fig. 5A). This is the first demonstration, to our knowledge, of a physical association between SK2 and α9/10-nAChRs.

**Expression of SK2 splice variants during chicken embryonic development**

Previous studies of posthatch chicken short (outer) hair cells identified an SK2 splice variant, containing a 3-residue “ARK” insertion within the C terminus (Fig. 3A), referred to here as SK2-ARK. We and others have also detected the ARK splice insertion in mammalian SK2. The same insertion is constitutively present in trout SK2, and a similar AQK sequence is found in the same region in mammalian SK1 (Fig. 3A). Because the ARK insertion localizes to the domain that binds CaM, a protein that regulates SK2 surface expression and Ca$^{2+}$-gating, we speculated that SK2 isoforms may exhibit differences in protein interactions and functional properties.

First, we determined whether SK2-ARK is expressed during the period of olivocochlear synapse formation, from E12-E20, in the embryonic chicken cochlea. To quantify the relative abundance of SK2 (refers here to the isoform that lacks the ARK insert) and SK2-ARK transcripts, we used RT-PCR with specific primers that flanked the splice insertion site, followed by subcloning and restriction endonuclease digestion of individual clones with Hpy188I, a restriction endonuclease that cuts at a unique site within the sequence encoding the ARK splice insertion site, but not in SK2 lacking the insert (Fig. 3B and C). From the 150–180 clones analyzed for each developmental age, we found a significant increase in the relative abundance of the SK2-ARK variant between ages E12-E14 and E20 (Fig. 3C; 22.69 ± 4.01% of SK2 clones at E12–14; 36.63 ± 4.21% at E20, n = 3 separate experiments with 50–60 clones per experiment, P < 0.01, Student t test).

In addition, SK2-ARK transcripts show a substantial developmental increase in mouse forebrain regions. The relative levels of SK2-ARK to SK2 transcripts increased by approximately 4-fold in both the hippocampus (2.9 ± 0.2; 11.9 ± 1.3; n = 6) and the cortex (6.9 ± 0.4; 30.5 ± 2.7 n = 6) from postnatal day 8 to adult ages (3 mo) (Fig. 3D). Thus, cochlear sensory hair cells and central neurons both exhibit increases in the relative levels of SK2 containing the ARK insertion. The chicken, mouse and human SK2 clones are 89% identical in sequence (Fig. 3A), suggesting that they are likely
to have conserved molecular interactions. Similarly, the chicken α-actinin-1 amino acid sequence is 97% homologous to human α-actinin-1.

Functional characterization of the SK2-ARK isoform

We tested for differential properties between SK2 and SK2-ARK in order to gain insights into the functional significance of the increased developmental expression of SK2-ARK. We utilized heterologous expression in Xenopus laevis oocytes because: (1) no reagents are available to distinguish between the two SK2 isoforms, which differ by only 3 amino acids and (2) exogenous SK2 forms functional channels on the oocyte surface. Since the ARK splice insertion is located within the Ca²⁺-dependent CaM binding domain of the SK2 C-terminus, we tested for effects on CaM-mediated Ca²⁺ gating. We used patch-clamp recording to look for differences in Ca²⁺ sensitivity between channels composed of SK2-ARK vs. SK2 subunits. SK2 and SK2-ARK isoforms were separately expressed in oocytes, and Ca²⁺ responses were recorded from inside-out patches to generate Ca²⁺ dose-response curves. Ca²⁺-activated potassium currents recorded from SK2-expressing patches had an apparent KD of 0.48 ± 0.0067 μM and a Hill coefficient of 4.90 ± 0.40 (n = 13). SK2-ARK channels demonstrated a steeper, right-shifted Ca²⁺ response curve caused by a significantly reduced normalized response to 0.5 μM Ca²⁺, with an increased apparent KD of 0.61 ± 0.019 μM and a Hill coefficient of 10.14 ± 1.84 (Fig. 4A, n = 13). These results demonstrate that the ARK insertion alters the response of the assembled channels to Ca²⁺, which could suggest altered interactions with CaM.

To assess this possibility, we tested for differences in CaM binding to SK2 and SK2-ARK C-termini in recombinant peptide binding assays. We found no difference in binding of Ca²⁺-bound CaM between the two isoforms (Fig. 4B). In contrast, we observed a modest significant increase in binding of Ca²⁺-bound CaM when Ca²⁺ was chelated with 5mM BAPTA (Fig. 4B, 141.84 ± 19.44% co-precipitated CaM compared with SK2; n = 3). Our results demonstrate that the ARK splice insertion alters both SK2 interactions with CaM and sensitivity to Ca²⁺.

SK2 ARK splice insertion modulates interactions with α9/10-nAChRs and surface levels

Native SK2 channels interact with α-actinin-1 (Fig. 1D), and recombinant SK2 (lacking the ARK insert) interacts with both α-actinin-1 and α9/10-nAChRs (Figs. 1E and 2B). We next tested whether the ARK splice insertion affects these interactions.

SK2-ARK or SK2 was co-expressed with exogenous α-actinin-1 in oocytes and immunoprecipitated from membrane fractions. SK2-ARK also co-precipitated with α-actinin-1 from oocyte membranes (Fig. 5A; n = 3) but displayed decreased...
binding to α-actinin-1 in recombinant peptide binding assays (Fig. 5B, 81.25 ± 2.89% bound α-actinin-1 compared with SK2; n = 3), suggesting that the ARK splice may modulate SK2 channel interactions with α-actinin-1.

Intriguingly, SK2-ARK showed a significant increase in co-precipitation with α9/10-nAChRs (Fig. 5C, 172.55 ± 35.06% compared with SK2; n = 4). SK2 null mouse studies suggest that SK2 is required for surface expression and/or stable retention of α9/10-nAChRs on cochlear hair cells.8,9 Thus, the difference that we found in their co-precipitation with α9/10-nAChRs (Fig. 5C) raises the possibility that the SK2 isoforms may have differential effects on nAChR trafficking.

We therefore tested for differences in surface membrane levels of α9/10-nAChRs co-expressed with either SK2-ARK or SK2 in oocytes. We used standard cell surface biotinylation assays with a membrane-impermeant biotinylation reagent. For each protein of interest, the levels on the surface (biotinylated) were normalized to their total expression levels. The two SK2 isoforms showed similar total expression levels; however their differential effects on nAChR trafficking.

We tested first for effects of Ca2+ concentration induced by synaptic activity lead to Ca2+-dependent binding of CaM to the Ca2+-dependent CaM binding domain (CaMBD) of an adjacent SK2 subunit, resulting in conformational changes that mediate channel gating.9,21 The ARK insert is located within the CaMBD of SK2 (Fig. 3A), suggesting the possibility that Ca2+ and CaM interactions with the different SK2 isoforms will exhibit differential modulation. In particular, Ca2+ and CaM may affect interactions of SK2 channels with the actin binding protein α-actinin-1 and with the Ca2+-permeant α9/10-nAChRs. As support for this hypothesis, α-actinin-2 interacts with the CaMBD of SK2 from cardiac muscle,25,28 and α-actinin-1 interactions with the NMDAR NR1 subunit are inhibited by Ca2+-bound CaM.35

We tested first for effects of Ca2+ alone on the binding of α-actinin-1 to SK2 and SK2-ARK using recombinant peptide binding assays. Ca2+ strongly promoted α-actinin-1 binding; we found a 3.2-fold increase in binding of α-actinin-1 to SK2 and a 6.8-fold increase in binding to SK2-ARK in the presence of 1mM CaCl2, compared with 5mM BAPTA (Fig. 7A; compare lanes 1 and 3, 5 and 7 with no CaM; n = 3). Similarly, α-actinin-1 co-precipitation with SK2 was reduced by loading oocytes with BAPTA-AM to chelate intracellular Ca2+ prior to SK2 immunoprecipitation (Fig. 7B; 78.72 ± 9.98% compared with untreated controls; n = 3). These data suggest that increased intracellular Ca2+ levels likely strengthen α-actinin-1 interactions with both SK2 and SK2-ARK subunits. Such surface levels were 50% lower with SK2-ARK vs. SK2 (Fig. 6B, n = 3–4), whereas the receptors exhibited increased co-precipitation with SK2-ARK from total membrane fractions (Fig. 5A). These findings suggest that receptor association with SK2-ARK, compared with SK2, leads to their greater intracellular localization. In summary, SK2-ARK, compared with SK2, exhibits reduced association with α-actinin-1 (Fig. 5B), and greater association with α9/10-nAChRs (Fig. 5C).

Based on the reduced SK2-ARK surface levels, we tested for increased turnover by measuring retention of the biotinylated protein on the cell surface over time after labeling. The 2 isoforms showed similar rates of endocytosis, as evidenced by similar percentages of initial surface biotinylated SK2 and SK2-ARK that remained at several time points after labeling (Fig. 6C). Thus, although surface membrane insertion of SK2-ARK is reduced compared with SK2, the 2 isoforms display similar surface stability (Fig. 6A and C). Taken together, our findings indicate that alternative splicing of SK2 likely modulates surface levels and ability to facilitate α9/10-nAChR surface membrane expression.

**SK2 interactions are regulated by Ca2+ and calmodulin**

Ca2+ is a key regulator of SK2 channel activation. Increases in local Ca2+ concentration induced by synaptic activity lead to Ca2+-dependent binding of CaM to the Ca2+-dependent CaM binding domain (CaMBD) of an adjacent SK2 subunit, resulting in conformational changes that mediate channel gating.9,21 The ARK insert is located within the CaMBD of SK2 (Fig. 3A), suggesting the possibility that Ca2+ and CaM interactions with the different SK2 isoforms will exhibit differential modulation. In particular, Ca2+ and CaM may affect interactions of SK2 channels with the actin binding protein α-actinin-1 and with the Ca2+-permeant α9/10-nAChRs. As support for this hypothesis, α-actinin-2 interacts with the CaMBD of SK2 from cardiac muscle,25,28 and α-actinin-1 interactions with the NMDAR NR1 subunit are inhibited by Ca2+-bound CaM.35

Consistent with the SK2 null mouse studies that suggest co-trafficking, α9/10-nAChR surface levels were significantly lower when expressed alone compared with co-expression with SK2 (Fig. 6B, 37.16 ± 10.61% of surface levels co-expressed with SK2, n = 3–4). Unexpectedly, α9/10-nAChR surface levels were not different when expressed alone vs. co-expressed with SK2-ARK, suggesting that SK2-ARK, in sharp contrast to SK2, does not augment receptor surface levels. α9/10-nAChR
changes may occur upon olivocochlear synaptic activation in sensory hair cells. Because CaM exhibits both Ca\textsuperscript{2+}-dependent and independent binding to SK2, we tested whether CaM, in the presence or absence of Ca\textsuperscript{2+}, alters α-actinin-1 interactions with SK2 isoforms. To test for competition between the interactors, we preincubated recombinant SK2 isoforms with CaM in buffer containing either 1mM CaCl\textsubscript{2} or 5mM BAPTA, and then measured the binding of α-actinin-1. Preincubation of the SK2 isoforms with Ca\textsuperscript{2+}-bound CaM partially blocked α-actinin-1 binding (Fig. 7A; n = 3). Further, Ca\textsuperscript{2+}-free CaM (with 5mM BAPTA) did not affect α-actinin-1 binding to SK2, suggesting that, at low levels of Ca\textsuperscript{2+}, CaM and α-actinin-1 may bind simultaneously to this isoform. In contrast, Ca\textsuperscript{2+}-free CaM partially blocked binding of α-actinin-1 to SK2-ARK (Fig. 7A, 72.61 ± 4.47% α-actinin-1 bound in the presence of CaM compared with CaM-free control; n = 3). This result is consistent with our findings that Ca\textsuperscript{2+}-free CaM shows increased binding to SK2-ARK, compared with SK2 (Fig. 4B). Thus, Ca\textsuperscript{2+}-independent CaM binding may induce differences in the conformation of SK2-ARK vs. SK2 that, in turn, lead to differential modulation of their interactions with α-actinin-1.

Interestingly, SK2 and SK2-ARK showed different effects of Ca\textsuperscript{2+} chelation on interactions with α9/10-nAChRs. Co-precipitation of α9/10-nAChRs with SK2 from oocytes increased 2-fold after treating with BAPTA-AM (Fig. 8; n = 4), suggesting that Ca\textsuperscript{2+} influx may reduce linkage of SK2 to α9/10-nAChRs. In contrast, co-precipitation of α9/10-nAChRs with SK2-ARK was not significantly altered in response to BAPTA-AM treatment, suggesting that Ca\textsuperscript{2+} does not inhibit the association of α9/10-nAChRs with SK2-ARK. These findings suggest that the isoform composition of SK2 channels may provide a novel mechanism for modulating protein interactions at the SK2 postsynaptic complex in response to synaptic activity-induced increases in local Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-CaM binding.

**Discussion**

Our findings provide novel insights into molecular interactions of SK2 channels and the differential properties of SK2 alternative splice variants. We show that SK2 co-precipitates with α9/10-nAChRs, using heterologous expression. This is the first demonstration, to our knowledge, of a physical association between these 2 key functional channels of olivocochlear postsynaptic sites. Further, we show direct binding of SK2 to the actin-binding protein α-actinin-1 in vitro and in vivo. We also find developmentally regulated expression of SK2 splice variants, in vivo, in the avian cochlea and the mammalian cortex and hippocampus, suggesting widespread significance of SK2 molecular heterogeneity. The SK2-ARK isoform has been identified previously in the avian cochlea, but changes induced by the insert have not been defined prior to our study. We demonstrate that the two isoforms differ in surface membrane levels, interactions with α9/10-nAChRs, Ca\textsuperscript{2+} sensitivity, and modulation of their molecular interactions by Ca\textsuperscript{2+} and calmodulin (Fig. 9). Based on these differences, we speculate that the 2 SK2 isoforms provide an unanticipated complexity that may serve to differentially modulate...
responses to synaptic activity in sensory hair cells and neurons.

Normal sound sensitivity and frequency selectivity requires the functional coupling and co-localization of α9/10-nAChRs and SK2 channels at efferent olivocochlear presynaptic sites. α9/10-nAChRs mediate efferent olivocochlear synaptic transmission. Activation of these highly Ca\(^{2+}\) permeant receptors causes Ca\(^{2+}\) influx that, in turn, leads to SK2 channel activation and fast efferent inhibition in both avian and mammalian hair cells. SK2-null mouse studies demonstrate that these channels play key roles in the organization and function of olivocochlear synapses. In particular, the loss of functional α9/10-nAChRs on the surface of SK2-null hair cells, despite normal levels of receptor subunit mRNAs, suggests that SK2 may interact with the nAChRs in a molecular complex whose surface membrane expression is directed by SK2-specific mechanisms or binding partners. Consistent with this hypothesis, we show that SK2 channels co-precipitate with α9/10-nAChRs. Whether the interaction is direct or mediated via linker proteins has yet to be determined. Additionally, we demonstrate that the surface levels of α9/10-nAChRs correlates with that of SK2 in heterologous cells, further supporting the model in which surface membrane targeting and/or stable retention of α9/10-nAChRs is promoted by their physical association with SK2.

SK2 channels in cochlear hair cells may also be activated by increased Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from internal stores following nAChR activation. Local Ca\(^{2+}\) levels are thought to be tightly regulated and physically restricted by the subsynaptic cistern that lies closely opposed to the postsynaptic membrane. In previous studies, blockade of CICR by application of 100 mM ryanodine reduced the amplitude of ACh-evoked IPSCs in hair cells, suggesting that CICR is important for SK2 channel activation. Intriguingly, our findings of Ca\(^{2+}\)-induced changes in SK2, interactions with α-actinin-1, which links to the submembranous actin cytoskeleton, suggest that changes in local Ca\(^{2+}\) levels may alter the membrane stability or subcellular localization of SK2 channels. Our demonstration of a physical association between α9/10-nAChRs and SK2 channels suggests that both direct coupling to nAChRs and CICR likely mediate Ca\(^{2+}\) activation of SK2 channels that, in turn, is required for fast suppression of hair cell-mediated sound amplification, and to promote slow suppression of outer hair cell mechanics that protects against noise-induced hearing loss.

SK2 alternative splicing appears to modulate the interactions and surface levels of the SK2-nAChR complex in our heterologous expression studies. We find increased interaction of α9/10-nAChRs with SK2-ARK, relative to SK2. Surface membrane levels of the SK2-ARK variant were significantly reduced compared with SK2. Surface levels of α9/10-nAChRs were also decreased when co-expressed with SK2-ARK, vs. SK2, indicating the dependence of α9/10-nAChR trafficking on that of the SK2 isoforms.

Mechanisms that mediate the trafficking of the SK2-nAChR complex are undefined. SK2 interactions with α-actinin-1 may regulate its surface expression. As support for this hypothesis, cardiac muscle SK2 surface expression and endocytic recycling are affected by interaction with α-actinin-2. Our findings of reduced binding of SK2-ARK, compared with SK2, with α-actinin-1 and decreased SK2-ARK surface levels, raise the possibility that α-actinin-1 may play a similar role in the cochlea to that of α-actinin-2 in cardiac muscle, promoting SK2 surface membrane expression and/or stable retention. Interestingly, α-actinin-1 contains EF-hand domains that bind Ca\(^{2+}\). In contrast, α-actinin-2 EF hands do not. Consistent with this difference, we find Ca\(^{2+}\)-dependent modulation of SK2 interactions with α-actinin-1. This differs from the reported lack of Ca\(^{2+}\) effects on α-actinin-2 interactions with SK2.

We show that Ca\(^{2+}\) and CaM modulate SK2 interactions with α9/10-nAChRs and α-actinin-1. Ca\(^{2+}\) decreases the association of SK2 with α9/10-nAChRs. Further, Ca\(^{2+}\)- and Ca\(^{2+}\)-bound CaM exert opposing effects on SK2 interaction with α-actinin-1. Ca\(^{2+}\) alone strongly promotes SK2 interaction with α-actinin-1. In contrast, Ca\(^{2+}\)-CaM, but not Ca\(^{2+}\)-free CaM, competes with α-actinin-1 for binding to the SK2 C-terminus, suggesting that the Ca\(^{2+}\)-dependent CaMBD is the key region in SK2 for α-actinin-1 binding. Based on these findings, we propose that the balance between Ca\(^{2+}\)-CaM-induced displacement vs. Ca\(^{2+}\)-mediated enhancement of α-actinin-1 binding to SK2 may shift, depending on local Ca\(^{2+}\) concentrations, to increase or decrease SK2 interactions with α-actinin-1. Thus, synaptic activity may lead to local increases in Ca\(^{2+}\) that dynamically alter interactions within the SK2-nAChR postsynaptic molecular complex of cochlear hair cells.

The SK2-ARK splice insertion within the Ca\(^{2+}\)-dependent CaMBD changes the effects of Ca\(^{2+}\) and CaM on SK2 interactions with its binding partners. Ca\(^{2+}\)-free CaM showed an increased interaction with SK2-ARK, compared with SK2, and reduced the
binding of α-actinin-1 to SK2-ARK in peptide binding assays, suggesting that the ARK insert may introduce conformational changes within the CaMBD that favor interaction with CaM over α-actinin-1. Competition by CaM for binding of α-actinin-1 and Ca2+ modulates the interactions. SK2-ARK, compared with SK2, channels exhibit reduced binding to α-actinin-1, and increased association with α9/10-nAChRs. However, SK2-ARK surface membrane levels are lower than those of SK2. Interactions between α-actinin-1 and SK2 (A) and SK2-ARK (B) are similarly modulated by Ca2+ (enhanced, arrow) and Ca2+-CaM (reduced). Ca2+ decreases SK2 interactions with α9/10-nAChRs, possibly serving as a mechanism to regulate efferent inhibition. In comparison, Ca2+ does not alter or modestly enhances the SK2-ARK:α9/10-nAChR interactions (dotted arrow), suggesting that they are less prone to inhibition by Ca2+. Additional differences, such as Ca2+ sensitivity (see text), suggest that developmental increases in SK2-ARK relative levels may modulate synaptic activity in cochlear hair cells and neurons.

whereas it has little effect or weakly promotes the association of α9/10-nAChRs with SK2-ARK. These findings indicate that the physical coupling of α9/10-nAChRs to SK2-ARK, compared with SK2, is less prone to inhibition by Ca2+, which may be an important property as olivocochlear synapses mature.

We also demonstrate a modest but significant decrease in Ca2+ sensitivity (shift in the Ca2+ response curve) of SK2-ARK- vs. SK2-mediated potassium currents. Consistent with our findings, a recent report shows that mammalian SK2-ARK currents are less sensitive to Ca2+. The increased steepness of the Ca2+ dose response curve for SK2-ARK compared with SK2 (Fig. 4A) suggests that SK2-ARK containing channels respond in a more all-or-none manner, compared with the more graded responses of SK2 channels, to changes in local Ca2+ concentrations. Previous studies found similar Ca2+-sensitivity between mammalian SK2 channels, which lack the ARK splice insertion, and SK1 channels with an AKQ insert at the same position as ARK in avian SK2. It will be interesting to test whether the differences in charge of the amino acids inserted, neutral glutamine (Q) vs. positively charged arginine (R) may underlie the differential effects of AQK vs. ARK insertion on the modulation of Ca2+ sensitivity.

SK channels function as tetramers, either homomers or heteromers composed of different subunits. Since SK1–3 subunits can co-assemble to form functional heterotetramers, it seems likely that SK2 and SK2-ARK may also co-assemble into functional channels. SK2-ARK mRNA levels show developmental increases that are modest in the avian embryonic cochlea and substantial (4-fold) in the mammalian postnatal hippocampus and cortex. Based on the 23 to 37% increases in SK2-ARK transcripts in the cochlea from E12-E14 to E20, equal rates of mRNA translation and subunit co-assembly could potentially result in 60–80% of the channels containing at least one SK2-ARK subunit. The impact of ARK expression and population effects are important issues but are difficult to resolve and interpretation of results will be limited because of the lack of reagents that can distinguish between the SK2 and SK2-ARK isoforms. We lack information about protein expression levels, subunit co-assembly, post-translational modifications, trafficking, and localization in sensory hair cells and neurons. Regulatory effects of ARK on these properties would enhance the importance of its developmental expression changes. Intriguingly, the ARK insertion is immediately adjacent to a predicted phosphorylation site. It will be important for future studies to also define mechanisms that regulate the extent of splicing and whether the relative levels are modified by synaptic activity in cochlear hair cells and forebrain neurons.

Overall, our data suggest that SK2 alternative splicing serves as a novel mechanism for regulating SK2 channel protein interactions, surface levels, and response to Ca2+ and CaM. Developmental increases in SK2-ARK levels in vivo during embryonic cochlea hair cell synapse maturation (Fig. 3C) raise the possibility that alternative splice isoforms may play a role in modulating hair cell inhibitory responses. By altering the relative levels of SK2 and SK2-ARK during development, and possibly during plasticity in adults, cochlear hair cells may modulate the surface levels and functional coupling of α9/10-nAChRs and SK2.
channels, and thereby fine-tune the strength and efficiency of olivocochlear synaptic transmission that leads to hair cell hyperpolarization and suppression of afferent auditory transmission. Our demonstration of SK2-ARK expression in mammalian neurons suggests that the mechanisms uncovered here for avian SK2 channels may have widespread relevance to the mammalian nervous system. This complexity of SK2 isoforms may provide a mechanism for activity-dependent changes in synaptic function.

Materials and Methods

Antibodies

Primary antibodies used were: monoclonal anti-α-actinin (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/a5044?lang=en&region=US); monoclonal anti-α-actinin-1 (United States Biological, http://www.usbio.net/item/A0761-01C?highlight); polyclonal anti-MAGI-2/S-SCAM (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/m2441?lang=en&region=US); polyclonal anti-SK2 potassium channel (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/p0483?lang=en&region=US); monoclonal anti-SV2 (Developmental Studies Hybridoma Bank, http://dshb.biology.uiowa.edu/synaptic-vesicles); polyclonal anti-MBP (maltose binding protein; New England Biolabs, https://www.neb.com/catalog/e8030-anti-mbp-antiserum); monoclonal anti-GST (glutathione S-transferase, Santa Cruz Biotechnology, http://www.scbt.com/datasheet-138-gst-b-14-antibody.html); monoclonal anti-calmodulin (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/c5345?lang=en&region=US); monoclonal anti-HA (Roche Diagnostics, http://www.roche-applied-science.com/shop/products/anti-ha-biotin-high-affinity-3F10-); monoclonal anti-cortactin (Millipore, http://www.millipore.com/catalogue/item/05-180); monoclonal anti-sodium/potassium ATPase (Thermo Scientific, http://www.emdsdiasum.com/microscopy/products/histology/embedding.aspx). Ten-micrometer thick cryosections were blocked with 5% normal donkey serum (Jackson Immunoresearch, http://www.jacksonimmuno.com/Catalog/CatPages/normal.asp) or 0.25% teloseg gelatin (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/g7765?lang=en&region=US) in PBS. Sections were incubated with primary antibodies for 1 h at RT, rinsed 3 times with blocking buffer, incubated with secondary antibodies for 45 min, rinsed, and mounted with coverslips using Vectorshield (Vector Labs, http://www.vectorlabs.com/catalog.aspx?prodID=428).

Image analysis

Epifluorescence images were captured using a Zeiss Axioskop epifluorescence microscope and QImaging Retiga 200R CCD camera and analyzed using Nikon Instruments NIS Elements software. Confocal images were captured using a Leica TCS SP2 confocal microscope with HeNe (633 nm), Kr (568 nm), and Ar (488 nm) lasers and a 63 x 1.32 numerical aperture lens. Optical sections were taken in 0.5 μM steps. For each cell, 3 consecutive sections through a representative region were compressed for analysis. For labeled cochlea sections, epifluorescence images or confocal stacks from representative cells were used to assess co-localization of double-labeled proteins. Pixel intensities were measured along -3 μM vertical lines drawn across representative regions of the efferent olivocochlear synapse that were distinguished by clearly detectable markers of the presynaptic terminal or postsynaptic membrane at the basal synaptic pole of the short hair cells. This criterion distinguished the synapse and verified the penetration of antibodies to the region. Presynaptic vs. postsynaptic localization of the test protein was defined on the basis of the extent of overlap of the peak pixel intensity with that of the marker pre- or postsynaptic protein and on the juxtaposition (predominantly red and green staining) vs. overlap (yellow staining) of the fluorescent double labeling.

RT-PCR and α-actinin sequencing

Total RNA was isolated from E19 chicken cochlear ducts using TRIzol Reagent (Invitrogen, http://products.invitrogen.com/ivgn/enUS/adirect/invitrogen?cmd=catDisplayStyle&catKey=97901&filterDispName=AlexaFluor®&filterType=1&OP=filter&filter=ft_1601). Reverse transcription (RT) was performed using oligo (dT) primers and Total RNA was isolated from E19 chicken cochlear ducts using TRIzol Reagent (Invitrogen, http://products.invitrogen.com/ivgn/enUS/adirect/invitrogen?cmd=catDisplayStyle&catKey=97901&filterDispName=AlexaFluor®&filterType=1&OP=filter&filter=ft_1601). Reverse transcription (RT) was performed using oligo (dT) primers and SuperScript II reverse transcriptase (Invitrogen, http://tools.invitrogen.com/content/sfs/manuals/819520_Rev1008.pdf).

Chicken embryos

Embryonated White Leghorn chicken eggs were obtained from the University of Connecticut Poultry Farm (Storrs, CT) or Charles River Spafas. Embryos were kept at 37 °C in forced air-draft humidified incubators until embryonic day (E) 12–20.

Immunolabeling

Cochlear ducts were dissected from E19–20 chicken embryos. For SK2 immunolabeling, cochleae were fixed for 1 h in 2% paraformaldehyde at 4 °C, cryoprotected by immersion in increasing concentrations of sucrose, and embedded in 7.5% gelatin with 15% sucrose. For S-SCAM and α-actinin immunolabeling, cochleae were fixed in 4% paraformaldehyde with 15% picric acid, cryoprotected, and embedded in TissueTek OCT compound (Electron Microscopy Sciences, http://www.emsdiasum.com/microscopy/products/histology/embedding.aspx). Ten-micrometer thick cryosections were blocked with 5% normal donkey serum (Jackson Immunoresearch, http://www.jacksonimmuno.com/Catalog/CatPages/normal.asp) or 0.25% teloseg gelatin (Sigma, http://www.sigmaaldrich.com/catalog/product/sigma/g7765?lang=en&region=US) in PBS. Sections were incubated with primary antibodies for 1 h at RT, rinsed 3 times with blocking buffer, incubated with secondary antibodies for 45 min, rinsed, and mounted with coverslips using Vectorshield (Vector Labs, http://www.vectorlabs.com/catalog.aspx?prodID=428).

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Total RNA isolated from chicken cochlear ducts at selected developmental ages was used for RT-PCR as described above, using SK2-specific primers flanking the ARK splice insertion site: 5’ACTTACGGGATCCAATTTTCCATGGACACCCA3’ (forward), 5’CATGATGACATGATCTCTG (reverse), 5’GGAAATTCTTTATGCTGTTTCCCGGGGACAATGATGGACACCCA3’ (reverse). PCR products were subcloned into pcDNA3.1 (Invitrogen, http://tools.invitrogen.com/content/sfs/vectors/pcdna3.1.pdf) and grown on LB-ampicillin plates. SK2 clones were amplified by PCR from individual colonies, and PCR products were digested with the restriction enzyme Hpy188I (New England Biolabs, https://www.neb.com/products/r0617-hpy188i.html), which recognizes a unique site in the ARK containing, but not the ARK lacking, splice variant.

Presence or absence of digestion products was assessed by agarose gel electrophoresis. Identity was verified by sequencing the ARK containing, but not the ARK lacking, splice variant.

**SK2-ARK splice variant quantification**

Total RNA was extracted from chicken cochlear ducts at selected developmental ages was used for RT-PCR as described above, using SK2-specific primers flanking the ARK splice insertion site: 5’ACTTACGGGATCCAATTTTCCATGGACACCCA3’ (forward), 5’CATGATGACATGATCTCTG (reverse), 5’GGAAATTCTTTATGCTGTTTCCCGGGGACAATGATGGACACCCA3’ (reverse). PCR products were subcloned into pcDNA3.1 (Invitrogen, http://tools.invitrogen.com/content/sfs/vectors/pcdna3.1.pdf) and grown on LB-ampicillin plates. SK2 clones were amplified by PCR from individual colonies, and PCR products were digested with the restriction enzyme Hpy188I (New England Biolabs, https://www.neb.com/products/r0617-hpy188i.html), which recognizes a unique site in the ARK containing, but not the ARK lacking, splice variant.

Presence or absence of digestion products was assessed by agarose gel electrophoresis. Identity was verified by sequencing the ARK containing, but not the ARK lacking, splice variant.

**Q-PCR of Mouse Forebrain SK2 splice variants**

Total RNA was extracted (Qiagen, http://www.qiagen.com/Products/Catalog/Sample-Technologies/RNA-Sample-Technologies/Total-RNA/RNeasy-Mini-Kit) from the cortex and hippocampus of young (postnatal day 8) and adult (3 mo old) mice. cDNA was generated using reverse transcription (Life Technologies) with oligo (dT) primers. Q-PCR assays were performed using primers common to chicken skeletal muscle, smooth muscle, and non-muscle α-actinin isoforms: 5’GACAACAGCAACACACTACACCTAGGAGCA3’ (forward), 5’ATCTCTGGTGTCCCCCCAGGACATGATGGACACCCA3’ (reverse). PCR product of the expected size was extracted from agarose gel and sequenced.

**Electrophysiology**

cRNAs encoding the chicken SK2 and SK2-ARK subunits were microinjected (3ng) into Stage V Xenopus laevis oocytes. SK2 currents were recorded under voltage clamp from the oocytes between 2 and 5 d after injection. Recordings were made in the inside-out patch-clamp configuration using an Axon 200B patch-clamp amplifier, and a Macintosh-based computer system running PatchMaster Software (HEKA Inc.). Patch pipettes were made of borosilicate glass and had resistances of between 1 and 1.5 Mohms in our standard internal and external solutions.

The internal (bath) solution contained in mM: 140 KMeSO4, 20 HEPES, 2 KCl, 1 EGTA and CaCl2, as needed (see below). The external (pipette) solution contained in mM: 140 KMeSO4, 20 HEPES, 2 KCl, and 2 MgCl2. After patch excision, solutions containing differing concentrations of free Ca2+ were superfused onto the cytoplasmic face of each patch using a DAD12 (ALA Scientific Instruments) sewer-pipette style superfusion system. Solutions were exchanged in less than 1 s.

During each experiment a voltage step from 0 to +50 mV was applied for 20 ms 5 times consecutively. The resulting five current responses were averaged before analysis. Current responses were recorded from each patch at the following Ca2+ concentrations in mM: 0.01, 0.1, 0.2, 0.5, 0.7, 1, 3, and 10. Ca2+ solutions were prepared by adding to our standard internal solution, which contained 1 mM EGTA, the correct amount of CaCl2 (100 mM Orion Standard), as calculated with MaxChelator Software (http://maxchelator.stanford.edu/) to achieve the desired free [Ca2+]. We assumed our standard internal solution contains 10 mM total calcium when no Ca2+ was intentionally added, as measured previously.46 The amplitude of the SK2 current was measured as the difference between the current observed at 0 mV and that observed at +50 mV at the center of the voltage step. The difference in amplitude ΔI was then used to plot Ca2+ dose-response curves for each patch. Each curve was then fitted with human α-actinin-1 (97% homology to chicken α-actinin-1) (NM_001130004; gift of David Critchley, University of Leicester) were cloned into the pPOX oocyte expression vector.

In vitro cRNA synthesis was performed using the mMessage mMachine T3 transcription kit (Applied Biosystems, http://products.invitrogen.com/ivgn/product/AM1348). Oocytes (provided by Joel Richter, University of Massachusetts Medical School) were obtained from adult Xenopus laevis frogs and defolliculated in OR2 (in mM: 82.5 NaCl, 2 KCl, 0.5 MgCl2, 5 HEPES pH 7.6) containing 2mg/ml collagenase (Sigma) and 1mg/ml dispase II (Roche Applied Science, http://www.roche-applied-science.com/shop/products/dispace14301-i-neutral-protease-grade-ii-). Oocytes (Stage V) were microinjected with cRNAs: 14–15ng each of α9-nAChR and α10-nAChR-HA, 10–14ng of α-actinin-1, and 3–8ng of SK2 or SK2-ARK. Oocytes were maintained at 19 °C for 2–3 d in ND96 (in mM: 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, 5 HEPES,pH 7.6) supplemented with 2.5mM sodium pyruvate, 50μg/ml tetracycline, 50 μg/ml gentamicin, 100μg/ml amikacin, and 100μg/ml ciprofloxacin (all from Sigma).
the Hill equation below and normalized to the maximum of the fit:

$$\Delta I = \Delta I_{\text{Max}} + \Delta I_{\text{Max}} \left[\text{Ca}^\text{2+}\right]^n$$

$$[\text{Ca}]^\text{2+} + K D^\text{H}$$

Here $\Delta I_{\text{Max}}$ and $\Delta I_{\text{Max}}$ are the minimum and maximum of the curve respectively. $KD$ is the [Ca$^{2+}$] at half-maximal response, and $H$ is the Hill coefficient. Normalized dose-response curves were then averaged to produce dose-response curves.

**Recombinant peptide binding assays**

Maltose binding protein (MBP) fusions of chicken SK2 and SK2-ARK C-termini (aa 368–553) were created by cloning sequence verified PCR products into the pMalC2 vector (New England Biolabs). Glutathione S-transferase (GST) fusion of full-length human $\alpha$-actinin-1 was created by cloning into the pGex4T3 vector (GE Healthcare, https://www.gelifesciences.com/gehcls_images/GELS/Related%20Content/Files/1314716762536/litdoc28-9545-52PS_AA_06-2009_WEB_20110830172010.pdf). Fusion peptides were expressed in Rosetta-gami 2(DE3) cells (EMD Chemicals, http://www.emdmillipore.com/life-science-research/rosetta-gami-2de3-competent-cells-EMD_BIO-71351/p_w lab_s1O2sAAAEjRx9LX) and grown in LB media containing 0.5M NaCl and 2.5 mM betaine to an optical density (OD) of 500–700. Peptide expression was induced with T7 promoter (Roche Applied Science, http://www.roche.com/products/product-details.htm?type=product&id=111). Beads were washed 3 times with Tris-Tx buffer. Complexes were eluted, separated on SDS-PAGE gels, and transferred to nitrocellulose membranes. Pulldown of $\alpha$-actinin-1-GST was detected using (Thermo Scientific, http://www.piercenet.com/browse.cfm?fieldID=02040806) and phosphatase inhibitor cocktail (Roche Applied Science, http://www.roche.com/products/products/product-details.htm?type=product&id=111). Band densities were quantified using Nikon Instruments NIS Elements software and normalized to SK2 or SK2-ARK levels, detected with anti-MBP antibody, in the same lane. In each experiment, normalized levels of $\alpha$-actinin-1-GST or CaM co-precipitated with SK2-ARK-MBP were calculated as a percentage of co-precipitation with SK2-MBP (100%).

For $\alpha$-actinin-1/CaM competition assays, SK2- or SK2-ARK-MBP were incubated with amylose resin and $\alpha$-actinin-1-GST in Tris-Tx buffer. To assess the effects of Ca$^{2+}$, Tris-Tx buffer was supplemented with 5mM BAPTA or 1mM CaCl$_2$. Bound complexes were washed, and CaM added at 0, 0.5, 1, or 10μM. Alternatively, SK2 or SK2-ARK peptides and amylose resin were incubated first with 10μM CaM in BAPTA or CaCl$_2$ buffer, washed, and then incubated with $\alpha$-actinin-1-GST. Proteins were eluted and $\alpha$-actinin-1-GST pulldown was assayed as above.

**Co-immunoprecipitation from cochlear membrane fractions**

Membrane fractions were prepared from E19–20 chicken cochlear ducts. Tissue was homogenized on ice in sucrose buffer (in mM: 320 sucrose, 10 HEPES, 1 EGTA) with protease inhibitor cocktail and centrifuged at approximately 2,000 x g for 10 min. The supernatant was centrifuged at 100,000 x g for 1 h, and the resulting membrane pellet was resuspended in membrane buffer (in mM: 190 NaCl, 10 KCl, 1 EGTA, 10 HEPES pH 7.4) with protease inhibitors. The suspension was sonicated and centrifuged again at 100,000 x g for 30 min to remove insoluble material. The supernatant was pre-cleared for 1 h with protein G agarose beads (Roche Applied Science) and incubated overnight at 4 °C with 8 μg of anti-SK2 antibody. Immunocomplexes were precipitated with protein G agarose beads and washed 6 times with membrane buffer supplemented with 0.1% Triton X-100. Proteins were eluted, separated on SDS-PAGE gels, and immunoblotted using anti-$\alpha$-actinin-1 antibody, ECL Plus detection, and exposure to X-ray film. As a specificity control, equivalent membrane fractions were immunoprecipitated with 8μg anti-HA antibody. In some experiments, 5mM BAPTA was added to all buffers.

**Co-immunoprecipitation from oocytes**

*Xenopus* oocytes were injected with cRNAs encoding SK2 or SK2-ARK, HA-tagged $\alpha$10, $\alpha$9, and $\alpha$-actinin-1 as above. To show dependence of co-precipitation on exogenous SK2, this cRNA was excluded in separate oocyte injections. Membrane fractions were used for SK2 immunoprecipitation at 3days after injection. Oocytes (45–75 for each prep) were homogenized in ice cold buffer H (in mM: 83 NaCl, 1 MgCl$_2$, 5 EDTA, 5 EGTA, 10 HEPES pH 7.8, 4–6μL/oocyte) and centrifuged for 10 min at 750 x g. Pellets were resuspended in buffer H and re-centrifuged. Combined supernatants were layered onto 7mL 15% sucrose in buffer H and centrifuged for 90 min at 160,000 x g. Membrane pellets were resuspended in Tris-Tx buffer, 2–4μL/oocyte, with protease and phosphatase inhibitors and incubated for 1 h at 4 °C with gentle agitation to solubilize membrane proteins. Insoluble material was removed by centrifugation at 100,000 x g for 1 h. Soluble proteins were pre-cleared with protein G agarose and incubated overnight with 6–9μg anti-SK2 antibody. Immunocomplexes were precipitated with protein G agarose beads, and washed 3 times with Tris-Tx buffer. Bound proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. Co-precipitation of HA-tagged $\alpha$9/10-nAChRs and $\alpha$-actinin-1 was assessed by immunoblotting. Band intensities were normalized to precipitated SK2 in the same.
Biotin (Thermo Scientific, http://www.piercenet.com/browse.cfm?fildID=01021103) were added to precipitate biotinylated proteins and washed 4 times in Tris-Txs buffer. Bound proteins were eluted and separated on SDS-PAGE gels. Levels of biotinylated SK2 and α9/10-nAChR were assessed by immunoblotting and normalized to input membrane expression. Background precipitation from unbiotinylated samples was subtracted from each measurement. As a negative control, membranes were probed with antibodies against the intracellular actin-binding protein cortactin to confirm specific biotinylation of surface proteins only.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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