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## The large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel interacts with the apolipoprotein ApoA1

Bernd Sokolowski<sup>1,\*,!</sup> , R. Keith Duncan<sup>2,!</sup> , Stephanie Chen<sup>2,</sup> , Jörg Karolat<sup>1,</sup> , Thandavarayan Kathiresan<sup>1,</sup> , and Margaret Harvey<sup>1</sup>

<sup>1</sup>Otology Laboratory, Dept. of Otolaryngology-HNS, University of South Florida, College of Medicine, 12901 Bruce B. Downs Blvd., Tampa, FL 33612, USA

<sup>2</sup>Kresge Hearing Research Institute, University of Michigan, 1150 W. Medical Center Dr., Ann Arbor, MI 48109, USA

### Abstract

Owing to the multifaceted functions of the large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (BK), identification of protein-protein interactions is essential in determining BK regulation. A yeast two-hybrid screening of a cochlear cDNA library revealed a BK-ApoA1 interaction. Patch clamp recordings of excised membrane patches from transfected HEK293 cells showed that ApoA1 inhibits the BK  $\alpha$ -subunit by significantly increasing activation and deactivation times, and shifting half-activation voltage to more positive potentials. Reciprocal coimmunoprecipitations verified the BK-ApoA1 interaction using excised sensory epithelium and ganglia. Additionally, immunocolocalization studies revealed BK and ApoA1 expression in both receptor cells and auditory neurons. These data suggest new avenues of investigation, given the importance of apolipoproteins in neurological diseases.

### Keywords

Ion channels; Large-conductance calcium-activated potassium channels; Apolipoprotein; Protein-protein interactions; Cochlea; Hair cells

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Ion channels have various roles in a number of different cell types and thus may encounter many different protein partners. The function of the BK channel is especially multifaceted, as it plays a role not only in excitation but also in intracellular signaling and metabolism. This functional complexity is reflected in findings that show BK expression in different subcellular compartments, including the mitochondrion and nuclear envelope [1-3]. BK will likely interact with a variety of proteins that are indigenous to these compartments and potentially alter cellular homeostasis [3].

Apolipoproteins have a significant role in various neurological diseases such as Alzheimer's, where they are found in both senile plaques and neurofibrillary fibers, and Creutzfeldt-Jakob disease, where they are found in kuru plaque amyloid [4]. This role extends to audition, since

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\*Corresponding Author: Fax: 813-974-1483; bsokolow@health.usf.edu.

<sup>!</sup>These authors contributed equally to this work.

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knockout mice, deficient in apolipoprotein E1 (ApoE1), lose high frequency hearing with the loss of receptor cells at the basal turn of the cochlea [5]. There are very few reported studies of lipoprotein effects on channel activity, however, despite the prevalence of these proteins in neuronal systems. A recent report shows that ApoE4 alters the biophysical properties of delayed rectifier type channels when applied to excised patches from hippocampal neurons [6], whereas oxidized low density lipoproteins appear to affect the kinetics of BK channels in cultured endothelial cells [7].

In the present study, a yeast two hybrid screening of a cDNA library made from embryonic days 14-19 chicken cochlea revealed an interaction between BK and the apolipoprotein, ApoA1, a component of high density lipoproteins. Here, we provide functional evidence for this interaction in excised membrane patches, when coexpressed in HEK293 cells. Moreover, we demonstrate that ApoA1 and BK are coexpressed in both neuronal and receptor cells. To date, apolipoprotein effects on ion channels have used acute application techniques. Thus, to the best of our knowledge, this study is the first to show a direct apolipoprotein-ion channel association via cellular expression.

## Materials and methods

### Yeast two-hybrid screening

The full BK channel inserted into pBD-Gal4 *Cam* (Stratagene) at the EcoRI and SalI restriction sites was used as bait to screen a cDNA library made from embryonic days 14-19 *Gallus gallus* sensory epithelium from the basilar papilla (i.e., cochlea) as described previously [8]. Yeast AH109 cells (Clontech) were transformed sequentially, first with pBDGal-4 containing the bait construct and then with pADGal-4/cDNA library using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method [9]. Cells were plated on SD-His-Leu-Ade-Trp (SD-HLAT) medium and positive colonies selected and analyzed. Full-length cDNA fragments of ApoA1 were obtained and subcloned into the pCMS-EGFP vector (BD Bioscience) for co-expression experiments, to study the biophysical effects on the BK channel.

### Electrophysiology

Heterologous expression and biophysical characterization of BK channel constructs were performed as described previously [10]. BK channels, with and without ApoA1, were transiently expressed in HEK293T cells using standard  $\text{Ca}^{2+}$ -phosphate transfection techniques. HEK cells were maintained in a high-glucose DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin and cultured at 37 °C. To limit genetic drift, cells were passaged no more than 20 times. Plasmid constructs included the minimal variant of the chicken BK  $\alpha$ -subunit (*cslo1*) in a mammalian expression vector (pcDNA3.1) [10,11] and ApoA1 in pCMS-EGFP. When required, ApoA1 was added at two-fold molar excess to BK $\alpha$  in order to saturate potential stoichiometric interactions between these proteins. Transfections using BK $\alpha$  alone included pEGFP $\alpha$ Luc (BD Biosciences Clontech) for fluorescence detection of transfected cells.

Patch clamp recordings were obtained from excised membrane patches in the inside-out configuration [12]. Cells were obtained 24 to 48 h after transfection. Bath solutions, pipette solutions, and perfusate each consisted of a high  $\text{K}^+$  saline with (mM): 142 KCl, 0.5  $\text{MgCl}_2$ , and 5 HEPES at pH 7.2.  $\text{CaCl}_2$  and a suitable buffer were added to achieve desired free  $\text{Ca}^{2+}$  concentrations. All solutions were adjusted to a pH of 7.2 using KOH. After excision, the inside face of the patch was exposed to a high  $\text{K}^+$  perfusate calibrated to contain free  $\text{Ca}^{2+}$  concentrations of 1 or 20  $\mu\text{M}$  using the  $\text{Ca}^{2+}$  chelators  $\text{Br}_2\text{BAPTA}$  or NTA, respectively [13]. The pipette solution was identical to the 1  $\mu\text{M}$  free  $\text{Ca}^{2+}$  test solution in order to set the  $\text{K}^+$  reversal potential to 0 mV and minimize junction potentials.  $\text{Ca}^{2+}$  concentration was calibrated using a  $\text{Ca}^{2+}$ -sensitive electrode and multiple  $\text{Ca}^{2+}$  standards (WPI, Sarasota, FL).

Microelectrodes were pulled from borosilicate glass to resistances of 5-9 M $\Omega$  and coated with R6101 (Dow-Corning) to reduce stray capacitance. Recordings were made with a MultiClamp 700B amplifier, Digidata 1440A digitizer, and the pClamp 10.0 software suite (Molecular Devices, Sunnyvale, CA). Data were sampled at 10 to 20 kHz, and low-pass filtered at 4 kHz. Each patch was estimated to contain between 4 - 160 BK channels, assuming a single channel conductance of 250 pS and a maximum open probability near unity. Transient and steady-state BK properties were determined from ensemble-averaged current recordings of 5 to 20 stimulus presentations. Pre-pulses to -50 or -100 mV were used to deactivate the channels prior to activation steps and ascertain the linear leak currents, which were subtracted off-line. Residual capacitance currents at the K<sup>+</sup> equilibrium potential were fit with exponentials and piecewise linearly subtracted off-line to resolve fast deactivation kinetics.

Statistical analyses of individual pairs were performed using a Student's t-test, whereas multiple group comparisons were analyzed using an ANOVA. Activation and deactivation kinetics were estimated with least square fits to standard single-exponential curves in Clampfit (pClamp) using a Levenberg-Marquardt algorithm. Averaged values are reported as the mean  $\pm$  standard error of the mean (S.E.M.) with significance indicated by  $p < 0.05$ . All recordings were made at room temperature (22-25°C).

### Coimmunoprecipitation

Immunoprecipitation was accomplished using the immunocomplex-capture technique. Eight cochleae from 3 week-old chickens, including sensory epithelium, ganglion and supporting cells were removed and processed as described previously [14,15]. The sample was centrifuged and the pellet solubilized in lysis buffer containing 50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 5 mM EDTA, 50 mM NaF, 500  $\mu$ g/ml AEBSF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml pepstatin A, 2  $\mu$ g/ml aprotinin, 5  $\mu$ M okadaic acid, and 0.1% ASB-14 (Calbiochem), followed by vortexing and agitating on a rocker for 1 h at 4°C. The fraction was precleared using rec-Protein G Sepharose 4B beads (Invitrogen). Monoclonal anti-BK antibody (5 $\mu$ g) (BD Biosciences; aa residues 995 - 1113 of human KCNMA1) was added to the sample and incubated at 4°C for 1 h followed by immunocomplex capture using 25  $\mu$ l of protein G beads at 4°C. Beads were washed in lysis buffer, eluted in sample buffer (Sigma), and heated at 70°C. Samples were fractionated on a 10% SDS-PAGE gel then blotted, followed by blocking in PBST, containing 5% milk for 1 h. Blots were probed with either the monoclonal anti-BK $\alpha$  antibody (1:200; BD Biosciences) or a polyclonal anti-ApoA1 antibody (1:300; Abcam). HRP-conjugated donkey anti-rabbit or goat anti-mouse secondary antibodies were used for visualization of both ApoA1 (1:5000) and BK $\alpha$  (1:4000), respectively. Immunoreactive bands were developed using ECL (Amersham). Controls consisted of sample incubation with beads alone and IPs with antibodies. Antigens for preadsorption controls were not available.

### Fluorescent Confocal Microscopy

Colocalization of BK and ApoA1 was determined in receptor and ganglion cells using respective antibodies with frozen sections prepared as described previously [16]. Briefly, three-week old chick basilar papillae (cochlea) with lagena were dissected partially from the temporal bone and fixed overnight (O/N) at 4°C by immersion in 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. The remaining temporal bone was removed the next day and the tissues immersed in 30% sucrose O/N, and infiltrated in a 1:1 mixture of O.C.T. (Sakura Fine-Tek) and 30% sucrose under vacuum for 1 hr. Cryosections of 20  $\mu$ m thickness were collected and permeabilized with 0.3 % Triton X-100 followed by blocking with 10% goat serum. Primary antibodies consisted of a monoclonal anti-BK $\alpha$  antibody (1:7.5; Antibodies Inc.) and a polyclonal anti-ApoA1 antibody (1:4; Abcam). Immunoreactivity was visualized using secondary antibodies labeled with the chromogens AlexaFluor 488 (ApoA1) and AlexaFluor 594 (BK) (Invitrogen). Sections were imaged with a Leica SP5 AOBs tandem scanning

inverted confocal microscope (Lasos, San Jose, CA) at the Microscopy Core Facility, Moffitt Cancer Center, and Z-stacks were acquired using a step size of 0.2  $\mu\text{m}$ .

## Results and discussion

### ApoA1 affects BK channel characteristics

A Y2H screening using pBD-Gal-4/BK as bait resulted in yeast colonies containing full-length *Gallus ApoA1* (Acc. No. M18746). Patch clamp analyses showed that ApoA1 inhibited the channel kinetics of BK $\alpha$  (Fig. 1). In the presence of ApoA1, the BK steady-state conductance-voltage ( $G$ - $V$ ) curve shifted to more positive potentials by  $\sim 11.6$  mV. Moreover, in the presence of 1  $\mu\text{M}$   $\text{Ca}^{2+}$ , the half-activation voltage for BK channels increased, showing mean values, with and without ApoA1, of  $69.8 \pm 4.5$  mV ( $n = 9$ ) and  $58.2 \pm 1.6$  mV ( $n = 8$ ;  $p < 0.05$ ), respectively. The inhibitory effects of ApoA1 were seen again when measuring the activation and deactivation times of BK $\alpha$ , in the presence of 1  $\mu\text{M}$   $\text{Ca}^{2+}$  (Fig. 2). As expected, both in the presence and absence of ApoA1, activation time constants decreased significantly with increased membrane potential from 80 to 130 mV (ANOVA;  $F_{\text{MEMB. POTENTIAL}} = 2.9$ ,  $\text{df} = 6$ ,  $p < 0.05$ ). However, ApoA1 increased activation time constants by  $\sim 3$  ms at these potentials (ANOVA;  $F_{\text{TREATMENT}} = 4.1$ ,  $\text{df} = 1$ ,  $p < 0.05$ ). BK $\alpha$  deactivation responded similarly in the presence of ApoA1, showing a significant increase in the time constant from  $1.3 \pm 0.2$  ms ( $n = 4$ ) to  $2.6 \pm 0.3$  ms ( $n=9$ ;  $p < 0.5$ ). In contrast, ApoA1 had no statistically significant effect on  $G_{\text{max}}$ , and the Boltzman slope factor ( $V_c$ ).  $G_{\text{max}}$  values measured for cells transfected with and without ApoA1 were  $21.6 \pm 10.6$  nS ( $n = 9$ ) and  $16.9 \pm 4.8$  nS ( $n = 8$ ;  $p > 0.05$ ), respectively, whereas  $V_c$  measured  $17.1 \pm 0.77$  mV and  $15.2 \pm 1.0$  mV ( $p > 0.05$ ).

These results demonstrate that ApoA1 has an inhibitory effect on BK response properties at the membrane as opposed to acting as a chaperone that increases channel density. This inhibitory effect would alter the hyperpolarization characteristics of excitatory cells and more specifically, in hair cells, the tuning properties. Chick hair cells, as those in turtle [17,18] show receptor potential oscillations or electrical “tuning.” This resonance is a function of BK channel number and kinetics [19], which may be varied systematically along the cochlea by several mechanisms including splice variation and co-assembly with accessory proteins. Thus, with ApoA1 inhibition, one might expect a decrease in the resonant frequency or tuning of the receptor cell.

The BK channel is sensitive to  $\text{Ca}^{2+}$  concentrations, exhibiting as many as three  $\text{Ca}^{2+}$ -binding sites [20]. To test the possibility that inhibition by ApoA1 was due to a change in  $\text{Ca}^{2+}$  sensitivity, the effect of ApoA1 was tested in a higher  $\text{Ca}^{2+}$ -free concentration. When exposed to 20  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ , the half-activation voltage for channels with and without ApoA1 was  $-1.4 \pm 3.4$  mV and  $10.7 \pm 2.6$  mV ( $p < 0.05$ ,  $n = 5$ ), respectively ( $G$ - $V$  data not shown). This shift in  $V_{1/2}$  of  $\sim 11.1$  mV was similar to the shift measured when using 1  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ , suggesting that ApoA1 influences channel gating by a  $\text{Ca}^{2+}$ -independent mechanism.

Regulators of BK can be both  $\text{Ca}^{2+}$ -independent and dependent. For example, the addition of purified PKC $\delta$  activates BK channels, and alters the biophysical characteristics, as  $V_{1/2}$  decreases with increased  $\text{Ca}^{2+}$  concentrations [21]. Similarly, syntaxin 1A has a  $\text{Ca}^{2+}$ -dependent effect by increasing BK activity at low  $\text{Ca}^{2+}$  concentrations (1-4  $\mu\text{M}$ ), but not at high  $\text{Ca}^{2+}$  concentrations ( $>10$   $\mu\text{M}$ ) [22]. In contrast, the lipid, *N*-arachidonoyl L-serine, and the xenoestrogen, tamoxifen, show no  $\text{Ca}^{2+}$  dependency [23,24].

### ApoA1 is expressed in sensory and ganglion cells

Reciprocal coIPs, using whole cochlea, demonstrate that BK and ApoA1 interact in the cochlea, since each protein was able to immunoprecipitate the other (Fig. 3). ApoA1

immunoprecipitated BK $\alpha$ , which is seen as an ~120 kDa band. Similarly, BK immunoprecipitated ApoA1 as observed by the band at ~28 kDa (Fig. 3). Colocalization studies using fluorescent tags, showed that BK $\alpha$  and ApoA1 are colocalized in receptor and ganglion cells (Fig. 4). Previous studies of apolipoproteins in cochlea were restricted to ApoE1 and ApoD, where mice deficient in the former showed a hearing loss and substantial cochlear cell death [5,25], while ApoD knockouts had normal hearing thresholds [26]. Putative roles include cochlear ionic and oxidative homeostasis [25,26], a balance that may be connected to ion channels.

In summary, we demonstrated the effectiveness of ApoA1 in altering the kinetics of the BK channel. Previously, acute application of ApoE1, a component of intermediate-density lipoproteins, changed the kinetics of the delayed rectifier channel in hippocampal cells [6]. Interestingly, ApoA1 had no effect on this channel despite its similarity in weight and amino acid sequence to ApoE [6]. These discrepancies may be the result of methodologies or physical differences in the channels. Nonetheless, given the different types of apolipoproteins, their association with ion channels should be explored further in relation to neurological diseases.

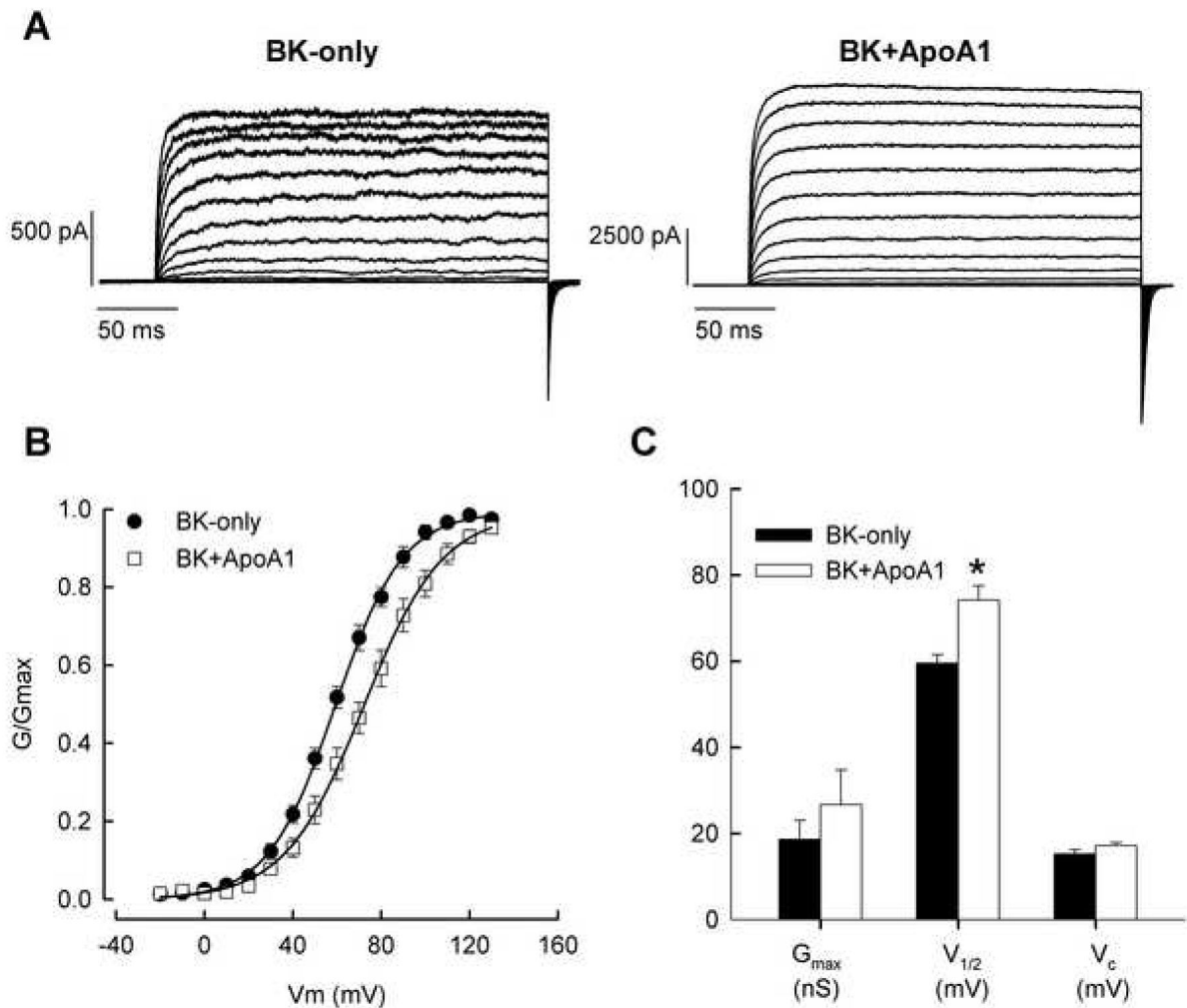
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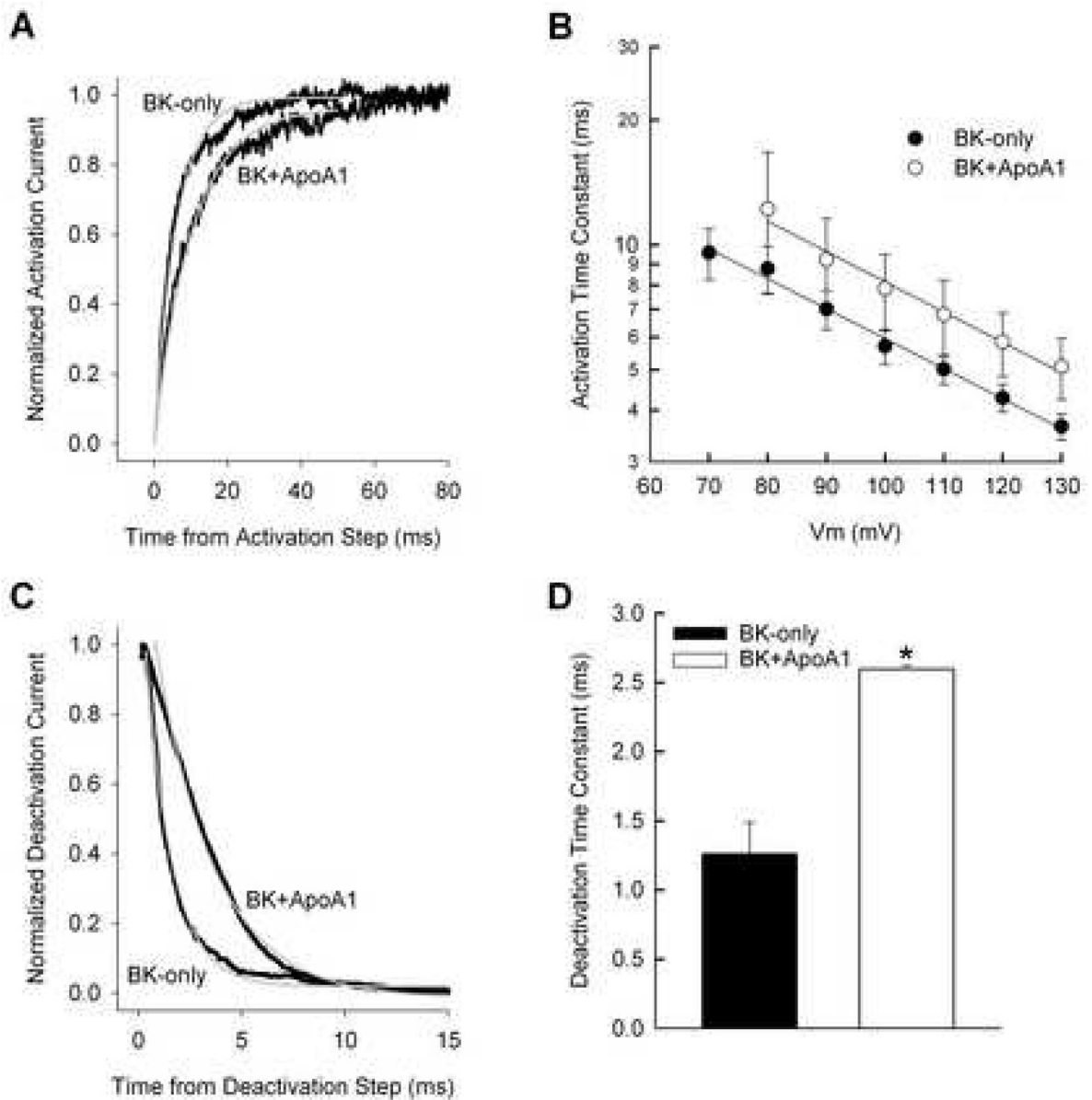
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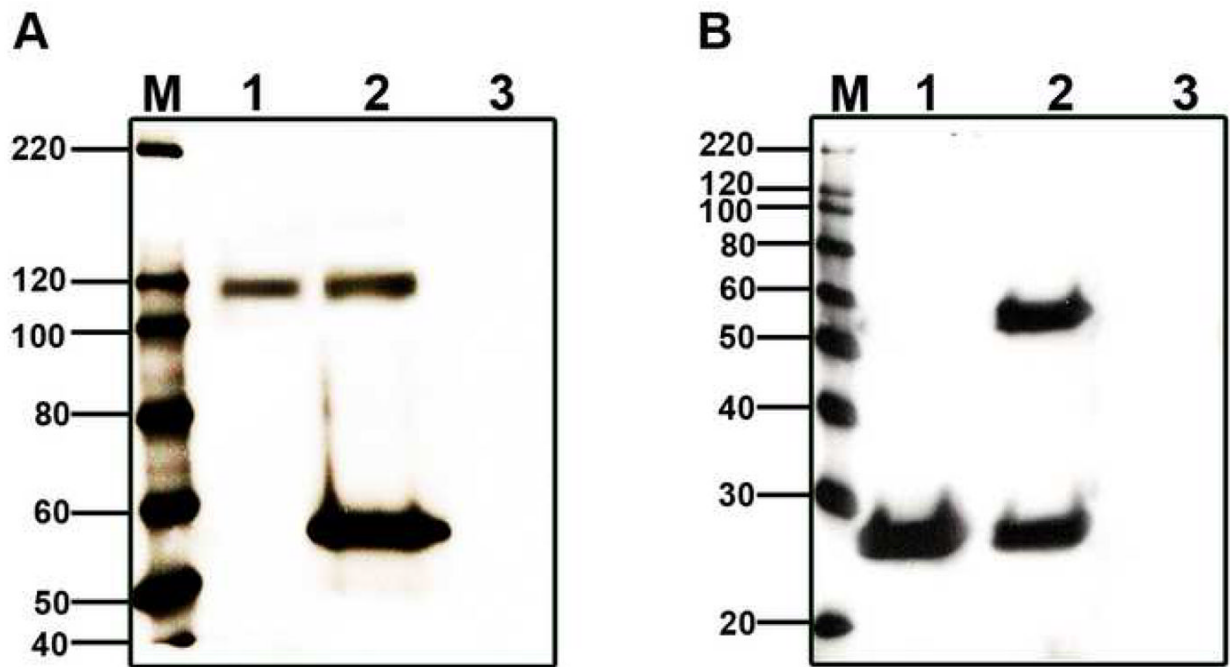
**Fig. 1.**

ApoA1 increases BK half-activation voltage. (A) Currents recorded from isolated inside-out patches of HEK293 cells transfected with BK only or BK+ApoA1. Patches were stepped from  $-20$  mV to  $130$  mV from a holding potential of  $-50$  mV. Tail currents were generated by a final step back to  $-50$  mV. (B) Average, normalized G-V curves were generated for patches containing BK-only ( $n = 8$ ) and BK+ApoA1 ( $n = 9$ ). Individual G-V curves were fit to Boltzmann equations and fit parameters averaged. (C) Plots of the average  $G_{\max}$ , half-activation voltage ( $V_{1/2}$ ), and Boltzmann slope factor ( $V_c$ ) are shown for the two-channel configurations. (\* $p < 0.05$ ).

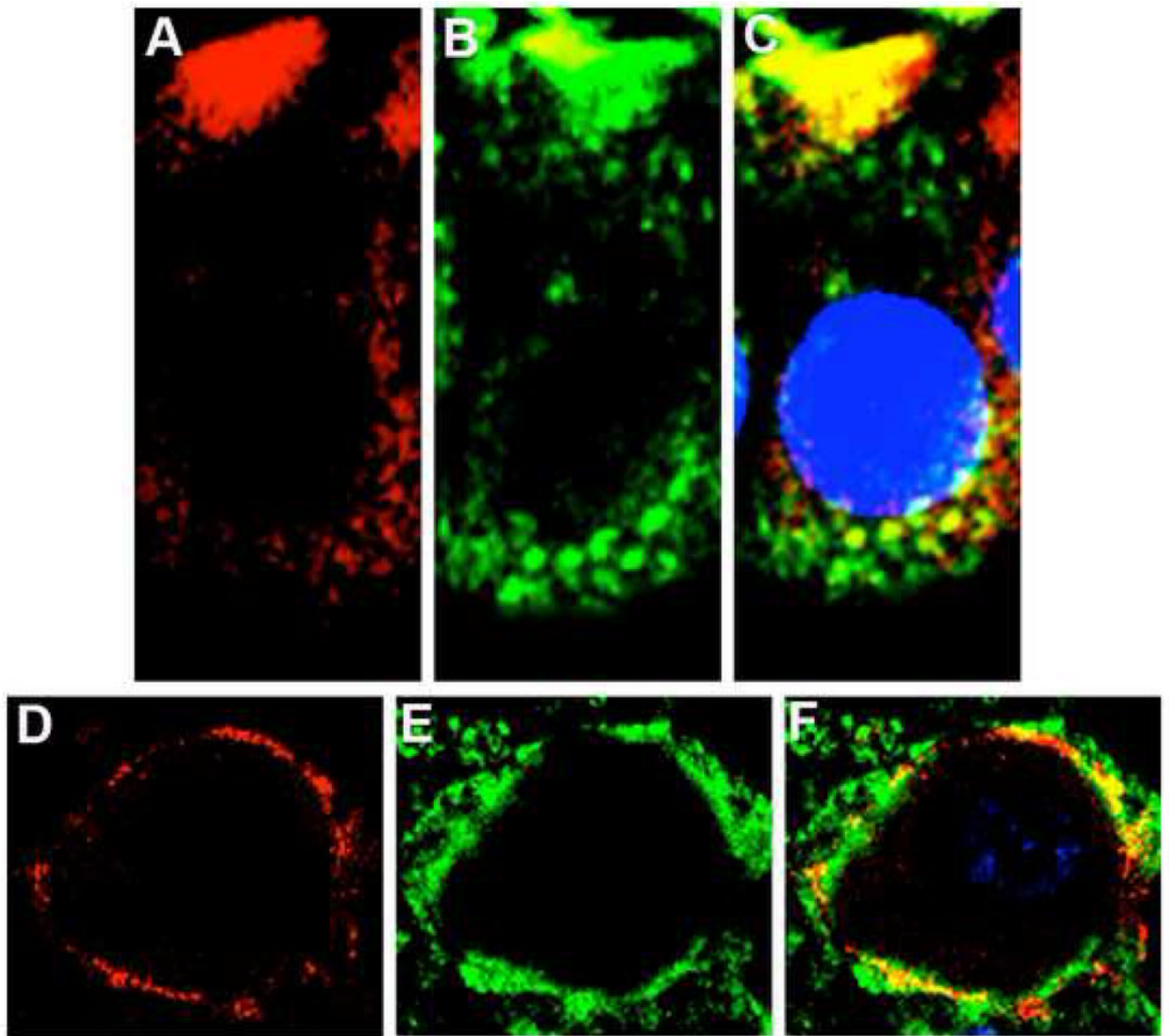
**Fig. 2.**

ApoA1 slows BK kinetics. (A) Exemplar of activation currents for a voltage step from  $-50$  mV to  $100$  mV. Traces were normalized to steady-state maxima after correcting for linear leak and capacitive transients. Single exponential fits (grey lines) are  $6.03$  and  $10.32$  ms for BK only and BK+ApoA1, respectively. (B) Average activation time constants plotted against the activation voltage command and fit with a single exponential show an  $\sim 3$  ms shift in BK activation with ApoA1 present. (C) Exemplar deactivation currents for a voltage step from  $130$  mV to  $-50$  mV. Traces were normalized and fit as in A. Single exponential fits (grey lines) are  $1.29$  and  $2.85$  ms for BK only and BK+ApoA1, respectively. (D) Deactivation kinetics at a single voltage level of  $-50$  mV were averaged from single exponential fits to tail currents elicited from a  $130$  mV activation step. Deactivation increased  $\sim 2$ -fold with ApoA1. Error bars indicate one SEM. ( $*p < 0.05$ )





**Fig. 3.** Verification of BK/ApoA1 interactions using reciprocal coIP. (A) CoIP of BK $\alpha$  with ApoA1 (lane 1) and IP of BK $\alpha$  (lane 2) reveal prominent bands at ~120 kDa, the expected weight of chick BK $\alpha$ . (B) Reciprocal coIP of ApoA1 with BK $\alpha$  (lane 1) and IP of ApoA1 alone (lane 2) reveal a prominent band at ~28 kDa, the expected weight of ApoA1. The control for both panels consisted of mixing IgG beads and lysate in the absence of an antibody (lane 3). The 55 kDa band is heavy IgG, and occurs when either monoclonal or polyclonal antibodies are used to both precipitate proteins and probe the blots. "M" denotes the standard.



**Fig. 4.** ApoA1 (green) and BK $\alpha$  (red) are colocalized in chick cochlea. The colocalization of ApoA1 and BK is visualized as punctate yellow fluorescence in (A) basolateral aspects of hair cells and (B) VIIIth nerve ganglion cell body. Images are from one section of a Z-stack acquired using a step size of 0.2  $\mu$ m.