Protein kinase A–induced internalization of Slack channels from the neuronal membrane occurs by adaptor protein-2/ clathrin–mediated endocytosis

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The sodium-activated potassium (K_{Na}) channel Kcnt1 (Slack) is abundantly expressed in nociceptor (pain-sensing) neurons of the dorsal root ganglion (DRG), where they transmit the large outward conductance I_{KNa} and arbitrate membrane excitability. Slack channel expression at the DRG membrane is necessary for their characteristic firing accommodation during maintained stimulation, and reduced membrane channel density causes hyperexcitability. We have previously shown that in a pro-inflammatory state, a decrease in membrane channel expression leading to reduced Slack-mediated I_{KNa} expression underlies DRG neuronal sensitization. An important component of the inflammatory milieu, PKA internalizes Slack channels from the DRG membrane, reduces I_{KNa}, and produces DRG neuronal hyperexcitability when activated in cultured primary DRG neurons. Here, we show that this PKA-induced retrograde trafficking of Slack channels also occurs in intact spinal cord slices and that it is carried out by adaptor protein-2 (AP-2) via clathrin–mediated endocytosis. We provide mass spectrometric and biochemical evidence of an association of native neuronal AP-2 adaptor proteins with Slack channels, facilitated by a dileucine motif housed in the cytoplasmic Slack C terminus that binds AP-2. By creating a competitive peptide blocker of AP-2–Slack binding, we demonstrated that this interaction is essential for clathrin recruitment to the DRG membrane, Slack channel endocytosis, and DRG neuronal hyperexcitability after PKA activation. Together, these findings uncover AP-2 and clathrin as players in Slack channel regulation. Given the significant role of Slack in nociceptive neuronal excitability, the AP-2 clathrin–mediated endocytosis trafficking mechanism may enable targeting of peripheral and possibly, central neuronal sensitization.

Activation of the PKA signaling cascade is a well established event in the milieu of inflammatory mediators leading to peripheral sensitization, a phenomenon characterized by increased excitability of nociceptive neurons of the dorsal root ganglion (DRG). Sodium-activated potassium (K_{Na}) channels are abundantly expressed in the cell bodies and axons of these sensory neurons of the DRG, where they conduct the prominent outward rectifying K^{+} current (I_{KNa}) that contributes to neuronal resting membrane potential (2) and basal membrane excitability (4). The K_{Na} channel Slack (sequence like a calcium-activated potassium channel; Kcnt1, K_{Na}1.1, Slo2.2) has been determined as critical to DRG neuron membrane excitability by siRNA knockdown of Slack channels, as well as in silico modeling studies that show reduced membrane channel expression and/or activity to result in loss of firing accommodation and hyperexcitability (4, 5). Recent work has identified PKA regulation of Slack channels to produce a consistent phenotype; namely, pharmacological activation of PKA induced a reduction in the K_{Na} component of I_{K} and membrane hyperexcitability (4). Although direct PKA phosphorylation produced no gating changes, significantly reduced Slack channel density at the DRG neuronal membrane following pharmacological activation of PKA points to an indirect trafficking mechanism likely involving intracellular endocytic proteins (4, 6). Given that DRG neuronal hyperexcitability is the first line of response and a hallmark of pro-inflammatory states, elucidation of the trafficking mechanism and the protein players involved in Slack channel internalization would be of value.

Among the cytoplasmic proteins identified heretofore as having a regulatory influence on Slack channels, intracellular protein kinases comprise a functionally significant fraction. The majority of these interactions can be attributed to the extensive intracellular C termini unique to K_{Na} channel sub-units, which house several consensus sites for phosphorylation by kinases (7–9) in the neighborhood of tandem regulators of K^{+} conductance (RCK) domains (10, 11). For example, PKC phosphorylation of Ser^{407} in the hinge region between the S6 transmembrane domain and the first RCK domain produces a constitutively active phenotype in Slack channels that mimics known KCNT1 mutations associated with malignant migrating

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The abbreviations used are: DRG, dorsal root ganglion; AP, adaptor protein; CME, clathrin-mediated endocytosis; RCK, regulator of conductance of potassium; AAK1, AP2-associated kinase 1; CID, collision-induced dissociation; ACSF, artificial cerebrospinal fluid.

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partial seizures of infancy (12). In the distal C terminus, phosphorylation by p38 MAPK is required for tonic conductance and surface expression of neuronal Slack channels, prevention of which causes significant reduction in total neuronal $I_{K}$ (9). Finally, the abovementioned indirect regulatory effect of PKA on Slack channels resulting in DRG neuronal hyperexcitability is bolstered by the fact that four of the five known PKA consensus sites are located outside the RCK domains (7). Taken together, this body of work on Slack channel regulation describes the Slack C terminus as a hub for intracellular protein interactions that directly impact neuronal membrane excitability.

The use of primary cultures of rat embryonic DRG neurons as a model system affords us with the unique ability to study native Slack channels in a state of endogenous abundance, because strong Slack immunolabeling has been demonstrated in $>90\%$ of intact DRG encompassing small- and medium-sized nociceptive neurons, as well as large-sized proprioceptive neurons (3). In culture, the required presence of NGF for survival of embryonic neurons, as well as the anti-mitotic agent arabinoside-C to eliminate non-neuronal cell types, produces a homogenous TrkA$^{+}$ nociceptive neuronal population that ubiquitously displays nearly identical membrane properties when electrophysiologically examined (13, 14). In the present study, we exploit these properties of primary DRG neurons to establish AP-2 as a primary player in Slack $K_{Na}$ channel trafficking and, hence, sensory neuronal excitability. We found Slack channels to be targeted for AP-2 clathrin-dependent endocytosis following activation of the PKA signal via a direct interaction at the conserved dileucine motif in the Slack C terminus. Using a short peptide inhibitor of Slack–AP-2 binding derived from the recognition motif sequence, we provide conclusive evidence for PKA-induced internalization of Slack channels by AP-2 clathrin–mediated endocytosis (CME).

**Results**

**Identification of AP-2 dileucine recognition motif in the Slack C terminus**

The AP complexes are the most extensively characterized intracellular endocytic proteins. There are five AP complexes (AP1–5) in the cytoplasm of the mammalian cell, where each adaptor complex participates in a distinct intracellular trafficking route (15). Removal of proteins from the plasma membrane is exclusively carried out by the AP-2 complex via recruitment and assembly of clathrin-coated vesicles, which can transport internalized cargo into intracellular compartments such as early or late endosomes. Much like the other AP complexes, AP-2 uses specific binding motif sequences in cargo proteins as recognition signals, canonically categorized as tyrosine-based sequences ($YXXΦ$ or NPXY, where $X$ represents any amino acid, and $Φ$ represents bulky hydrophobic amino acids) or dileucine-containing structures ($D/E$XXXL/I) (16). We asked whether the Slack channel is an AP-2 cargo protein by scanning the Slack C terminus protein sequence for these recognition motifs. We found a distally located dileucine motif ($D/E$XXXL/I) that conforms to the known AP-2 dileucine trafficking signal. Further, sequence alignment of the Slack C terminus across species revealed evolutionary conservation of the dileucine-binding motif, as well as complete conservation in the second $K_{Na}$ member subunit Slick (Fig. 1). Based on the high functional significance predicted by this observation, we hypothesized that the Slack $K_{Na}$ channel belongs to the repertoire of AP-2 cargo proteins.

**Biochemical association of Slack channels with the AP-2 complex in neurons**

cAMP activation of PKA results in significant loss of membrane Slack channel protein in $\sim 30$ min, suggesting that the underlying mechanism is likely trafficking involving cytoplasmic endocytic machinery (4). To identify interacting protein players downstream to the PKA signal that may serve endocytic functions, we performed a mass spectrometric analysis on a neuronal pulldown of Slack protein following treatment of primary DRG neurons with the potent PKA activator–cAMP analog Sp-cAMPs. Functional analysis of detected binding partners revealed the complete subset of adaptor protein subunits to comprise the AP-2 complex: $α$, $β$, $σ$, and $γ$-adaptin (Table 1). In addition, both heavy and light chain clathrin subunits as well as the clathrin coat assembly protein AP180 were detected, representing important components essential for clathrin coat assembly and vesicle formation. In comparison, the complete subset of proteins essential for AP-2 clathrin–mediated endocytosis was not detected in the vehicle-treated sample, demonstrating the dependence of endocytic machinery recruitment on the PKA signal.

**AP-2 binds to Slack channels at the C-terminal dileucine motif following PKA activation**

The ability of AP-2 to bind proteins destined for cytoplasmic internalization with high affinity and specificity while simultaneously recruiting clathrin molecules to phospholipid contacts at the membrane has been demonstrated as integral to the
Neuronal Slack channel endocytosis by AP-2

Table 1
Mass spectrometric identification of endocytic proteins associated with neuronal Slack channels

Proteins identified by LC-MS/MS mass spectrometry in immunoprecipitation-enriched sample of Slack protein from lysate of primary embryonic rat DRG neurons following treatment with vehicle (DMSO) or the PKA activator Sp-cAMPs (50 μM, 30 min, 37 °C). Listed are proteins functionally associated with endocytic/trafficking cellular processes, including protein name, National Center for Biotechnology Information accession number, molecular mass in kilodaltons, and percentage of protein sequence covered by the peptide.

| Protein | NCBI accession no. | Molecular mass (kDa) | Protein coverage (%) |
|---------|---------------------|----------------------|----------------------|
| + DMSO vehicle | 14-3-3 scaffolding proteins | | |
| 14-3-3 protein γ | 1433G_RAT | 28 | 15.4 |
| + Sp-cAMPs | AP-2 adaptor complex | | |
| AP-2 complex subunit α-2 | AP2A2_RAT | 104 | 13.50 |
| AP-2 complex subunit β | AP2B1_RAT | 105 | 13.30 |
| AP-2 complex subunit μ | AP2M1_RAT | 50 | 12.60 |
| AP-2 complex subunit σ | AP2S1_RAT | 17 | 21.10 |
| Clathrin-coated vesicle proteins | | | |
| Clathrin light chain B | CLH1_RAT | 192 | 30.00 |
| Clathrin coat assembly protein | CLCB_RAT | 25 | 12.20 |
| AP180 | AP180_RAT | 94 | 10.90 |
| 14-3-3 scaffolding proteins | | | |
| 14-3-3 protein ε | 1433E_RAT | 29 | 41.20 |
| 14-3-3 protein ζ/δ | 1433Z_RAT | 28 | 34.30 |
| 14-3-3 protein β/α | 1433B_RAT | 28 | 26.00 |
| 14-3-3 protein η | 1433F_RAT | 28 | 22.80 |
| 14-3-3 protein θ | 1433T_RAT | 28 | 33.50 |
| 14-3-3 protein γ | 1433G_RAT | 28 | 24.70 |
| ELKS/Rab6-iAST family member | RB6I2_RAT | 109 | 18.70 |
| Regulating synaptic membrane exocytosis protein 1 | RIMS1_RAT | 180 | 5.82 |
| Flotillin-1 | FLOT1_RAT | 47 | 32.20 |
| Vesicle-associated membrane protein-associate protein A | VAPA_RAT | 28 | 43.00 |
| Neurabin-2 | NEB2_RAT | 90 | 9.06 |
| ARF GTPase-activating protein GIT1 | GIT1_RAT | 85 | 11.00 |
| GTP-binding nuclear protein Ran | RAN_RAT | 24 | 29.60 |
| Coatomer subunit β’ | COPB2_RAT | 103 | 5.52 |
| ADP-ribosylation factor 5 | ARF5_RAT | 21 | 30.00 |
| Ras-related protein Rab-1B | RAB1B_RAT | 22 | 22.40 |
| Protein transport protein Sec31A | SC31A_RAT | 135 | 3.36 |
| DNA (cytosine-5)-methyltransferase 1 | DNMT1_RAT | 183 | 4.62 |
| Protein unc-13 homolog C | UN13C_RAT | 249 | 1.54 |
| Brefeldin A–inhibited guanine nucleotide-exchange protein 2 | BIG2_RAT | 202 | 3.13 |
| Exocyst complex component 4 | EXOC4_RAT | 111 | 3.18 |
| Syntaxin-6 | STX6_RAT | 29 | 21.20 |
| Syntaxin-binding protein 1 | STXB1_RAT | 68 | 5.05 |
| Synaptotagmin-1 | SYT1_RAT | 47 | 6.89 |
| Synaptotagmin-11 | SYT11_RAT | 48 | 10.00 |
| Exocyst complex component 3 | EXOC3_RAT | 86 | 5.17 |
| Membrane-associated guanylate kinase, WW, and PDZ domain–containing protein 3 | MAGI3_RAT | 161 | 3.67 |
| Tripartite motif–containing protein 3 | TRIM3_RAT | 81 | 3.09 |
| Vacular protein sorting–associated protein 4A | VPS4A_RAT | 49 | 6.18 |
| Coatomer subunit β | COPB_RAT | 107 | 3.88 |
| Synaptotagmin-5 | SYT5_RAT | 43 | 4.92 |

endocytic process (17). We tested the ability of the cargo recognition subunit α-adaptin to bind Slack when recombantly co-expressed in CHO cells via co-immunoprecipitation experiments and found significant association between the two proteins (Fig. 2A). To specifically ask whether the site of their interaction is the recognition motif in the Slack C terminus, the dileucine LI residues within the motif were substituted with alanines. Co-immunoprecipitation pulldowns performed on WT and mutant Slack channels showed significantly reduced AP-2 α-Slack binding upon disruption of the trafficking signal (Fig. 2, B and C). We note that significant but not complete abolishment of binding is seen with disruption of dileucine motif binding, indicating that the motif is presumably the primary, if not the only, site of AP-2–Slack interaction.

PKA-induced Slack channel internalization is mediated by the AP-2 complex

The observation of reduced membrane density of Slack channels as a consequence of CAMP-activation of PKA appears not to be limited to the neuronal membrane, because the same
We first confirmed endogenous AP-2 and Slack channel interaction following PKA activation in primary DRG neurons as pulldowns of core adaptin AP-2 ω contained significant Slack channel protein (Fig. 3C). Of note, we found tonic association of a small yet detectable density of channels with AP-2 (Fig. 3C, control lane), suggesting that a fraction of channels are likely in constant flux. Pharmacological treatments in primary neurons found that the inhibitory peptide disrupted binding, as confirmed by reduced amount of Slack pulled down with adaptin AP-2 ω subsequent to PKA activation. We also tested the peptide effect on intact spinal cord slices by preincubating the slices with peptide for ~2 h (to allow for maximum tissue permeation) before Sp-cAMPs treatment and found inconclusive effects on Slack channel endocytosis (data not shown). A likely explanation could be protease-mediated peptide degradation or dephosphorylation because intact tissue is favorable to robust enzyme activity.

Next, we used the inhibitor to ask whether PKA internalization of membrane Slack channels is indeed mediated by AP-2. Consistent with previous evidence from biotinylation assays (4), we found treatment of primary DRG neurons with the potent PKA activator Sp-cAMPs to result in gradual loss of surface Slack protein over a duration of 30 min (Fig. 3B). When preincubated with the inhibitor peptide for 24 h before PKA activation, not all but most of the membrane Slack protein was retained at the membrane (Fig. 3D). Prevention of AP-2 binding to Slack appears to attenuate internalization of Slack channels and retain them at the membrane, implicating AP-2 as a central player in Slack channel endocytosis. Together, this biochemical evidence validates the signal-dependent recruitment of AP-2, as well as the preventative potential of the inhibitor peptide.

**PKA internalization of membrane Slack channels occurs by AP-2 clathrin endocytosis**

If Slack channels were to undergo internalization by AP-2 clathrin–mediated endocytosis, membrane channels would be marked for inclusion in clathrin-coated vesicles by clathrin adaptors (20). We used immunofluorescence studies to investigate the involvement of clathrin in PKA-induced AP-2 internalization of Slack channels. In keeping with our findings thus
Neuronal Slack channel endocytosis by AP-2

Figure 3. PKA-induced Slack channel internalization is mediated by the AP-2 adaptor proteins. A, top panel, representative Western blot for membrane Slack protein following biotinylation assays on lysates of adult mouse spinal cord slices treated with 50 μM Sp-cAMPs (+) or DMSO vehicle (−) for 30 min at 37 °C. Actin was used as a loading control. Bottom panel, densitometric analysis of represented Western blots for Slack quantified as normalized to Actin. The data are expressed as means ± S.E. (n = 3 for both groups). The statistics were determined using unpaired t test. *, p < 0.05. B, representative Western blot for membrane Slack protein following biotinylation assays on lysates of primary embryonic rat DRG neurons following 10, 20, or 30 min of treatment with 50 μM Sp-cAMPs for 30 min at 37 °C. Actin was used as a loading control. C, representative Western blot of Slack protein from embryonic rat DRG neurons that were treated with 50 μM Sp-cAMPs (+) or vehicle (−) (30 min, 37 °C) following preincubation with Scrambled (Scr) or inhibitory (AP-2) peptide (10 μM, 24 h, 37 °C). The cells were subject to Western analysis for Slack following pulldown using an antibody specific to the AP-2 α subunit. D, left panel, representative Western blot of membrane Slack protein following biotinylation assays on lysates of embryonic rat DRG neurons that were treated with 50 μM Sp-cAMPs (+) or vehicle (−) (30 min, 37 °C) following preincubation with Scrambled (Scr) or inhibitory (AP-2) peptide (10 μM, 24 h, 37 °C). Actin was used as a loading control and gel splice seam is as indicated. Right panel, densitometric analysis of represented Western blots for Slack quantified as normalized to actin. The data are expressed as means ± S.E. (n = 3 for all groups). The statistics were determined using unpaired t test. *, #, p < 0.05. IP, immunoprecipitation.

far demonstrating PKA-dependent AP-2 recruitment to membrane Slack channels, we found significant AP-2 α-adaptin localization at the cell membrane following pharmacological activation of PKA in cultured primary DRG neurons (Fig. 4A). However, AP-2 recruitment to the membrane appears to be independent of cargo protein recognition, because nearly identical membrane immunolabeling of AP-2 α was observed in primary DRG neurons treated with control scrambled peptide (Fig. 4F). This could be explained by detectable amounts of AP-2 reported to always reside in the neighborhood of clathrin molecules and the cell membrane (21), and although we observe no detectable clathrin co-localization under control conditions, AP-2 α membrane localization could be independent of intracellular and/or extracellular signals.

More importantly, we found significant differences in localization of Slack channels and clathrin proteins between conditions of control and inhibitor peptide pretreatment (Fig. 4, A–M). Consistent with previous work, PKA activation dislocated Slack channels from the membrane into the cytoplasm (Fig. 4C). As would be predicted, pretreatment with the inhibitor peptide abolished internalization of Slack channels as observed by strong absence of cytoplasmic labeling and retention of channels at the membrane (Fig. 4H). Finally, we observed clear clathrin localization at the membrane upon PKA activation in the absence of inhibitor peptide but not in its presence, suggesting that AP-2 binding to Slack channels necessarily precedes clathrin recruitment to the membrane (Fig. 4, B and G). This appears to be an extension of previous studies showing a severe reduction in formation of clathrin coats following RNAi depletion of AP-2 because of AP-2 μ being an integral part of the clathrin coat itself (21). Taken together, our immunofluorescence findings show simultaneous AP-2 and clathrin localizing to the neuronal membrane and Slack channel redistribution into the cytoplasm following PKA activation, indicating that PKA internalization of Slack channels occurs via AP-2 clathrin endocytosis.

Pharmacological inhibition of AP-2–Slack channel binding alleviates PKA-induced neuronal hyperexcitability

cAMP acts as an important second messenger during inflammation in mammalian neurons, actively initiating downstream signaling cascades via classical cAMP-dependent PKA phos-
phorylation of protein targets (22). Consistent with previous findings, treatment of primary DRG neurons with the PKA activator Sp-cAMPs induced membrane hyperexcitability, defined as repetitive firing of more than two action potentials in whole cell current clamp recordings upon injection of a suprathreshold current (Fig. 5, A and B). Preincubation of neurons with the inhibitor peptide prior to activation of PKA appeared to produce a state of firing accommodation and prevent hyperexcitability, as would be expected in the event that AP-2–mediated trafficking of Slack channels from the neuronal membrane is the mechanism underlying PKA-induced hyperexcitability. Similar experiments performed on acutely dissociated adult mouse DRG neurons recapitulated these findings to a large extent (Fig. 5C), although less robust in response to pharmacological treatments of the cAMP analog and the inhibitor peptide. Although the latter result validates the physiological relevance of our embryonic neuronal culture model, as well as the reproducible pharmacological effect of the peptide across model systems, we attribute the lack of statistical significance to well documented heterogeneity and perils associated with acutely dissociated adult DRG neurons (23).

**Discussion**

Slack channel function at the neuronal membrane appears to rely heavily on surface channel density, which studies thus far have shown to be susceptible to influence by intracellular protein interactions. A strong example of this is provided by PKA regulation of Slack in that activation of the kinase causes internalization of channels in primary DRG neurons, causing them to become hyperexcitable (4). In the present study, we reveal AP-2 CME to be the trafficking mechanism underlying PKA internalization of Slack channels and add AP-2 to the growing list of cytoplasmic proteins interacting with Slack in a functionally significant manner. Based on the presence of an evolutionarily conserved dileucine motif, known recognition sequences for AP-2 binding, in the Slack C terminus, we hypothesized that PKA-induced changes in membrane channel density occurs via AP-2 endocytosis. Using mass spectrometry and co-immunoprecipitation assays, we found both recombinantly expressed and endogenous Slack channels to directly bind AP-2 adaptins at the predicted dileucine-binding motif in the C terminus. Pharmacological inhibition of AP-2 binding to Slack had striking functional consequences in primary DRG neurons in that it

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**Figure 4. PKA internalization of Slack channels occurs by AP-2 clathrin-mediated endocytosis.** A–J, representative immunofluorescent staining of primary embryonic rat DRG neurons treated with 50 μM Sp-cAMPs (30 min, 37 °C) following preincubation with 10 μM Scrambled (A–E) or Inhibitor AP-2 (F–J) peptide (24 h, 37 °C). The cells were immunolabeled for α-adaptin (anti-mouse), clathrin heavy chain (anti-rabbit), and Slack (anti-chicken). All cells were nuclear counterstained with DAPI (E and J). Scale bar represents 10 μm. K and L, scatter plots depicting pixel intensities (PI) of Slack and clathrin heavy chain immunofluorescence (left panels), α-adaptin, and clathrin heavy chain immunofluorescence (center panels) and Slack and α-adaptin immunofluorescence (right panels) in embryonic DRGs treated with Scrambled peptide (K) or AP-2 inhibitor peptide (L). M, bar graph representing Pearson’s correlation coefficients values for Slack and α-adaptin, α-adaptin, and clathrin heavy chain and clathrin heavy chain and Slack, with control peptide or AP-2 inhibitor peptide (n = 5 cells). *p < 0.05.
prevented surface channel internalization and the resultant loss of firing accommodation. Finally, immunofluorescence studies revealed recruitment of clathrin to the neuronal membrane following activation of the PKA signal. Taken together, these results indicate that PKA internalization of neuronal Slack channels occurs via AP-2 clathrin–mediated endocytosis.

Despite AP-2 recognition of Slack at the dileucine motif being a novel addition to the repertoire of signals that modulate Slack channel activity, AP-2 regulation of K^+ currents is preceded by the inward rectifying potassium channel Kir2.3 (24). Similar to Slack channels, Kir2.3 is endocytosed by CME following AP-2 recognition of a dileucine motif when expressed in heterologous cells. Interestingly, the study showed preferential binding of AP-2 at the dileucine motif despite an upstream canonical tyrosine motif, suggesting specificity in AP-2 recognition of binding sites. We report unequivocally that PKA-induced AP-2 binding to Slack channels occurs primarily at the dileucine motif. However, the possibility of alternative binding sites cannot be excluded. Speculation by Mason et al. (24) that binding specificity may be governed by physiological context appears plausible in the case of Slack channels as well. Recent work recognizes a tightly regulated balance on channel density by intra- or extracellular signals that insert channels into the membrane (such as p38 MAPK phosphorylation) (9) and route channels into the endocytic pathway (such as PKA activation) to subsequently determine neuronal excitability, requiring sophisticated recognition specificity by intracellular transport mechanisms. Although not well understood, factors determining recognition by different AP complexes could include complex-specific contacts with phosphatidylinositol lipids at the membrane (25) and sequence local to the binding motif in the cargo protein (26). Further, adaptin subunit characterization dictates distinct conformations for distinct binding motif recognition by AP-2. Specifically, the μ2-adaptin is reported to recognize the tyrosine motif (27), whereas there is a lack of consensus on dileucine motif binding to the δ2-adaptin despite strong evidence (28). Although it is certain that unique conformation changes underlie the two recognition events, a clearer understanding of evidently varying structural contexts remains elusive.

Akin to the Slo channel, the K_{Na} channel subunit is predicted to have an extensive intracellular C termini in addition to six

Figure 5. Pharmacological inhibition of AP-2–Slack channel binding alleviates PKA-induced neuronal hyperexcitability. A, representative traces of whole cell recordings from embryonic rat DRG neurons that were either (top left panel) untreated or treated with 50 μM Sp-cAMPs (30 min, 37 °C, bottom left panel), 50 μM Sp-cAMPs (30 min, 37 °C, top right panel) following preincubation with 1 μM inhibitory AP-2 peptide (24 h, 37 °C), or 50 μM Sp-cAMPs (30 min, 37 °C, bottom right panel) following preincubation with 1 μM scrambled peptide (24 h, 37 °C). B and C, scatter plot of number of action potentials fired in response to a suprathreshold stimulus of 400 pA for 1000 ms by embryonic rat (B) or adult mouse (C) DRG neurons that were either untreated or treated with Sp-cAMPs, Sp-cAMPs following preincubation with inhibitory AP-2 peptide, or Sp-cAMPs following preincubation with scrambled peptide. Data points reflect the firing frequency of individual cells. Bars represent mean value per group (n = 8–12/group). The data are expressed as means ± S.E. via unpaired t test. *, #, p < 0.05.
transmembrane domains and a short N terminus (29). PKA phosphorylation is putatively predicted to occur at five consensus sites located within the Slack C terminus but not within the RCK domains, supporting effects of phosphorylation at these sites beyond channel gating (7). Indeed, neither pharmacological activation nor direct application of the PKA catalytic subunit acutely affected activation kinetics and open probabilities of Slack channels (6). That PKA activation does produce significant trafficking effects may be related to the location of the site of phosphorylation; mass spectrometric evidence exists for phosphorylation of Ser1088 in close proximity to the dileucine motif for AP-2 binding in the rat Slack protein sequence (30). In the Slack channel protein sequence, the dileucine motif is entirely conserved (Fig. 1), and an analogous PKA phosphorylation event is reported at Ser1100 (31). Considering recent evidence that KCNT2 is transcriptionally regulated by the pro-inflammatory transcription factor NF-κB, it is conceivable that Slack channels are subject to regulation by PKA as well (32). Future studies examining Slack channel modulation by PKA would provide a broader picture of PKA-KNa channel interactions with a critical role for AP-2.

The proteomics analysis reported in the present study is not only valuable proof of the PKA-dependent association of the AP-2 clathrin endocytic machinery with Slack channels but also provides a brief snapshot into the specific trafficking mechanisms favored for regulation of Slack channel density within the DRG primary neuronal system. Indeed, a similar mass spectrometric investigation into cAMP-activated HEK cells gave us no overlapping and/or non-overlapping unique endocytic binding partners (data not shown). We suspect that the absence of such trafficking machinery underlies the previously reported lack of PKA regulation of Slack channels in heterologous cell systems (4, 6). Alternatively, it could be conceived that PKA is unable to mediate its regulatory effect because of the absence of certain intracellular accessory scaffolding proteins. We detected the full subset of the well-characterized 14-3-3 scaffolding proteins in our neuronal proteomic analysis, but whether they are involved in the PKA-AP-2-clathrin trafficking axis for neuronal Slack channels is a question of future interest. Investigating these possibilities would help overcome current limitations on functionally testing the predicted PKA sites in recombinantly expressed Slack channels by conventional molecular biology approaches, because we have previously reported heterologously expressed Slack channels to not exhibit PKA-dependent regulation of currents (4). Additionally, a more detailed future study beyond the scope of the present one involving genomic editing of predicted PKA sites would shed light on the contribution of one or more sites to neuronal Slack channel trafficking.

Substantial literature on their role and regulation illustrates the neuronal Slack channel as an increasingly promising candidate in targeting processes of nociception. They are abundantly expressed in the cell bodies and axons of small- and medium-sized nociceptive DRG neurons, wherein empirical as well as in silico evidence demonstrates their significance in maintenance of resting membrane potentials (2–5, 33). Reduced Slack channel function at the DRG neuronal membrane results in loss of firing adaptation, hyperexcitability, and sensitized primary afferent nociceptors. Recent significant insights on channel regulation have revealed that nociceptive sensitization can be profoundly counteracted by modulating surface channel density toward maintenance of stable membrane expression. For example, association with the ion channel TMEM16C was shown to increase membrane expression of Slack channels in DRG neurons such that neuronal excitability was alleviated (34). p38 MAPK phosphorylation-induced insertion of Slack channels into the DRG membrane is critical to tonic maintenance of firing accommodation (9). The present work demonstrates PKA-induced trafficking of channels from the DRG membrane to result in loss of firing accommodation, which, when prevented, restores it. As these results would predict, severe reduction in vivo Slack channel function exclusively in DRG neurons leading to nociceptive sensitization has been deemed as causal to pro-nociceptive behaviors (34, 35). These findings substantiate the Slack channel as an arbiter of nociceptive neuronal excitability. Given that PKA is part of the pro-inflammatory milieu that initiates sensitization of nociceptors (36), identification of novel downstream players involved in PKA regulation of Slack channels such as AP-2 and clathrin discerns novel potential targets with which to intervene in the neuronal pathophysiological response. Interestingly, recent in vivo evidence of potential AP-2 involvement in chronic pain has been presented in the form of AP2-associated kinase 1 (AAK1) knock-out mice and with AAK1 inhibitor small molecule anti-nociceptive effects in multiple mouse models of nerve injury (37). This provides a promising context for AP-2 endocytosis-mediated neuronal responses to nociceptive stimuli, likely involving multiple endocytosed molecules including Slack channels.

**Experimental procedures**

**DRG neuronal culture**

Timed pregnant Sprague–Dawley rats (Harlan Laboratories) were used for all experiments. The animals were housed singly in a temperature- and humidity-controlled animal facility on a 12-h:12-h light:dark schedule with food and water freely available. All procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health guidelines for the use of laboratory animals in research. On the day of the dissection, the rats were euthanized by CO2 asphyxiation, and embryonic day 15 embryos were extracted. DRGs were dissected from the embryos and enzymatically digested with trypsin (2.5 mg/ml) at 37 °C for 50 min, followed by mechanical dissociation and plating. DRG neurons were plated onto poly-d-lysine–coated (Sigma; 100 mg/ml) and laminin-coated (Invitrogen; 3 mg/ml) coverslips. The cells were maintained at 37 °C in a 7% CO2 humidified incubator in serum-free C2 medium, comprised of the trophic factors N2 (Gemini Bio products; 1%), l-glutamine (Invitrogen; 200 mg/ml), and NGF (Harlan Laboratories; 100 ng/ml) in 50% DMEM and 50% F-12 (4). For 2 days after the day of the dissection, the cells were cultured in medium containing the anti-mitotic cytosine β-d-arabinofuranoside hydrochloride (Sigma; 3 mM), followed by 2 days of recovery before use in experiments.
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**Molecular biology**

CHO cells were cultured at 37 °C in 5% CO$_2$ in an Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, 1% HT supplement (Life Technologies), and 1% penicillin-streptomycin. One day after plating at ~70% confluence, CHO cells were singly or co-transfected transiently with 0.5 μg of rat Slack in the expression plasmid pTRACER and/or FLAG-tagged σ AP-2 via Lipofectamine (Invitrogen) and cultured for 48 h. The cells were plated in 35-mm dishes for all electrophysiology and in 6-well plates for all biochemistry experiments. Site-directed mutagenesis was performed using the QuickChange site-directed mutagenesis kit (Stratagene). The mutations L1200A, I1201A, and L1200A/I1201A were engineered into rat Slack cDNA in pTRACER using specific primer sets. Pfu Turbo-mediated PCR incorporation of the mutation(s) was followed by DpnI digestion to eliminate non-amplified cDNA. Remaining PCR products were transformed into XL-10 Gold Ultracompetent Cells (Agilent Technologies) and mini-prepped (Qiagen Spin mini-prep kit). The final products were sequence verified to confirm the mutations.

**Mass spectrometry**

*Sample preparation for mass spectrometry precipitation/on-pellet-digestion—Immunoprecipitation-enriched protein mixture samples were reduced by addition of 5 mM DTT at 56 °C in dark for 30 min and then further alkylated by addition of 20 mM iodoacetamide for 30 min at 37 °C in dark. The reduction and alkylation of proteins were both conducted with rigorous oscillation in an Eppendorf Thermomixer (Eppendorf) at 500 rpm. The proteins were precipitated by stepwise addition of 6 volumes of cold acetone with continuous vortexing and then incubated overnight at −20 °C. Then proteins were centrifuged at 20,000 × g for 30 min at 4 °C, the supernatant was removed, and the pellet was allowed to air dry. For the on-pellet digestion, Tris-FA buffer (50 mM, pH 8.5) containing trypsin at an enzyme/substrate ratio of 1:20 (w/w) was added and incubated at 37 °C overnight with vortexing at 500 rpm in the Thermomixer, and the final volume was controlled at 50 μl. The digestion was terminated by adding 1% (v/v) formic acid and centrifuged at 20,000 × g for 30 min at 4 °C. The resultant supernatant was used for mass spectrometry analysis.*

*LC-MS/MS—4 μl of the digest solution was analyzed by nanospray LC-MS/MS on an Orbitrap Fusion Trubrid mass spectrometer with collision-induced dissociation (CID) (Thermo Fisher Scientific) coupled to an ultra-high pressure Eksigent ekspert NanoLC 425 system (Dublin, CA) with a autosampler of Eksigent NLC 400 (made in the Netherlands). A nano-LC/nanospray setup was used to obtain a comprehensive separation of the complex peptide mixture. Mobile phases A and B were 0.1% formic acid in 1% acetonitrile and 0.1% formic acid in 88% acetonitrile, respectively. The samples were loaded onto a large ID trap (300-μm inner diameter × 5 mm, packed with Zorbax 5 μm C18 material) with 1% B at a flow rate of 10 μl/min for 3 min. A series of nanoflow gradients was used to back-flush the trapped samples onto the nano-LC column (75-μm inner diameter × 100 cm, packed with Pepmap 3-μm C18 material). The column was heated to 52 °C to improve both chromatographic resolution and reproducibility. The gradient profile was as follows: (i) a linear increase from 3 to 8% B over 5 min; (ii) an increase from 8 to 27% B over 117 min; (iii) an increase from 27 to 45% B over 10 min; (iv) an increase from 45 to 98% B over 20 min; and (v) isocratic at 98% B for 20 min.*

The mass spectrometry was operating under data-dependent product ion mode. For the identification of the protein, a 3-s scan cycle included an MS1 scan followed by MS2 scanned with CID activation. The parameters used for MS and MS/MS data acquisition under the CID mode were: FTMS for MS1: scan range (m/z) = 400–1600; resolution = 120 K; AGC target = 5 × 105; maximum injection time (ms) = 50; filter: precursor selection range = 400–1600; include charge state = 2–7; dynamic exclusion after n times = 1, duration time of 45 s; decision: data-dependent mode: top speed, precursor priority = most intense; ddMS2 (CID): isolation mode = quadrupole; isolation window = 1.6; collision energy (%) = 30; detection type: ion trap; iontrap scan rate: rapid; AGC target = 1 × 104; maximum injection time (ms) = 35; microscan = 1; AGC target = 1 × 104.

*Database searching and methylation calculation—CID activation spectra were processed using Proteome Discoverer 1.4 (Thermo Scientific). Briefly, raw files were searched against the human or rat forward-decoyed database based on the sample species. All database was subset from Swiss UniProt released in January 2015, with the precursor mass tolerance of 20 ppm, the peptide fragment mass tolerance of 1 Da, the static side chain modifications of oxidation (15.995), the protein false discovery rate was controlled at 1%.*

**Spinal cord slice preparation**

Under isoflurane anesthesia, adult mice were decapitated, and a ventral laminectomy was performed to access the spinal cord. The lumbar portion was dissected and embedded in low-gelling temp agarose (Sigma–Aldrich) and bathed in ice-cold modified artificial cerebrospinal fluid (ACSF) of the following composition: 110 mM choline chloride, 2.5 mM KCl, 0.5 mM CaCl$_2$, 7 mM MgSO$_4$, 1.25 mM NaH$_2$PO$_4$, 26.2 mM NaHCO$_3$, 11.6 mM sodium l-ascorbate, 3.1 mM sodium pyruvate, and 25 glucose. Transverse slices of the lumbar spinal cord were obtained using a vibrating blade microtome (Lancer series 1000; Leica Biosystems, St. Louis, MO). The slices were incubated at 35–37 °C in regular ACSF of the following composition: 119 mM NaCl, 2.5 mM CaCl$_2$, 1.3 mM MgSO$_4$, 1 mM NaH$_2$PO$_4$, 26.2 mM NaHCO$_3$, 15 mM glucose, and 1.0 mM sodium l-ascorbate. With continuous bubbling with 95% O$_2$, 5% CO$_2$, slices were sequentially incubated in ACSF containing peptide and Sp-cAMPs and then lysed for biotinylation assays.

**Pharmacology**

Inhibitor and Scrambled peptide compounds were custom ordered from GenScript. Following 5 days of culture, DRG neurons were preincubated in peptide (10 μM) for 24 h, followed by treatment for 30 min with the cAMP analog Sp-cAMPs (50 μM) for PKA activation at 37 °C.
Membrane biotinylation assay

Cell surface biotinylation on primary DRG neurons was performed with the Pierce cell surface protein isolation kit (Thermo Scientific) according to the manufacturer’s protocol. Briefly, the cells were washed with PBS and incubated with EZ-LINK Sulfo-NHS-SS-biotin for 30 min at 4 °C followed by quenching solution. The cells were homogenized in lysis buffer (500 μl) containing protease inhibitor mixture. An aliquot of the lysate was saved for Western blotting for total protein. Biotinylated Slack was isolated with NeutrAvidin-agarose gel, eluted by sample buffer containing dithiothreitol. The resulting eluate was loaded onto a Ready Gel, and Western analysis was performed to detect membrane Slack with monoclonal anti-Slack. Signal was detected using horseradish peroxidase-conjugated mouse secondary antibody and chemiluminescent substrate kit (KPL). β-Actin was used as a loading control.

Co-immunoprecipitation experiments

CHO cells transiently co-transfected with WT or mutated Slack pTRACER and FLAG-tagged AP-2 σ DNA or cultured DRG neurons preincubated with peptide and treated with Sp-cAMPS were used. The cells plated into 6-well plates were lysed with 100 ml/well of cold buffer containing radioimmune precipitation assay buffer and protease inhibitor mixture (Sigma). 60 ml/well of protein G-linked Sepharose bead slurry (GE Healthcare) was washed three times with cold lysis buffer and incubated on a rotator overnight at 4 °C with 4 mg of anti-FLAG or anti-AP-2 α (Abcam) in PBS with 0.1% Tween and cell lysate. On the following day, the samples were centrifuged, and supernatants were stored separately. The pellets were washed three times with cold lysis buffer, and bound protein was eluted via boiling three times at 95 °C for 8 min each. The samples were centrifuged to separate proteins into the supernatant, which was then denatured with SDS and loaded onto a Ready Gel (Bio-Rad) (4–15% Tris-HCl) as the immunoprecipitate. Supernatants collected earlier and whole cell lysate (total input) were also denatured with SDS and run as controls. After electrophoresis, the gel was then transferred onto a nitrocellulose membrane for 1 h and incubated in 5% nonfat dry milk as a blocking agent for 1 h. The monoclonal anti-Slack (NeuroMab) primary antibody was used to probe the blot for Slack channel, and signal was detected using horseradish peroxidase-conjugated mouse secondary antibody and chemiluminescent substrate kit (KPL).

Immunofluorescence

After 5 days in culture, rat embryonic DRG neurons were washed with PBS, fixed with fresh 4% paraformaldehyde for 10 min, and permeabilized in blocking solution that contained 1% BSA for 1 h at 4 °C. Neurons were triple-labeled with Slack anti-chicken (1:250, NeuroMab), AP-2 anti-mouse, and Clathrin anti-rabbit (1:1000, Abcam) antibodies overnight at 4 °C, followed by secondary antibodies AlexaFluor 633 anti-mouse and 488 anti-goat (1:1000; Life Technologies) for 2 h at room temperature. Coverslips were mounted on slides using Prolong Gold antifade reagent with DAPI (Life Technologies). Images were taken using a Zeiss Axio-Imager at 60× magnification. Quantification of the degree of association between Slack, AP-2, and Clathrin immunolabeling within embryonic DRG neurons was performed using the coloc 2 plugin from the Fiji version of the free image processing software ImageJ (National Institutes of Health) as previously described (38).

Electrophysiology–whole cell patch clamp

Glass electrodes were pulled using a horizontal pipette puller (Sutter Instrument Company) and fire-polished to be of 5–12 MΩ resistance. Whole cell voltage and current clamp recordings were performed on primary DRG neurons. Currents were recorded in voltage clamp mode at a holding potential of −70 mV, and voltage steps from −120 to +120 mV were applied in 20-mV steps. A current clamp protocol consisting of depolarizing steps in increments of 10 pA from −10 to 200 pA (20-ms duration) was used to record action potentials. Firing frequency was examined by measurement of repetitive discharge of each cell upon injecting a supra threshold stimulus of 400 pA for 1000 ms. A pipette solution consisting of 124 mM potassium gluconate, 2 mM MgCl2, 13.2 mM NaCl, 1 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, and 0.3 mM Na-GTP, pH 7.2, was used. A bath solution consisting of 140 mM NaCl, 5.4 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 15.6 mM HEPES, and 10 mM glucose, pH 7.4, was used. All data were acquired using the Axopatch 200B amplifier (Molecular Devices) and Multiclamp-700B (Molecular Devices), digitized, and filtered at 2 kHz. Data acquisition was monitored and controlled using pClamp 10.2 and analyzed using Clampex (Molecular Devices).

Statistics

All electrophysiological analysis was performed using Clampfit 10 (Axon Instruments) and Origin 8.0 (Microcal Software Inc.). All statistical tests were performed using Prism (GraphPad). The data are shown as means ± S.E. Confidence levels were calculated using Student’s t test and analysis of variance. Sample size for all experiments was least recordings required to detect significant differences and ensure reproducibility.

Author contributions—S. G. conducted most of the experiments, analyzed the results, and wrote the paper. K. M. E. prepared the adult mouse spinal cord slices. K. D. P. performed the immunofluorescence experiments. A. B. conceived the idea for the project and supervised manuscript preparation by S. G.

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