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The Appearance of a Protein Kinase A-regulated Splice Isoform of *slo* Is Associated with the Maturation of Neurons That Control Reproductive Behavior*

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In response to brief synaptic stimulation that activates protein kinase A (PKA), the bag cell neurons of *Aplysia* trigger the onset of reproductive behaviors by generating a prolonged afterdischarge. In juvenile animals, such afterdischarges are inhibited by a high density of Ca^{2+}-activated K^+ (BK) channels, encoded by the *slo* gene. An increase in this current also follows an afterdischarge in mature animals, contributing to a subsequent refractory state that limits reproductive behaviors. Using a bag cell cDNA library, we have isolated two alternative transcripts of the *slo* gene, differing in the presence (*slo*-a) or absence (*slo*-b) of a consensus phosphorylation site for PKA. Expression of either isoform in Chinese hamster ovary cells produced Ca^{2+}- and voltage-dependent channels with macroscopic and unitary properties matching those in bag cell neurons. The isoforms differed, however, in their response to application of the catalytic subunit of PKA, which reduced the open probability of Slo-a, an effect that was reversed by a PKA inhibitor. In contrast, PKA had no effect on Slo-b. By immunocytochemistry, we determined that the PKA-regulated Slo-a subunit is present in adult, but not juvenile, bag cell neurons. Patch clamp recordings from adult and juvenile bag cell neurons confirmed that PKA decreases BK channel activity only in adults. Our findings suggest that a change in the identity of Slo isoforms expressed during development allows mature neurons to generate afterdischarges that are required for reproduction.

During development of the nervous system, the pathways that underlie reproductive behaviors are the last to mature (1, 2). In adult *Aplysia*, a series of reproductive behaviors is controlled by the bag cell neurons within the abdominal ganglion. Brief stimulation of the bag cell neurons triggers a period of repetitive firing termed an afterdischarge (3, 4). Secretion of neuropeptides during the afterdischarge initiates a sequence of reproductive behaviors culminating in egg laying behavior. Although juvenile animals synthesize the same neuropeptides as adults and can be induced to secrete them by pharmacological manipulations, electrical stimulation of the neurons in juvenile animals fails to trigger the normal afterdischarge characteristic of adults (5).

The onset of an afterdischarge in adult bag cell neurons is associated with an elevation of cyclic AMP levels and the activation of cAMP-dependent protein kinase (PKA)[1] (4, 6, 7). PKA suppresses several species of K^+ currents, which enhances excitability and increases action potential broadening (8, 9). Activation of PKA also indirectly shifts the gating mode of non-selective cation channels through the activation of a protein tyrosine phosphatase, such that a long lasting closed state of the channel is eliminated, resulting in the sustained depolarization underlying the afterdischarge (10, 11).

Previous electrophysiological work has shown that the density of a Ca^{2+}- and voltage-dependent K^+ current in juvenile neurons is 3-fold greater than that in adults (12) and that it falls during maturation. The properties of this current in the bag cell neurons have been shown to match those of the large conductance BK channels that are encoded by the *slo* gene (13). We have now isolated two isoforms of the *slo* gene, encoding BK Ca^{2+}-activated K^+ channels, from the bag cell neurons. The channel encoded by one of the isoforms is inhibited by PKA, whereas the other is unaffected by this kinase. The PKA-sensitive isoform appears in development during the transition from juvenile to mature neurons. The selective presence of this isoform in adults may allow PKA to decrease current in response to stimulation and permit afterdischarges to occur.

MATERIALS AND METHODS

Cloning—Aplysia Slo cDNAs were first isolated from a whole central nervous system λ Zap II cDNA library that was screened at low stringency using DNA probes corresponding to the amino and carboxyl cytoplasmic coding regions of the *Drosophila* Slo channel. Five independent clones were isolated. Two of these, F7 and F11, encoded the fifth transmembrane domain of Slo up to the stop codon and appear to represent alternatively spliced cDNAs. When compared with F11, F7 lacks two exons in the region encoding the carboxyl terminus and has an alternative 3′-end coding region. Another clone, F6, encodes the 5′-end of the mRNA and has partial overlap with F7 and F11. F7 and F11 full-length cDNAs were constructed by joining F6 to either F7 or F11 at a common restriction site.

To isolate Slo isoforms that are expressed in the bag cell neurons, we designed eight pairs of PCR primers, four forward and four reverse, based on the F7 and F11 sequences expressed in the total central

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AY359443.

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[1] The abbreviations and trivial terms used are: PKA, protein kinase A; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; NPo, number of channels multiplied by probability of opening; I-V, current voltage.

nervous system. These were used for PCR or nested PCR on a bag cell neuron λ Zap II cDNA library. PCR products were purified and cloned into the T/A cloning vector pCR2.1 (Invitrogen). The products were directly sequenced by the HHMI Biopolymer-Keck Foundation Biotechnology Resource Laboratory at Yale University. Both coding and untranslated regions were identified for two distinct Slo isoforms. New PCR primers based on the 5′ and 3′ untranslated regions were then used for nested PCR to obtain full-length Slo-a and Slo-b fragments, which were subcloned into the pCDNA3 vector and sequenced in their entirety. Slo-a is identical to the AsloF11 cDNA isolated from the whole central nervous system.

Electrophysiological Recordings—CHO cells were cultured in Iscoves modified Dulbecco's medium supplemented with 10% fetal bovine serum, 0.1 mM hypoxanthine, 100 units/ml penicillin, and 100 units/ml streptomycin (Invitrogen) and maintained in a 5% CO_2 incubator at 37 °C. Before transient transfection of CHO cells with *Aplysia* Slo cDNA, cells were grown to ~60–70% confluence on 35-mm dishes or coverslips. Cells were transfected using LipofectAMINE (Invitrogen) with 1 μg of *Aplysia* Slo cDNA. Recordings were made from fluorescent cells 1–2 days later. Electrodes had a resistance of 3–5 MΩ for whole cell recordings and 5–10 MΩ for single channel recordings. For whole cell recordings, the bath solution consisted of 140 mM NaCl, 1.0 mM $CaCl_2$, 3 mM KCl, 29 mM glucose, and 25 mM HEPES (pH 7.2). The pipette (intracellular) solution contained 32.5 mM KCl, 97.5 mM K^+ gluconate, 5 mM EGTA, 4.27 mM $CaCl_2$, and 10 mM HEPES (pH 7.2). For single channel patch recordings, pipettes were filled with an extracellular solution and the cytoplasmic side of the membrane was perfused with intracellular solution buffered to 1, 10, or 100 μM free Ca^{2+} (as determined by the computer program CaBuffer, courtesy of Dr. L. Schlichter, University of Toronto, Toronto, Canada). Data were acquired with an EPC-8 amplifier (HEKA Electronics, Mahone Bay, Nova Scotia, Canada), a Digidata 1200 analog-to-digital converter (Axon Instruments, Foster City, CA), and the clampex acquisition program of pClamp (version 6.02; Axon Instruments). Current was sampled at 10 kHz and filtered at 1 kHz with a Bessel filter (Frequency Devices, Haverhill, MS). Data were gathered in 1–3 min intervals while holding the patch at +30 mV.

Single BK channel current in bag cell neurons was measured as for CHO cells using the excised, inside-out patch clamp method. Pipettes had resistances of 5–10 MΩ when filled with normal artificial sea water; see below for composition but lacking glucose, penicillin, and streptomycin). After excision, the cytoplasmic face was bathed with artificial intracellular saline composed of (in mM): 470.4 KCl, 66 $MgCl_2$, 15 HEPES, 5 EGTA, pH 7.3, with a calculated free [Ca^{2+}] of ~1 μM. The catalytic subunit of PKA and the peptide inhibitor PKA_{6-22} were obtained from Calbiochem.

For single channel analysis, pClamp 9.0 (Axon Instruments) was used. Data are presented as the mean and S.E. Statistical analysis was performed using Instat (version 2.01; Graph Pad Software Inc., San Diego, CA). Student's *t* test was used to test for differences between two means. Data were considered significantly different at $p < 0.05$.

Dose-response curves for Ca^{2+} were fit with the equation: $I = I_{min} + (I_{max} - I_{min})/(1 + 10 \hat{} (\log EC_{50}\text{-}C)*n$, where I is the measured channel open probability (NPo), I_{min} is the minimal channel open probability, I_{max} is the peak channel open probability, C is the concentration of agonist used, C_{50} is the concentration of agonist required to achieve half-maximal activation (*i.e.* EC_{50}), and n is the degree of cooperativity.

Animals and Bag Cell Neuron Culture—Adult *Aplysia Californica* weighing 100–200 g were obtained from Marinus Inc. (Long Beach, CA). Juvenile *Aplysia* weighing between 5–10 g were obtained from the Aplysia Resource Facility (University of Miami). Primary cultures of isolated bag cell neurons were prepared as described previously (13) and maintained in normal artificial sea water (in mM: 460 NaCl, 10.4 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 15 HEPES, 1 mg/ml glucose, 100 units/ml penicillin, and 0.1 mg/ml streptomycin, pH 7.8) for 1–3 days in a 14 °C incubator.

Antibody Production and Purification—Two peptides were synthesized to generate antibodies against the amino acid sequences encoded by the cloned Slo-a and Slo-b cDNAs. Peptide 1 (CXRFRDTNESVRSPSSKR) included the carboxyl-terminal 15 amino acids of both identified bag cell Slo isoforms. Peptide 2 (CXPEKRKPQSRRKPSTTLK) was selective for the Slo-a isoform and coincided with the consensus PKA phosphorylation site. Both were synthesized at the W. M. Keck Biotechnology Resource Center, Yale University. The X in each sequence represents aminocaproic acid, which acted as a spacer molecule between the specific sequence and a nonspecific amino-terminal cysteine, which allowed conjugation of peptides to the keyhole limpet hemocyanin (KLH) peptide. KLH, in turn, acted as a carrier for antigenic peptide in the generation of chicken polyclonal IgY, which was carried out by Aves Labs, Inc. (Tigard, OR).

For affinity purification of IgYs, the synthetic peptides were conjugated to Sulfolink coupling gel (Pierce) by the amino-terminal cysteine. Each of the two prepared columns was incubated with IgYs for 1 h at room temperature, and then the columns were washed three times with phosphate-buffered saline (PBS). Specific antibodies were eluted with 100 mM glycine buffer, pH 2.5, and then immediately neutralized with 1 M Tris, pH 8.5. Fractions containing proteins (measured by the optical density at 280 nm) were pooled and dialyzed against PBS using Slide-A-Lyzer cassettes (Pierce) and concentrated using a spin column.

Immunocytochemistry—Staining of 1–2-day-cultured bag cell neurons was performed on coverslips coated with 1 μg/ml poly(D)lysine. Fixation with 4% paraformaldehyde in 400 mM sucrose/artificial sea water and preparation were carried out as described by (14). Coverslips were washed twice with PBS and blocked with 5% goat serum/PBS before incubation with primary antibodies. They were inverted on 100 μl of primary antibody solution (0.75 μg/ml (Slo-a) or 0.5 μg/ml (Slo-b) in 5% goat serum/PBS) and placed in a humidified chamber at 4 °C overnight, washed extensively with PBS, and then incubated for 2 h at room temperature with either fluorescein- or Texas Red-conjugated goat anti-chicken IgG secondary antibodies (Texas Red-GαCIgG or fluorescein isothiocyanate-GαCIgG).

CHO cells transiently transfected with *Aplysia* Slo were grown on glass coverslips to ~60–70% confluence and fixed in PBS containing 4% paraformaldehyde for 10 min. After washing with PBS, cells were permeabilized in PBS containing 1% bovine serum albumin and 0.2% Triton X-100 for 10 min. This buffer was used for all subsequent incubations. Primary affinity-purified anti-*Aplysia* Slo was added to the coverslips for 1 h at room temperature. Coverslips were then washed, incubated with fluorescein isothiocyanate-GαCIgG (1/1,000; Molecular Probes) for 30 min, washed again, and mounted on glass slides with Citifluor mounting medium (Ted Pella, Inc., Redding, CA) for fluorescence microscopy.

RESULTS

Cloning of Slo Isoforms in Bag Cell Neurons—Using a bag cell neuron cDNA library as described under "Materials and Methods," we isolated two full-length cDNAs encoding Slo channels with open reading frames encoding proteins of 1070 and 1060 amino acids (Fig. 1*A*). We termed these isoforms Slo-a and Slo-b, respectively. Both isoforms have characteristics of Slo channels in other species, including seven transmembrane domains (S0-S6), RCK domains, and long cytoplasmic carboxyl-terminal domains (15–18). The amino acid sequences of the *Aplysia* Slo channels are 56 and 62% identical to hSlo and dSlo, the human and Drosophila orthologs. The sequences of both Slo-a and Slo-b contain multiple consensus sites for phosphorylation by protein kinase C. In contrast, there are no consensus sites for PKA on Slo-b, whereas Slo-a has a single consensus PKA site that is located at the carboxyl-terminal end of the 10-amino acid insert that is unique to this isoform (Fig. 1*B*). The *Aplysia* Slo-a sequence has been submitted to GenBank™ with the accession number AY359443.

Properties of Slo-a and Slo-b Channels in Transfected Cells—To examine the properties of the *Aplysia* Slo channels, we expressed both isoforms in CHO cells and carried out whole cell voltage clamp recordings on the second day after transfection. When intracellular Ca^{2+} was set at 1 μM, currents that activated at potentials >0 mV and did not inactivate over several hundred milliseconds were detected (Fig. 2*A*). No currents were found in CHO cells transfected with vector alone. The mean current-voltage (*I-V*) relationships for Slo-a-transfected CHO cells is shown in Fig. 2*B*. The kinetics and *I-V* relation of Slo-b expressed in CHO cells were similar to those of Slo-a (data not shown).

We also examined the unitary properties of the *Aplysia* Slo channels in excised inside-out patches with symmetrical K^+ (Fig. 2*C*). The amplitudes of unitary currents were determined from Gaussian fits of amplitude histograms at different voltages and were used to construct a single channel *I-V* relationship for Slo-a (Fig. 2*D*). The slope of the *I-V* relationship gave a single channel conductance of 117 ± 16 pS (range 101–133 pS,

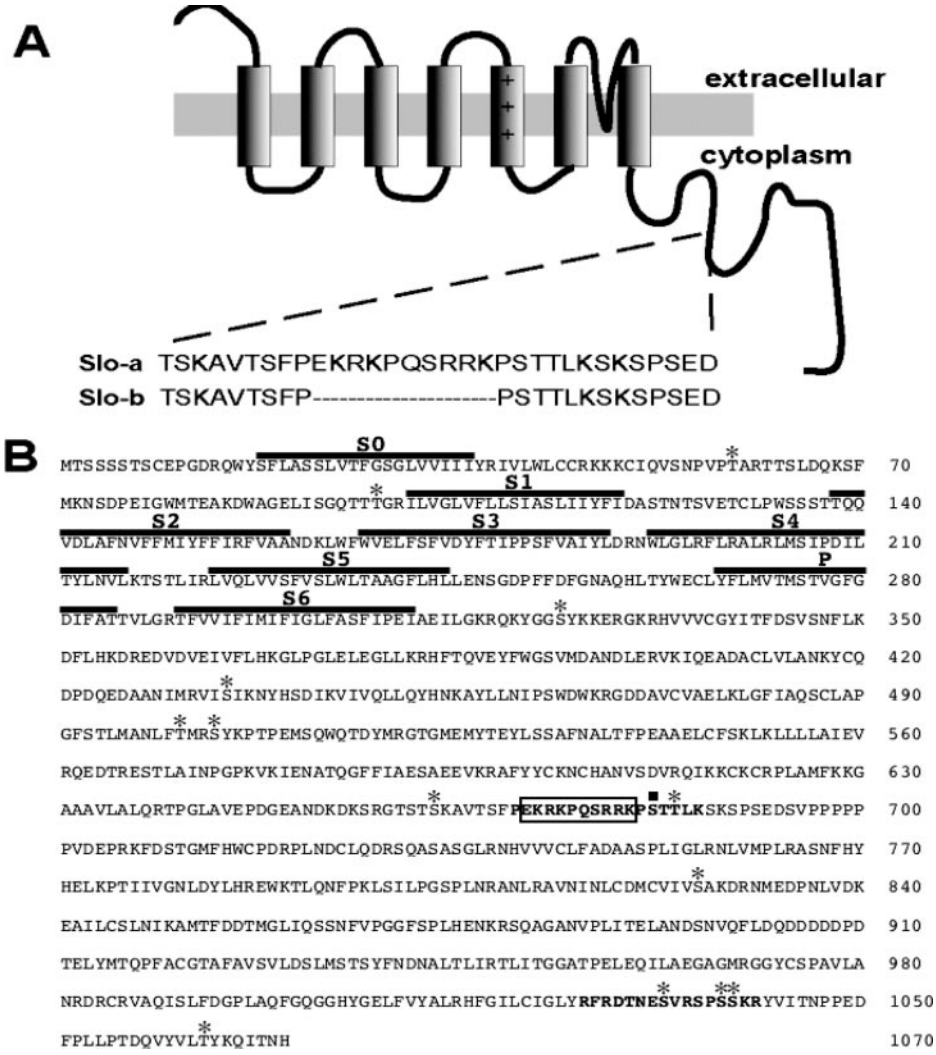

FIG. 1. *A*, diagram of the transmembrane organization of Slo subunits showing the difference in Slo-a and Slo-b isoforms. *B*, deduced amino acid sequence of the *Aplysia* Slo-a isoform. PKA consensus sequences are indicated by a *black square*, protein kinase C consensus sequences are indicated by *stars*. *Bars* over a sequence show putative transmembrane segments S0-S6. The sequences in *bold* in the carboxyl-terminal region correspond to peptides used to generate α-Slo-a and α-Slo-b antisera. *Boxed* sequence corresponds to the insert in Slo-a.

$n = 5$). We also determined the unitary conductance for the Slo-b isoform, which was 114 ± 15 pS, (range 99–129 pS, $n = 5$). The conductances were not statistically different (unpaired Student's t test, $p > 0.05$), indicating that the splice insert in Slo-a does not affect unitary conductance.

To measure the Ca^{2+} sensitivity of the *Aplysia* Slo channels, we carried out excised inside-out patch clamp recordings from Slo-a- and Slo-b-transfected CHO cells using different Ca^{2+} concentrations at the cytoplasmic face of the patch. As expected for BK channels, raising the intracellular Ca^{2+} concentration from 1 μM up to 100 μM resulted in a pronounced increase in the open probability of these channels (Fig. 2, *E* and *F*). There was no significant difference between the Ca^{2+} sensitivity of the Slo-a and Slo-b isoforms (data not shown).

Modulation of Slo Isoforms by PKA in CHO Cells—Channels encoded by the Slo-a and Slo-b isoforms differed in their response to the catalytic subunit of PKA. When the cytoplasmic face of inside-out patches from CHO cells expressing Slo-b was exposed to the PKA catalytic subunit in the presence of 1 mM Mg-ATP and 1 μM free Ca^{2+}, no significant effect on open probability was observed (Fig. 3, *B* and *E*). In contrast, application of the catalytic subunit to patches from Slo-a-expressing CHO cells under the same conditions produced a significant reduction in open probability (Fig. 3, *A* and *E*). In patches containing only one channel, the mean open time under control conditions at +30 mV was 2.5 ± 0.4 ms and was unaltered by treatment with the PKA catalytic subunit (2.3 ± 0.5, $n = 5$, $p > 0.05$), but the mean closed time changed significantly from 4.4 ± 0.2 ms to 6.7 ± 0.5 ms ($n = 5$, $p < 0.001$). By grouping true single channel recordings with those containing more than one channel, we found that the mean reduction of NPo (number of channels multiplied by probability of opening) by the PKA catalytic subunit was 31 ± 2%, ($n = 10$, $p < 0.001$). The effect of the catalytic subunit could be blocked fully by co-application of PKA_{6-22}, a specific peptide antagonist of PKA (55) (Fig. 3, *C* and *E*). We also tested the effect of PKA on cells transfected with both Slo-a and Slo-b. When the cytoplasmic face of inside-out patches from CHO cells expressing Slo-a and Slo-b was exposed to the PKA catalytic subunit in the presence of 1 mM Mg-ATP and 1 μM free Ca^{2+}, the NPo was reduced by 30 ± 2.4% ($n = 5$, $p < 0.001$) (Fig. 3, *D* and *E*), a degree of suppression similar to that observed for Slo-a alone. This suggests that, if heteromers of Slo-a and Slo-b form in such transfected cells, the heteromers remain sensitive to PKA.

Effect of PKA on BK Channels in Adult and Juvenile Bag Cell Neurons—To compare the properties of *Aplysia* Slo channel isoforms expressed in CHO cells with BK channels in native bag cell neurons of adult and juvenile animals, we carried out single channel recordings on isolated cultured bag cell neurons. Inside-out patch clamp recordings from these neurons, using symmetrical K^+ solutions, typically exhibited little channel activity at +30 mV in a Ca^{2+}-free medium at the cytoplasmic face. When the Ca^{2+} concentration at the cytoplasmic face of the patch was raised to 1 μM, channel activity increased markedly and reverted to control upon return of the patch to a Ca^{2+}-free medium (Fig. 4*A*). Further raising the cytoplasmic

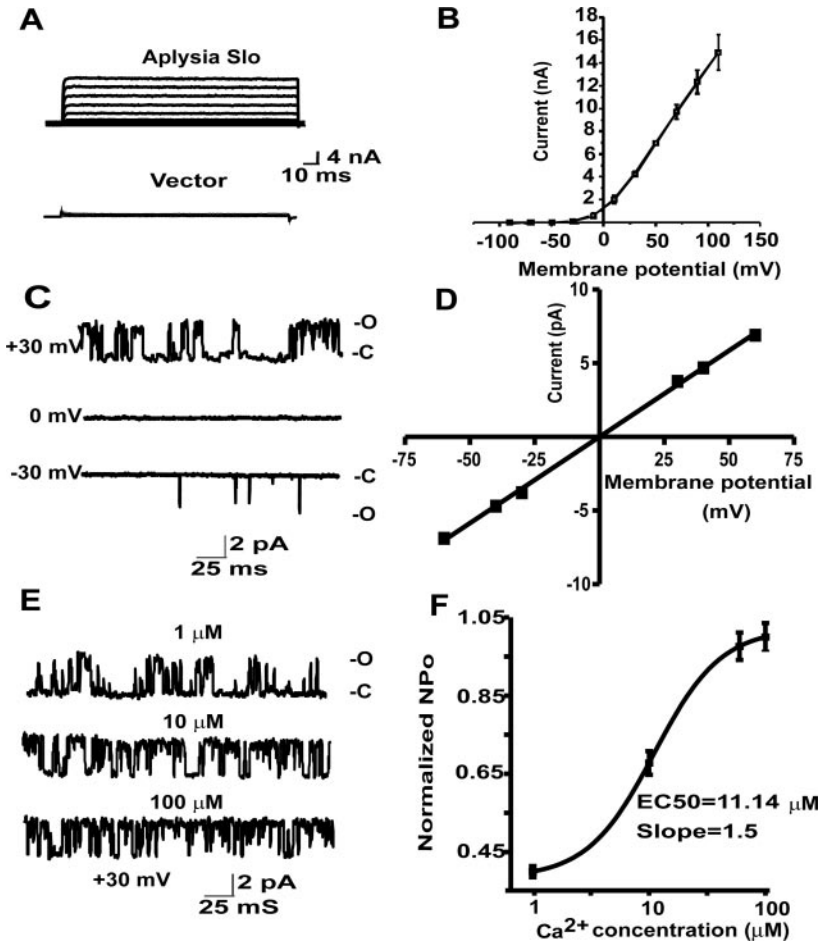

FIG. 2. *A*, whole cell recordings of Slo-a in CHO cells. Currents were recorded with 1 μM intracellular Ca^{2+} by depolarizing the membrane potential from −80 mV to test potentials between −80 and +110 mV in 20-mV increments. *B*, current-voltage relationship for Slo-a. *C*, single channel recordings of Slo-a in CHO cells with symmetrical solutions containing 130 mM K-gluconate and 1 μM free Ca^{2+} at different holding potentials. Open and closed states are marked by the letters *O* and *C*, respectively. Data were filtered at 1 kHz. *D*, single channel *I-V* relationship for Slo-a. Current amplitudes at different potentials were obtained by Gaussian fits of amplitude histograms. The resulting *I-V* relationship was fit by using linear regression, and channel conductance was obtained from the slope of the fitted line. *E*, representative single channel recording of Slo-a channels in 130 mM symmetrical K-gluconate at three different free Ca^{2+} concentrations. Data were filtered at 1 kHz. *F*, Ca^{2+}-dependence of Slo-a channel gating. Currents were recorded at 30 mV in inside-out patches during rapid superfusion with solutions buffered to free Ca^{2+} concentrations ranging from 1 to 100 μM. NPo were normalized to the NPo obtained at 100 μM Ca^{2+}. Each point represents the average value from four experiments. *Error bars* represent S.E. Data were fitted as described under "Materials and Methods." EC_{50} was determined to be 11.14 μM with a Hill coefficient of 1.5.

Ca^{2+} concentration from 1 μM to 100 μM resulted in a further increase in open probability of the channels (Fig. 4*F*). The unitary conductance of endogenous BK channels was 98 + 10 pS (range 88–108 pS, n = 5, Fig. 4*B*), which is consistent with that of *Aplysia* Slo in CHO cells (see Fig. 2*D*).

To examine PKA-dependent regulation of native BK channels, the catalytic subunit of PKA was applied to the cytoplasmic face of inside-out patches that contained BK channels excised from adult bag cell neurons. In all cases (n = 5), application of the catalytic subunit in the presence of 1 mM ATP (MgATP) produced a significant reduction in NPo (Fig. 4, *C* and *E*). The calcium dependence of channel opening was also examined after application of the PKA catalytic subunit. The normalized Ca^{2+} dependence of channel opening was not different from that prior to application of the catalytic subunit (Fig. 4*F*). In contrast to adults, application of the PKA catalytic subunit to patches excised from bag cell neurons prepared from juvenile bag cell neurons failed to produce any change in open probability (Fig. 4, *D* and *E*).

Expression of the Two Splice Variants Is Differentially Regulated during Bag Cell Neuron Development—To examine the localization and development of Slo subunits in bag cell neurons, two antibodies against the *Aplysia* Slo isoforms were raised in chickens. The first (α-Slo-ab) was generated against 15 amino acids that are conserved in the carboxyl-terminal region of both Slo-a and Slo-b (Fig. 1). The second (α-Slo-a) was generated against a 15-amino acid peptide corresponding to the insert that is specific to the Slo-a isoform and contains the single consensus site for phosphorylation by PKA. Characterization of the antibodies was performed using immunofluorescence. CHO cells transfected with either the Slo-a or Slo-b isoforms were strongly labeled with the α-Slo-ab antibody directed against the conserved region (Fig. 5, *A* and *C*). In contrast, the α-Slo-a antibody selectively stained Slo-a, but not Slo-b-transfected CHO cells (Fig. 5, *B* and *D*). No staining was detected with preimmune serum, and staining with each antibody was eliminated by preabsorption with its corresponding immunogen peptide (data not shown).

To examine the localization of the Slo isoforms in bag cell neurons, we prepared isolated bag cell neurons from adult and juvenile animals. Using either the α-Slo-ab or the α-Slo-a antibodies, intense staining was detected in the membranes of the somata and in the growth cones of adult neurons (Fig. 5, *E* and *F*). The staining pattern with both antibodies appeared very similar, and we could find no significant differences in apparent localization of channels. This indicates that the Slo-a isoform, which contains the PKA consensus phosphorylation site, is expressed in adult bag cell neurons. In contrast to adults, juvenile neurons were stained only very faintly with the α-Slo-a antibody (Fig. 5*H*), whereas robust staining was obtained using α-Slo-ab directed against the carboxyl-terminal region conserved in both isoforms (Fig. 5*G*). These findings indicate that the Slo-a isoform is expressed in bag cell neurons only late in development.

DISCUSSION

Large conductance Ca^{2+}-activated K^+ channels (BK channels) are ubiquitous in neurons (19) and play a critical role in regulating neuronal firing patterns and neurotransmitter release (20, 21). Although there is only one gene, *slo*, that encodes these channels, diversity in the properties of BK channels is generated by the association of the Slo subunit with ancillary proteins (21–23) or by alternative splicing of *slo* RNA (24, 25). RNA splicing at several different sites can give rise to multiple

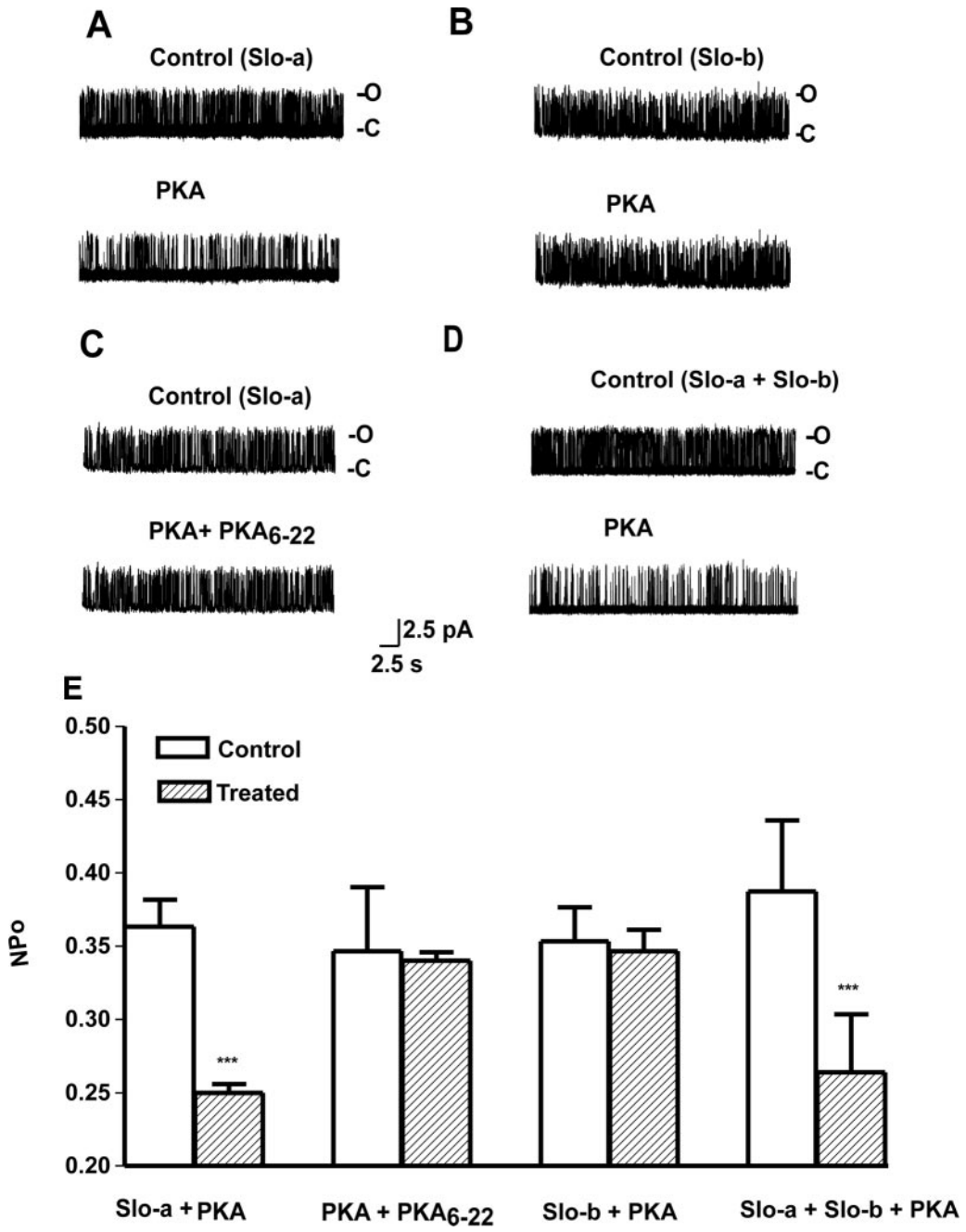

FIG. 3. *A* and *B*, representative traces from excised inside-out patches from CHO cells expressing Slo-a or Slo-b channels before and after application of 10 units/ml PKA catalytic subunit to the patches. *C*, 10 μM specific PKA inhibitor PKA_{6-22} was applied to the intracellular face of an inside-out patch from a Slo-a-expressing CHO cell before application of PKA catalytic subunit. *D*, representative traces from excised inside-out patches from CHO cells expressing Slo-a plus Slo-b channels before and after application of 10 units/ml PKA catalytic subunit to the patches. *E*, *bar graphs* showing group data for the effects of PKA on Slo-a and Slo-b channels in CHO cells. All currents were recorded at a holding potential of +30 mV during superfusion with solutions containing 1 mM ATP (MgATP) and buffered to 1 μM free Ca^{2+}. Data are expressed as mean ± S.E. *** indicates a significant difference from control at $p < 0.001$.

distinct ion channels from the *slo* gene (26, 27). Some of the splice variants differ from each other in their biophysical properties (27, 28), thereby allowing fine-tuning of BK currents to the needs of a particular cell. For example, alternative splicing seems to play a particularly critical role in the tonotopic organization of the cochlea (29, 30).

We have isolated the *slo* gene from the bag cell neurons of *Aplysia* and determined that two specific splice isoforms, Slo-a and Slo-b, are found in these cells. They differ only in the presence or absence of a unique consensus phosphorylation site for PKA, located in the large carboxyl tail domain that plays a role in Ca^{2+} sensing and acts as a partner for protein-protein interactions (31–34). Previous work has shown that Slo channels in other species are modulated by a number of different protein kinases (35–42) and the nature of this modulation can differ between tissues. For example, PKA activates BK channels in smooth muscle as well as in neurons but inhibits channel activity in endocrine cells of the anterior pituitary (43–46). Regulation of the channels can occur either by phosphorylation of the ion channel protein itself or by influencing regulatory components intimately associated with channel subunits (40, 47). Moreover, in some cases, BK channel modulation occurs through dephosphorylation by phosphoprotein phosphatases (37, 41, 45, 48, 49).

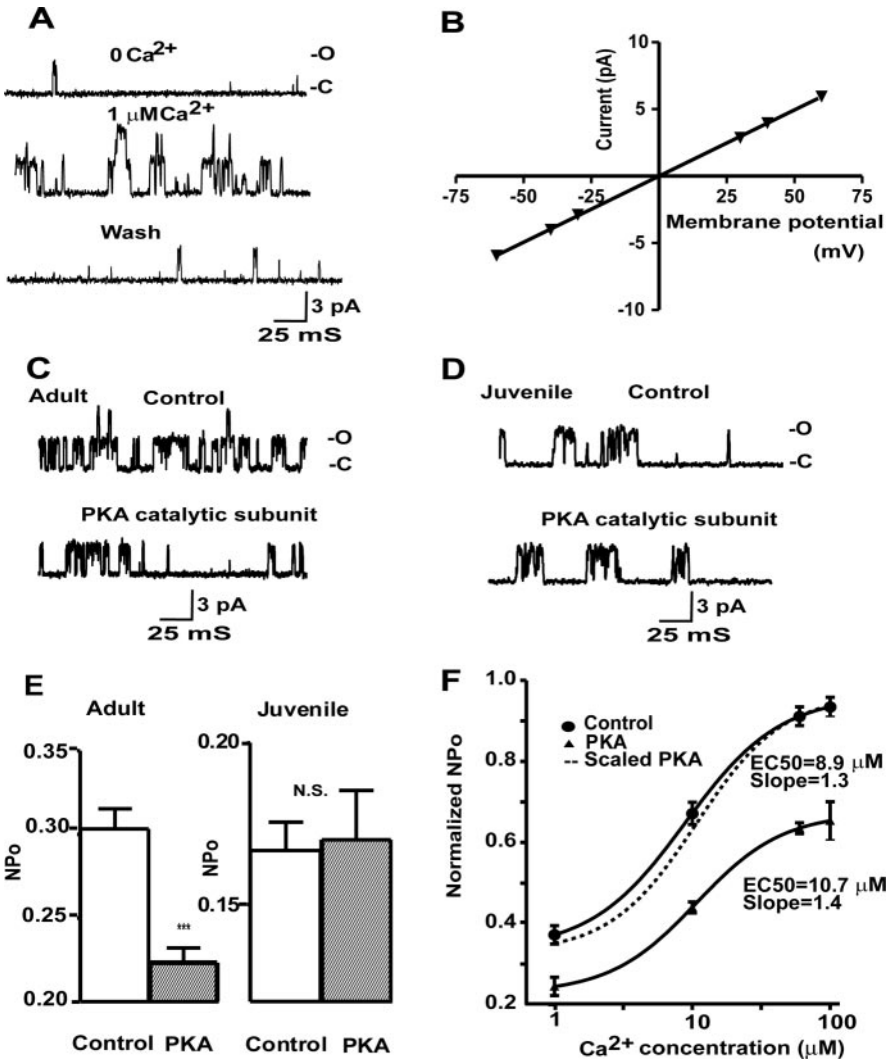

FIG. 4. *A*, representative single channel traces from an inside-out patch containing BK channels excised from isolated adult bag cell neurons in symmetric K^+ solutions. Activity was recorded at +30 mV in the presence of 1 mM ATP (MgATP) and either nominally 0 or 1 μM free Ca^{2+} at the cytoplasmic face of patches as described under "Materials and Methods." *B*, single channel *I-V* relationship for BK channels from native bag cell neurons. Current amplitudes at different potentials were obtained by Gaussian fits of amplitude histograms. The resulting *I-V* relationship was fit by using linear regression, and single channel conductance was calculated from the slope of the fitted line. *C*, representative excised patch recordings before and after application of the catalytic subunit of PKA to the cytoplasmic face of patches from adult bag cell neurons. *O* and *C* represent single openings and closed states, respectively. *D*, representative single channel traces before and after application of PKA catalytic subunit to the intracellular face of patches from a juvenile bag cell neuron. *E*, summary of the modulation by PKA of Slo channels in excised patches from adult and juvenile bag cell neurons. Data are expressed as the mean ± S.E. *** indicates a significant difference from control at $p < 0.001$. N. S., not significant. *F*, the effect of PKA catalytic subunit on Ca^{2+}-dependence of channel opening. NPo values are shown for patches before and after treatment with PKA catalytic subunit ($n = 4$). NPo were then normalized to the NPo obtained at 100 μM Ca^{2+}. Each point represents the average value from four experiments. *Error bars* represent S.E. Data were fitted as described under "Materials and Methods." EC_{50} was determined to be 8.9 μM with a Hill coefficient of 1.3 and 10.7 μM with a Hill coefficient of 1.4 before treatment with PKA catalytic and after treatment with PKA catalytic subunit. *Dotted line* shows the NPo *versus* Ca^{2+} relationship for patches after PKA treatment normalized to that of controls at 100 μM free Ca^{2+}, demonstrating that Ca^{2+} dependence is apparently not altered by PKA.

The afterdischarge of adult bag cell neurons is evoked by brief stimulation of an afferent input from the head ganglia and endures for ~30 min. Onset of the afterdischarge is associated with the activation of both PKA and protein kinase C (4, 50). Cyclic AMP levels increase rapidly during the first few minutes of afterdischarge and thereafter decline to control values (6). In isolated cells, elevations of cyclic AMP or direct injection of the catalytic subunit of PKA produces broadening of action potentials that mimics the action potential broadening that occurs during the first 2 min of the afterdischarge. This spike broadening is associated with a decrease in the amplitude of K^+ currents (4, 51). Our findings that the PKA-sensitive Slo-a isoform is expressed in adult bag cell neurons and that BK channel activity in adult neurons is attenuated by PKA suggest that decreases in BK current may contribute to the early increase in excitability and/or spike broadening that occurs in the first few minutes of the afterdischarge.

The Slo-a isoform appears to be absent, or expressed at very low levels, in bag cell neurons of juvenile animals. Although neurons from juvenile animals synthesize the neuroactive peptides that trigger reproductive behaviors and can be induced to release these peptides by pharmacological manipulations (12), their electrical properties differ from those of adults. In particular, although juvenile neurons fire normal action potentials, repetitive stimulation of these neurons fails to increase their excitability and trigger the afterdischarge that normally evokes neuropeptide release (5). The inhibition of the Slo-a isoform in adult neurons by elevated cyclic AMP and PKA will promote the increased excitability necessary for afterdischarge generation. Furthermore, the absence of Slo-a from juvenile

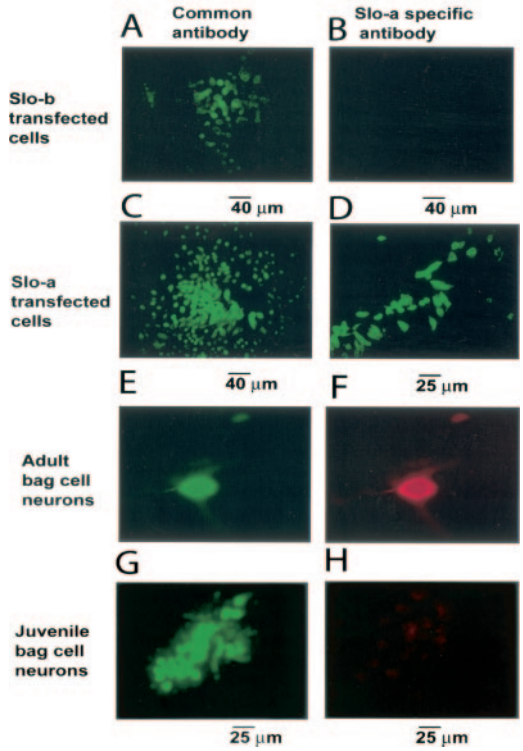

FIG. 5. **Immunostaining of CHO cells transfected with Slo-a and Slo-b cDNAs and of untransfected bag cell neurons.** *A* and *C*, CHO cells transiently transfected with either Slo-a or Slo-b show strong staining with the common antibody (α-Slo-ab antibody), which recognizes both channel isoforms. *B*, no staining with the specific antibody is seen in CHO cells transiently transfected with Slo-b cDNA. *D*, CHO cells transiently transfected with Slo-a cDNA show strong staining with specific α-Slo-a antibody. *E* and *F*, bag cell neurons from adult *Aplysia* are immunoreactive with both α-Slo-a and α-Slo-ab. *G* and *H*, cultured bag cell neurons from juvenile *Aplysia* can be labeled with the common α-Slo-ab antibody (*G*), but not with the specific α-Slo-a (*H*).

neurons is consistent with this difference in excitability between the two neuronal types. Although the mechanism for the change in expression of *slo* splice variants during development is not known, it has been shown in *Drosophila* that there are four promoter regions for the *slo* gene and their recruitment appears to be tissue- and development stage-specific (52, 53). Changes in promoter activity may therefore also be coupled to alterations in the pattern of splicing of Slo channel RNA, both during development and in response to external stimuli, providing a general mechanism for modulation of cellular excitability (54).

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