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 Ca2+-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 Ca2+- 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) (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 Ca2+- 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 Ca2+-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 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 mRNAs 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 nervous system. Cloning and sequencing of the forward and reverse primers was performed using the above methods. A putative 5′-UTR sequence was identified in the region encoding the carboxyl terminus of Slo and was used as a probe in Northern blot analysis.

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

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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. PKA-regulated Isoform of slo

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% CO2 incubator at 37 °C. Before transient transfection of CHO cells with Aplysia Slo cDNAs, 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 CaCl2, 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 CaCl2, 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 Ca2+ (as determined by the computer program CaBuffer, courtesy of Dr. L. Schlichter, University of Toronto, Toronto, Canada). Data were acquired with an EPC-9 amplifier (List Biological Laboratories, Campbell, CA) and 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, MA). 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 described 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 sea water see below for composition but lacking glucose, penicillin, and streptomycin. 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/BSA before incubation with primary antibodies. They were incubated on 100 μl of primary antibody solution (0.75 μg/ml of Slo-a or 0.5 μg/ml of 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-GoClG or fluorescein isothiocyanate-GoClG).

Immunocytochemistry—Staining of 1–2-day-cultured bag cell neurons was performed on coverslips coated with 1 μg/ml poly(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 incubated on 100 μl of primary antibody solution (0.75 μg/ml of Slo-a or 0.5 μg/ml of 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-GoClG or fluorescein isothiocyanate-GoClG).

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. 1A). 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 carboxy-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-a, whereas Slo-b has a single consensus PKA site that is located at the carboxy-terminal end of the 10-amino acid insert that is unique to this isoform (Fig. 1B). The Aplysia Slo-a sequence has been submitted to GenBankB with the accession number AJ359443.

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 Ca2+ was set at 1 μM, currents that activated at potentials >0 mV and did not inactivate over several hundred milliseconds were detected (Fig. 2A). 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. 2B. 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. 2C). 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. 2D). The slope of the I-V relationship gave a single channel conductance of 117 ± 16 pS (range 101–133 pS,

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**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 correspond to the insert in Slo-a.

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 Ca²⁺ 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 Ca²⁺ concentrations at the cytoplasmic face of the patch. As expected for BK channels, raising the intracellular Ca²⁺ 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 Ca²⁺ 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 Ca²⁺, 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 PKA₆₋₂₂, 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 Ca²⁺, 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 Ca²⁺-free medium at the cytoplasmic face. When the Ca²⁺ 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 Ca²⁺-free medium (Fig. 4A). Further raising the cytoplasmic
Ca\textsuperscript{2+} concentration from 1 \(\mu\)M to 100 \(\mu\)M resulted in a further increase in open probability of the channels (Fig. 4F). The unitary conductance of endogenous BK channels was 98 ± 10 pS (range 88–108 pS, \(n = 5\), Fig. 4B), which is consistent with that of Aplysia Slo in CHO cells (see Fig. 2D). 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. 4C and E). The calcium dependence of channel opening was also examined after application of the PKA catalytic subunit. The normalized Ca\textsuperscript{2+} dependence of channel opening was not different from that prior to application of the catalytic subunit (Fig. 4F). 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 (\(\alpha\)-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 (\(\alpha\)-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 \(\alpha\)-Slo-ab antibody directed against the conserved region (Fig. 5, A and C). In contrast, the \(\alpha\)-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 \(\alpha\)-Slo-ab or the \(\alpha\)-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 \(\alpha\)-Slo-a antibody (Fig. 5H), whereas robust staining was obtained using \(\alpha\)-Slo-ab directed against the carboxyl-terminal region conserved in both isoforms (Fig. 5G). These findings indicate that the Slo-a isoform is expressed in bag cell neurons only late in development.

**DISCUSSION**

Large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} 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
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. 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 $p < 0.001$. 

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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
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 are tissue- and development stage-specific (52, 53). Alterations in the pattern of splicing of slo channel RNA, both during development and in response to external stimuli, provide a general mechanism for modulation of cellular excitability (54).

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