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Calcium-activated potassium channel of the tobacco hornworm, *Manduca sexta*: molecular characterization and expression analysis

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Summary

Large-conductance calcium- and voltage-gated potassium channels (BK or Slowpoke) serve as dynamic integrators linking electrical signaling and intracellular activity. These channels can mediate many different Ca^{2+}-dependent physiological processes including the regulation of neuronal and neuroendocrine cell excitability and muscle contraction. To gain insights into the function of BK channels *in vivo*, we isolated a full-length cDNA encoding the alpha subunit of a Slowpoke channel from the tobacco hornworm, *Manduca sexta* (*msslo*). Amino acid sequence comparison of the deduced *Manduca* protein revealed at least 80% identity to the insect Slo channels. The five C-terminal alternative splice regions are conserved, but the cloned cDNA fragments contained some unique combinations of exons E, G and I. Our spatial profile revealed that transcript levels were highest in skeletal muscle when compared with the central nervous system (CNS) and visceral muscle. The temporal profile suggested that *msslo* expression is regulated developmentally in a tissue- and regional-specific pattern. The levels of *msslo* transcripts remain relatively constant throughout metamorphosis in the CNS, transiently decline in the heart and are barely detectable in the gut except in adults. A dramatic upregulation of *msslo* transcript levels occurs in thoracic but not abdominal dorsal longitudinal body wall muscles (DLM), suggesting that the msSlo current plays an important role in the excitation or contractile properties of the phasic flight muscle. Our developmental profile of *msslo* expression suggests that msSlo currents may contribute to the changes in neural circuits and muscle properties that produce stage-specific functions and behaviors.

Key words: Slowpoke, developmental regulation, gene expression, tissue-specific expression.

Introduction

Large-conductance calcium-activated potassium channels, BK_{Ca} or Slowpoke (Slo), respond to both membrane potential and intracellular calcium levels (Latorre, 1989; McManus, 1991). This class of ion channels serves as dynamic integrators linking electrical signaling and intracellular activity and plays critical roles in regulating cell excitability. *Slowpoke* was first identified from a mutant phenotype in *Drosophila melanogaster* that exhibited abnormal muscle physiology (Elkins et al., 1986) and subsequently was genetically isolated and characterized (Atkinson et al., 1991). Slo channels are found in species as divergent as nematodes (Wang et al., 2001) and humans (Pallanck and Ganetzky, 1994; Tseng-Crank et al., 1994) and in excitable and non-excitable cells, suggestive of diverse cellular and physiological functions. For example, Slo currents repolarize action potentials, modulate neurotransmitter release (Elkins et al., 1986; Gho and Mallart, 1986; Robitaille and Charlton, 1992; Robitaille et al., 1993; Warbington et al., 1996) and hormone secretion (Petersen, 1986; Shipston et al., 1996), regulate vascular tone and blood pressure (Nelson et al., 1995; Perez et al., 1999) and are critical for antimicrobial activity in neutrophils (Ahluwalia et al., 2004).

The functional diversity of Slowpoke channels is thought to arise from extensive regulation of a single *slo* gene and its gene products. Distinct transcripts can shape the channel properties to the requirements of the cells, tissue, developmental stage or physiological state. In *Drosophila*, tissue-specific transcriptional promoters (Atkinson et al., 2000; Brenner et al., 1996) and alternative splicing (Adelman et al., 1992; Derst et al., 2003; Lagrutta et al., 1994) generate a large number of transcripts that could modify channel properties such as single-channel conductance, calcium sensitivity and mean open time (Adelman et al., 1992; Lagrutta et al., 1994). In vertebrates, alternatively spliced BK transcripts are expressed in distinct patterns in the brain (Tseng-Crank et al., 1994), cochlea (Jiang et al., 1997; Langer et al., 2003), kidney (Bravo-Zehnder et al., 2000) and smooth muscle of arteries, esophagus and uterus (Knaus et al., 1994; Salapatek et al., 2002; Zhou et al., 2000), suggesting isoform-specific functions. Alternate exon selection in mammals can fluctuate depending upon the physiological state; for example, pregnancy (Benkusky et al., 2000, 2002) and stress (Xie and McCobb, 1998). The functional consequences of reversible post-translational modification of Slo channels by serine/threonine and tyrosine kinases are complex and can be

isoform- and tissue-specific. For example, protein kinase A (PKA) activates the ZERO splice variant of BK channels expressed in HEK (human embryonic kidney) cells but inhibits the activity of the stress axis regulated exon (STREX) isoform (Tian et al., 2001). In glial cells, PKA phosphorylation enhances BK channel activity whereas protein kinase C (PKC) reduces channel gating (Schopf et al., 1999). Tyrosine kinase phosphorylation of BK channels can lead to smooth muscle vasoconstriction due to inhibition of channel activity (Alioua et al., 1998) while enhancement of calcium-sensitive gating can occur in heterologous expression systems (Ling et al., 2000). BK channels also are modulated by phosphatases, which can lead to enhanced channel activity in pituitary tumor cells (White et al., 1991) and in HEK cells by reducing PKA inhibition (Tian et al., 2001). In addition, association with beta and other accessory subunits or binding proteins can profoundly alter channel properties such as calcium sensitivity and gating (Xia et al., 1998; Zhou et al., 1999).

Far less is known about how developmental changes in *slo* expression levels alter cellular excitability and contribute to synaptic and behavioral plasticity (Becker et al., 1995; Brenner et al., 1996; Lhuillier and Dryer, 2000; Muller and Yool, 1998). Metamorphosis of the tobacco hornworm, *Manduca sexta*, provides the opportunity to analyze the effects of changing ion channel gene expression and channel density *in vivo* at the level of identified neurons or muscles whose developmental fates and behavioral roles are known (Truman, 1992; Weeks et al., 1997). Developmental changes occur in calcium and potassium currents that could tailor the electrical properties of neurons, glia or muscles to permit postembryonic changes in behavior (Duch and Levine, 2002; Hayashi and Levine, 1992; Mercer and Hildebrand, 2002a,b). However, none of the genes or gene products producing these currents in *Manduca sexta* are known. Only one potassium channel gene has been cloned from this insect: *Manduca sexta ether à-go-go*, or *mseag* (Keyser et al., 2003). The *ether à-go-go* (*eag*) gene was isolated from *Drosophila melanogaster* based on its ether-induced leg-shaking mutant phenotype (Kaplan and Trout, 1969; Ganetzky and Wu, 1983) and is the prototype of a subfamily of voltage-gated potassium channels that includes *ether à-go-go related* (*erg*) and *ether à-go-go like* (*elk*) (Warmke and Ganetkzy, 1994).

Here, we present the isolation of the cDNA that encodes the alpha subunit of a calcium-activated potassium channel, *Manduca sexta slowpoke* (*msslo*), and its postembryonic temporal profile in the CNS, visceral muscles and two sets of skeletal muscles. Our data show that *msslo* gene expression is regulated developmentally in a tissue-specific pattern suggesting that the msSlo currents may contribute to the changes in neural circuits and muscle properties that produce stage-specific functions and behaviors.

Materials and methods

Animals, rearing and developmental staging

Manduca sexta L. larvae were raised on an artificial diet at 26°C (Bell and Joachim, 1976) under a long-day photoperiod (17 h:7 h L:D). Under these rearing conditions, metamorphosis begins approximately 14 days after hatching. Pupation occurs about 4 days later, and the fully developed adult emerges in about 20 days.

We use abbreviations to indicate the different developmental stages of the insect: roman numeral V for the final or fifth instar larvae, W for the wandering stage, P for pupal animals, PA for pharate adults (insects on the day of emergence but still within the pupal cuticle), and A for emerged adults. Insects are staged using discrete developmental markers: larval, pupal and adult ecdysis (V0, P0 and A0, respectively) and the onset of 'wandering' behavior by the fifth-stage larvae (W0). The number following the stage designates the number of days past the molt. For example, V2 indicates a larval animal that molted 2 days previously. Cuticular and pigmentation markers were used to stage developing adults and adults near eclosion (Schwartz and Truman, 1983; Curtis et al., 1984).

Reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from larval CNS and skeletal muscle and pharate adult thoracic dorsal longitudinal muscles (DLM) by mechanical homogenization in TRIzol reagent (GIBCO-BRL, Gaithersburg, MD, USA), pooled, its integrity confirmed on a 1% non-denaturing agarose gel stained with ethidium bromide, and quantified by UV spectrophotometry. Complementary DNA was synthesized from 1 μg of total RNA with 200 units of Superscript II reverse transcriptase (GIBCO) and 150 ng of random hexamer primers. The cDNA reaction was diluted 2.5-fold with double-distilled H_2O (ddH_2O) in preparation for PCR amplification. The cDNA fragments were amplified using 0.5 units of Taq polymerase (Promega, Madison, WI, USA) in PCR buffer B (Promega), pH 8.0, containing 2.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} dNTPs (Promega), 0.4 μmol l^{-1} of each *Manduca* specific primer, 0.8 μmol l^{-1} of each degenerate primer (The Great American Gene Company, Ramona, CA, USA) and 1 μl of diluted cDNA as template in a thermocycler (MJ Research, Waltham, MA, USA) using the following touchdown PCR paradigm (Don et al., 1991) to increase specificity: (94°C, 1 min; 65°C–0.8°C/cycle, 2.5 min; 72°C, 1 min) for 19 cycles followed by (94°C, 1 min; 55°C, 1 min; 72°C, 1.5 min) for 35 additional cycles.

Three primer pairs designed to regions of high conservation and low degeneracy between *Drosophila melanogaster* Slo (dSlo) and *Rattus norvegicus* Slo amino acid sequences were used to PCR amplify the *msslo* cDNA. To obtain the large cytoplasmic tail region, a degenerate primer pair was designed to the conserved S7 domain [MQYHNKA (5′-TGCARTAY-CAYAAYAARGC-3′)] and to the S9 domain [NFHYHEL (5′-AGCTCRTGRTAGTGRAARTT-3′)]. The degenerate forward [NFHYHELK (5′-AAYTTYCACTAYCAYGAGCT-3)] and reverse KRYVITNPP (5′-ATRCAKTADTGGTT-RGGK GG-3′)] primers were designed to a conserved region 3′ to the S10 domain. To obtain the S1–S6 domains, we

designed a degenerate primer to a conserved region 5′ of the S1 domain [KDWAGE (5′-WGYGAARGACTGGGCWGG-3′)] and a *Manduca* specific primer within S7 (5′-TGG-GATATTCAGCAGGTAG-3′). All PCR products were separated on a 1.0% agarose gel, cloned into the pGEM-T Easy vector (Promega), and sequenced.

5′ rapid amplification of cDNA ends (5′ RACE)

Total RNA was extracted from pharate adult thoracic DLM as described above and reverse transcribed with 150 units of Omniscript reverse transcriptase (Qiagen, Valencia, CA, USA) primed with the *Manduca* specific oligonucleotide primer 5′-GAATGCTACTAGCGAACATTGC-3′. Excess primer was removed from the cDNA reaction on a MinElute DNA purification column (Qiagen) prior to modification in a $(dATP)_n$ tailing reaction using terminal transferase (Promega) and diluted fivefold with ddH_2O for PCR amplification. The 5′ RACE products were PCR amplified (reaction components described earlier) by using the adapter primers GACT-CGAGTCGACATCG$(T)_{17}$ and GACTCGAGTCGACATCG (Sambrook and Russell, 2001), designed to the synthesized polyA tail at the 3′ end of the cDNA (5′ end of mRNAs), and the specific primer 5′-CACCAACACTACCAGTATTCG-3′ under the following touchdown PCR paradigm: (94°C, 5 min; 55°C, 5 min; 72°C, 40 min) one cycle, followed by (94°C, 40 s; 58°C – 0.2°C/cycle; 72°C, 3 min) for 35 cycles followed by a final extension at 72°C for 15 min. The PCR reaction was diluted four-fold and re-amplified using the same set of primers [minus the $(T)_{17}$ modified primer] using the following paradigm (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) for 30 cycles to increase yield and specificity of the PCR products. The PCR products were separated on a 1.5% agarose gel, cloned into the pGEM-T Easy vector and sequenced.

3′ RACE

PolyA mRNA extracted from 100 μg DLM total RNA using an Oligotex mRNA purification system (Qiagen) was reverse transcribed with 150 units of Omniscript reverse transcriptase (Qiagen) and then the oligonucleotide primer $(T)_{17}$ diluted fivefold with ddH_20 in preparation for PCR amplification. The 3′ RACE products were amplified using the adapter primers 5′-GACTCGAGTCGACATCG$(T)_{17}$-3′ and 5′-GACTCGAG-TCGACATCG-3′ and the specific primer 5′-CGACAC-CTCCTCCTCCTGC-3′ under the following touchdown paradigm: (94°C, 5 min; 55°C, 5 min; 72°C, 40 min) one cycle, followed by (94°C, 1 min; 68°C–0.6°C/cycle, 1 min; 72°C, 1 min) for 19 cycles and (94°C, 1 min; 58°C, 1 min; 72°C, 1 min) for 35 cycles with a final cycle of (94°C, 40 s; 55°C, 1 min; 72°C, 15 min). To increase yield and specificity, the reaction was diluted fourfold with ddH_2O and re-amplified using the nested specific primer 5′-CGACACCTCCTCC-TCCTGC-3′ and the adapter primer [minus the $(T)_{17}$ modified primer] under the same paradigm used to re-amplify the 5′ RACE products. The PCR products were separated on a 1% agarose gel, cloned into the pGEM-T Easy vector and sequenced.

Sequence analysis

The plasmid clones containing the isolated cDNA fragments were sequenced using Big Dye technology and ABI Model 377 Prism DNA Sequencers (Foster City, CA, USA) at the Iowa State DNA Sequencing and Synthesis Facility (Iowa State University, Ames, IA, USA). Universal vector- and sequence-specific primers were used to generate overlapping sequence products from sense and antisense ssDNA strands. The sequencing fragments were assembled using ContigExpress DNA sequence analysis software (Informax, Inc., Frederick, MD, USA), and amino acid alignments between Slo family members were made using a modification of the ClustalW algorithm within the AlignX sequence alignment software (Informax, Inc.). Consensus sequences for enzymatic modifications were performed using Prosite (Swiss Institute of Bioinformatics, Geneva, Switzerland) and NetPhos2.0 (CBS, Technical University of Denmark).

Northern blot analysis

Total RNA (100 μg) was extracted from staged CNS, skeletal muscles and visceral muscles from 5–15 animals per stage through mechanical homogenization and two successive extractions in TRIzol reagent. RNA integrity was verified by non-denaturing gel electrophoresis on an ethidium bromide-stained 1% agarose gel, and the concentration of RNA quantified by UV spectrophotometry. RNA samples were adjusted to a concentration of 1 μg μl^{-1} in RNA storage solution (1 mmol l^{-1} sodium citrate, pH 6.4; Ambion, Austin, TX, USA). 1 μg of CNS and skeletal muscle and 5 μg of midgut and heart total RNA were separated by electrophoresis on a 1.2% denaturing agarose gel (1× MOPS and 2.2 mol l^{-1} formaldehyde), transferred to a positively charged nylon membrane (Roche, Indianapolis, IN, USA), UV cross-linked and hybridized to digoxigenin (DIG)-labeled RNA probes.

The RNA probes were transcribed from linearized pBSK plasmid clones (Stratagene, La Jolla, CA, USA) containing either a 680 bp PCR fragment of *msslo* amplified from cDNA using the specific primers 5′-GCCCTTCAAACAGGCTA-CAGAG-3′ and 5′-GACGACCAGTCGAAAGATTTC-3′ or containing the coding sequence for *Manduca sexta* ribosomal protein S3 (rpS3; Jiang et al., 1996; generously provided by Dr Michael Kanost, Kansas State University). The probes were purified on an RNeasy RNA purification column (Qiagen) and visualized on a non-denaturing 1.5% ethidium bromide-stained agarose gel. Incorporation rate of DIG-UTP was assessed by dot blot analysis and was used to estimate probe concentration. Membranes were hybridized with probes at a concentration of 100 pg ml^{-1} for *msslo* and 25 pg ml^{-1} for *rpS3* in preheated Ultrahybe hybridization buffer (Ambion) overnight at 68°C in a Little Shot hybridization oven (Boekel, Festerville, PA, USA). The membranes were washed sequentially at 68°C in 2× SSC/0.1% SDS for 45 min, 0.5× SSC/0.1% SDS for 30 min, and 0.1× SSC/0.1% SDS for 30 min. The hybridized probe was detected using anti-DIG:alkaline phosphatase (1:5000) and its chemiluminescent substrate CDP-Star as described in the protocol provided by

Roche. Exposure time to Biomax light film (Eastman Kodak, Rochester, NY, USA) varied between 30 s and 10 min depending on signal intensity.

The number of replicates for the northern blots was as follows: CNS *N*=5, visceral muscle (heart and gut) *N*=3, and skeletal muscle *N*=5. Densitometric analysis was performed using GeneTools software (Synoptics, Frederick, MD, USA) and plotted using GraphPad Prism Software (San Diego, CA, USA).

Results

Isolation of a Manduca slo *cDNA*

PCR amplification of CNS and DLM cDNA and its 5′ and 3′ RACE products yielded seven cDNA fragments showing sequence similarity to calcium-activated potassium channels through BLAST comparison (NCBI; Figs 1, 2). Three cDNA fragments, *mssloA-1* (1065 bp), *mssloA-2* (1032 bp) and *mssloA-3* (1131 bp), were obtained with the degenerate forward primer MQYHNKA (5′-TGCARTAYCAYAAYA-ARGC-3′) and the degenerate reverse primer NFHYHEL (5′-AGCTCRTGRTAGTGRAARTT-3′), which were designed to the S7–S9 region of the channel (Fig. 1). These three fragments share 100% amino acid identity, with the exception of regions corresponding to alternate exon splice sites E, G and I, which are also found in *Drosophila slo* (*dslo*) (Adelman et al., 1992; Brenner et al., 1996) and *Periplaneta slo* (*pslo*) (Derst et al., 2003) (Fig. 2). A fourth cDNA fragment (852 bp) 3′ of *mssloA*, which we called *mssloB*, was amplified using the forward degenerate primer NFHYHELK (5′-AAYTTYCACTAYC-AYGAGCT-3′) and the reverse degenerate primer KRYVITNPP (5′-ATRCAKTADTGGTTRGGKGG-3′). The deduced amino acid sequence from this fragment spanned from the end of S9 to just 5′ of S10 (Figs 1, 2). A fifth cDNA fragment (1118 bp) 5′ of *mssloA*, *mssloC*, was isolated with the degenerate forward primer KDWAGE (5′-WGYGAARGACTGGGCWGG-3′) and a *Manduca* specific reverse primer (5′-TGGGATATTCAGCAGGTAG-3′) designed from *mssloA*. This fragment spanned from S1 to S6 and contained sequences analogous to exons A and C found in other insects (Figs 1, 2) (Adelman et al., 1992; Brenner et al., 1996; Derst et al., 2003).

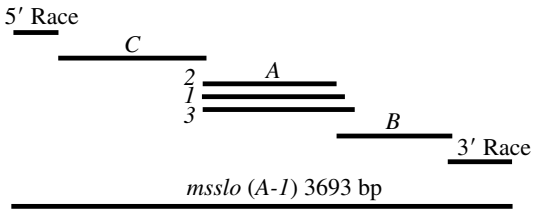

Fig. 1. Schematic of strategy used to isolate *Manduca slowpoke* cDNA. Degenerate oligonucleotide PCR primers designed to conserved regions of the Slo protein were used to amplify cDNA fragments *msslo A-1, A-2* and *A-3*. Each of these cDNA fragments varied slightly in size due to alternate splicing. *Manduca sexta* specific PCR primers were used in conjunction with degenerate PCR primers upstream and downstream of the *A* fragments to amplify cDNA fragments *msslo B* and *msslo C*. 5′ and 3′ RACE was performed to attain and confirm the ends of the cDNA sequence. When assembled, an *msslo* cDNA containing alternate exons E1, G1 and I1 totals 3693 bp in length.

5′ RACE analysis revealed six putative cDNA fragments, each of which was cloned into a plasmid vector (see Materials and methods). Sequence analysis revealed only one cDNA fragment (366 bp) containing an ATG start codon, which, when translated and aligned to dSlo and pSlo 5′ sequences, was found to encode a polypeptide of similar length and identity. Furthermore, stop codons in the UTR upstream from this ATG confirm its identity as the start codon. 3′ RACE analysis revealed a single cDNA fragment (462 bp) that contained a putative polyA tail that upon translation revealed a conserved NKDDXS amino acid sequence found within the 3′ terminus of both fly and cockroach Slo polypeptides. These features suggest that this 366 bp cDNA fragment is the intact 3′ end of the *msslo* cDNA.

Sequence fragments obtained from plasmid clones containing *mssloA–C* cDNAs and from plasmid clones containing 5′ and 3′ RACE-generated cDNAs were assembled using ContigExpress DNA sequence analysis software (Informax, Inc). When *mssloA-1* was included into the sequence assembly, the fragments generated a 3693 bp nucleotide sequence containing the open reading frame and encoding a 1129 amino acid polypeptide (Fig. 2). The *Manduca slo* sequence was entered into GenBank (AY644784). Analysis of the deduced *Manduca* protein revealed conserved domains common to all voltage-gated potassium channels including the six transmembrane domains (S1–S6), the voltage sensor (S4) and a pore lining region between S5 and S6 characterized by the GYG K^+ specificity filter motif. Conserved domains specific to large-conductance calcium-dependent voltage-gated K^+ channels were also identified, including an S0 transmembrane domain, cytoplasmic hydrophobic segments S7–S10, the regulation of conductance of potassium (RCK) domain-containing sites critical for Mg^{2+} binding, a calcium bowl, and multiple C-terminal alternative splice regions (Fig. 2).

Five alternative splice sites within the C-terminus, A, C, E, G and I, have been described within *dslo* (Adelman et al., 1992; Lagrutta et al., 1994) and within *pslo* (Derst et al., 2003) as sites for insertion of an array of small exons encoding different polypeptides. Alignment of *msslo* cDNA fragments *A1–A3, B* and *C* to *dslo* and *pslo* reveals that the location of each alternate splice site is conserved: site A is within the S6 transmembrane domain, site C is within the RCK domain, site E lies partly within the S8 domain, while sites G and I are within variable regions between the S8 and S9 domains (Fig. 2). *Msslo* cDNA fragments contained some unique combinations of exons E, G and I: *mssloA-1* contained exons E1 (111 bp; AY644784), G1 (63 bp; AY644784) and I1 (42 bp; AY644784), *mssloA-2* contained exons E2 (111 bp; AY644785), G2 (48 bp; AY644787) and I2 (32 bp; AY644788), *mssloA-3* contained exons E2, G3 (147 bp; AY644786) and I2, while *mssloB*

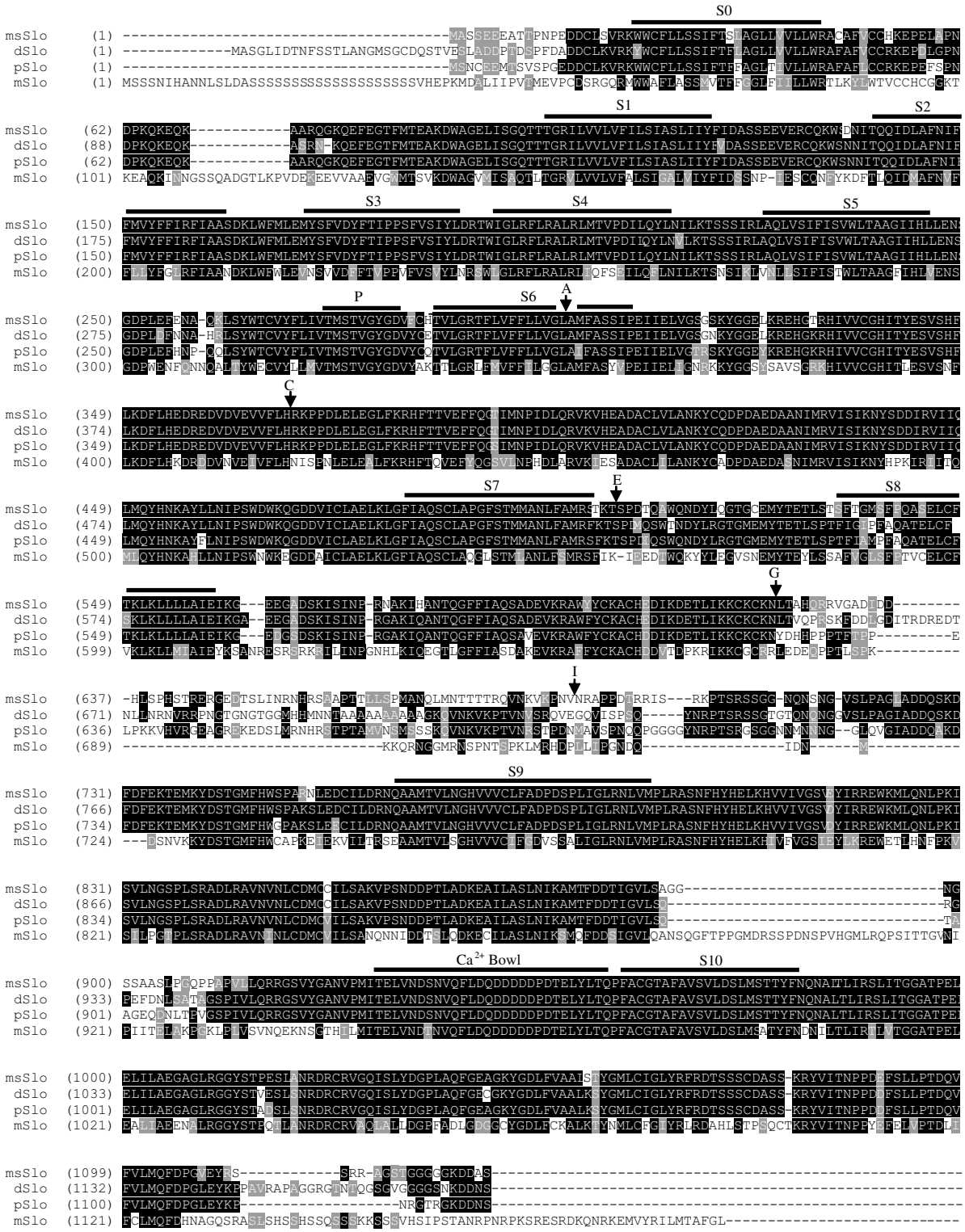

Fig. 2. Deduced amino sequence of msSlo and its alignment to insect and mammalian Slowpoke proteins. The sequences were compared using AlignX software (Informax, Inc.). The black shading indicates identical residues; the gray shading indicates similar residues. The dashes indicate gaps in the alignment. The approximate location of the seven membrane-spanning regions (S0–S6), the pore region (P), the four hydrophobic C-terminal cytoplasmic regions (S7–S10) and the calcium bowl are marked by black bars. The beginning of the five alternate splice sites (A, C, E, G, I) found in the insect sequences are marked with an arrowhead. GenBank accession numbers for sequences: *Manduca sexta* Slo (AY644784), *Drosophila melanogaster* Slo (M96840), *Periplaneta americana* Slo (AF452164), *Mus musculus* Slo (NM010610). Abbreviations: dSlo, *Drosophila melanogaster* Slowpoke; msSlo, *Manduca sexta* Slowpoke; pSlo, *Periplaneta americana* Slowpoke; mSlo, mouse Slowpoke.

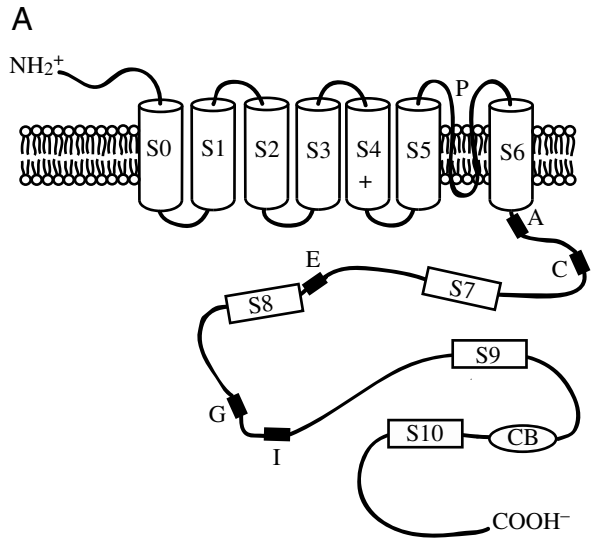

Fig. 3. Alternative RNA splicing of some of the *msslo* gene products. (A) Schematic diagram of the Slowpoke protein. The hydrophobic core (S0–S6), S4 voltage sensor (+) and hydrophobic tail (S7–S10) containing the RCK domain and calcium bowl are evolutionarily conserved among Slo proteins. Approximate locations of the five alternate splice regions within the tail region that are conserved among insects (A, C, E, G, I) are indicated by black boxes. (B) Alternative forms for *msslo* regions E, G, I. While some of the exon sequences are conserved among insects, those that are unique to *Manduca sexta* are denoted with an asterisk (*).

contained exons A1 (87 bp; AY644784) and C1 (105; AY644784) (Figs 1–3).

Sequence comparison of the deduced *Manduca* protein (NCBI, protein BLAST) revealed the highest amino acid identity to insect Slo channels (>80%), fly (*Drosophila melanogaster*), cockroach (*Periplaneta americana*) and mosquito (*Anopheles gambiae*), with less identity to nematode (60%; *Caenorhabditis elegans*) or mammalian (55%; rat, mouse or human) Ca^{2+}-activated K^+ channels. The regions of highest conservation between the insects have the following amino acid identities: segments S0–S6, 93–95%; the RCK domain, 97–98%; and segments S9–S10, 86%. The only region exhibiting low identity to Slo insect family members other than the 5′ and 3′ ends lies within the cytoplasmic tail region between S7 and S9 (58–64%). Part of the divergence among insect Slo proteins can be attributed to alternate exons E, G and I, which reside within this segment. While exons E1, E2 and G3 are conserved among insects (69–94% identity), exons G1, G2, I1 and I2 are unique to *Manduca* (0% identity) and could endow msSlo channels with a different range of biophysical properties to suit species-specific behaviors. Thus, the *Manduca* cDNA that we isolated is likely to be an ortholog of the *slo* family of calcium-activated potassium channels and will be referred to as *msslo*.

Msslo *gene expression is developmentally regulated in tissue-specific patterns*

In *Drosophila*, transcriptional regulation of *slo* directs expression of gene products in neurons and other cell types including muscles, midgut and trachea at embryonic and postembryonic life stages (Becker et al., 1995). Developmental and tissue-specific expression levels are not easily quantified in *Drosophila.* We used a highly sensitive non-isotopic northern blot assay to quantify *msslo* expression during development in the nervous and several muscle systems to complement and extend the molecular genetic analysis in *Drosophila*. The spatial distribution of the *slo* transcript in *Manduca* was also widespread, but the developmental expression patterns were tissue specific, with the most dramatic changes occurring in one set of skeletal muscles.

In all tissues, we detected a major mRNA of 4 kb. The size of this mRNA is similar to the size of the *msslo* cDNA, suggesting that the cDNA we isolated was full length. A much larger mRNA (11 kb) that may represent pre-processed RNA was observed at a few developmental stages, primarily in the CNS (Fig. 4).

CNS levels of msslo *mRNA do not change dramatically during postembryonic development*

The DIG-labeled RNA probe was hybridized to blots containing 1 μg of total RNA isolated from the CNS of day 2 fifth instar larvae (V2), at the onset and end of the wandering stage (W0 and W3, respectively), the day of pupal ecdysis (P0) and then approximately every two days during adult development (Figs 4, 5). These larval and early pupal developmental time points were chosen because they are temporally correlated to periods of major steroid-mediated reorganization of the CNS during metamorphosis (Truman, et al., 1992; Weeks and Levine, 1995). Our northern blot analysis did not reveal dramatic changes in the CNS expression of *msslo* throughout these metamorphic transitions, although levels do fluctuate during the larval–pupal transition and are higher in the adult than in the larva (Figs 4, 5). However, total CNS levels, as detected at the level of northern blot analysis, could be masking neuronal-specific developmental changes in *msslo* expression

levels. *In situ* hybridization analysis will aid in resolving the cell-specific *msslo* expression profile within the CNS.

Developmental changes in msslo *expression occur in visceral muscle*

Northern blot expression analysis of the visceral muscles, heart and midgut was performed at three stages of the animal's life: larval on V2, pupal on P6 and in adults on the day of their emergence (pharate adults, PA). We observed marked muscle-specific developmental regulation of *msslo* gene expression in the heart and midgut (Figs 6, 7). In the heart, levels of *msslo* mRNA transiently declined in pupae from larval levels then returned to similar levels of expression in the adult (Figs 6A, 7A). In the midgut, levels of the transcript are barely detectable in larvae and pupae but are present in the pharate adult (Figs 6B, 7B). Our hybridization analysis also suggests that *msslo* mRNA levels are generally lower at all stages in the heart and midgut than in the CNS because five times more total RNA from these visceral muscles was needed for transcript detection (see Materials and methods).

Msslo *expression is dramatically upregulated in skeletal muscle during metamorphosis*

We analyzed the expression pattern of *msslo* mRNA in the abdominal and thoracic DLMs in larvae (V2), developing pupae (P6) and pharate adults (PA). We chose to compare this regional set of skeletal muscles because the adult thoracic DLMs that are formed from a larval template as well as newly generated muscle (Duch et al., 2000) change from tonic to phasic flight muscles during metamorphosis (Rheuben and Kammer, 1980), while the abdominal muscles persist until after the moth ecloses and their muscle properties are thought not to change (Rheuben and Kammer, 1980; Truman et al., 1992). Although transcript levels in the DLMs in both regions transiently declined at P6, there was a marked regional difference in *msslo* mRNA levels in the adult tissue (Figs 8, 9). In the pharate adult thoracic DLMs, the levels of *msslo* transcript increased dramatically compared with levels in the abdominal DLMs, suggesting that the msSlo current plays an important role in the excitation or contractile properties of the phasic flight muscle (Figs 8, 9).

To obtain a more comprehensive developmental profile for the upregulation of *msslo* in the thoracic DLMs, we analyzed transcript levels starting with the molt to the fifth instar, throughout the wandering stage and every two days during adult development (Figs 10, 11). *Msslo* transcript levels are high after the molt to the fifth instar (V0), then appear to decline slightly during the feeding stage of the fifth instar (V2) and into the first day of wandering (W0). Transcript levels appear to increase slightly during wandering (W2, W3) but then decline at pupal ecdysis (P0). *Msslo* mRNA levels remain low throughout most of adult development, except for a transient increase at P8–P10 back to larval levels, and then abruptly and dramatically increased on P15 (Figs 10, 11). Transcript levels remain high throughout the adult molt (PA, A0) and then appear to gradually decline after emergence (A2; Figs 10, 11).

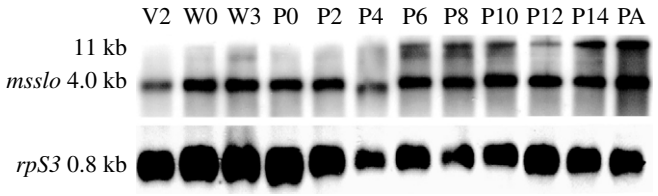

Fig. 4. Developmental profile of *msslo* mRNA within the CNS. No dramatic changes in *msslo* gene expression are detected within the CNS during metamorphosis. Total RNA (1 μg per lane) was isolated from day 2 fifth instar larvae (V2), days 0 and 3 wandering larvae (W0, W3), days 0, 2, 4, 6, 8, 10, 12, 14 after pupation (P0, P2, P4, P6, P8, P10, P12, P14) and pharate adults (PA) and hybridized to the digoxigenin-labeled *msslo* 680 bp cDNA insert or loading control *M. sexta* ribosomal protein S3 (rpS3) cDNA insert (Jiang et al., 1996). The sizes (kb) of the hybridizing RNAs are shown on the left. The major mRNA is 4 kb and is similar in size to the *msslo* cDNA. The 11 kb band is probably pre-processed *msslo* mRNA.

Discussion

We have isolated and molecularly characterized the spatial distribution and developmental expression profile of a new member of the calcium-activated K^+ channel family, *Manduca sexta slowpoke* (*msslo*). Slowpoke or BK channels show strong evolutionary sequence conservation, sharing seven transmembrane-spanning regions and a large cytoplasmic C-terminus with putative binding sites for Mg^{2+} and Ca^{2+} (refer to Fig. 3A). While dSlo is encoded by a single gene, differential promoter usage, alternative splicing and

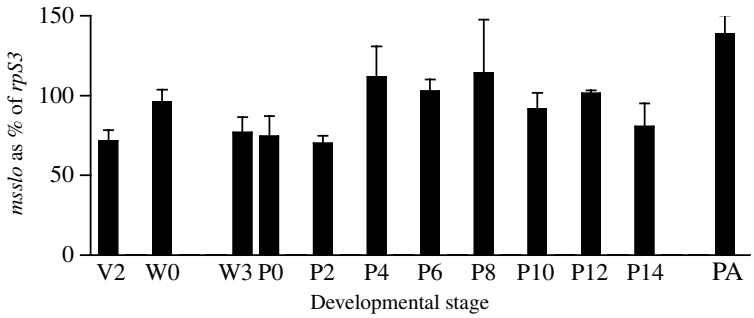

Fig. 5. Semi-quantitative analysis of *msslo* mRNA developmental profile within the CNS. Densitometric analysis of CNS northern blots using GeneTools software showed relatively constant levels of *msslo* transcripts throughout larval life, with levels fluctuating during the larval–pupal transition and increasing in adults. Semi-quantification of the chemiluminescence was done by comparing the intensity of the *msslo* mRNA band with that of the internal control, *rpS3*. Mean and S.E.M. are plotted (*N*=5). Abbreviations: day 2 fifth instar larvae (V2), days 0 and 3 wandering larvae (W0, W3), days 0, 2, 4, 6, 8, 10, 12, 14 after pupation (P0, P2, P4, P6, P8, P10, P12, P14) and pharate adults (PA).

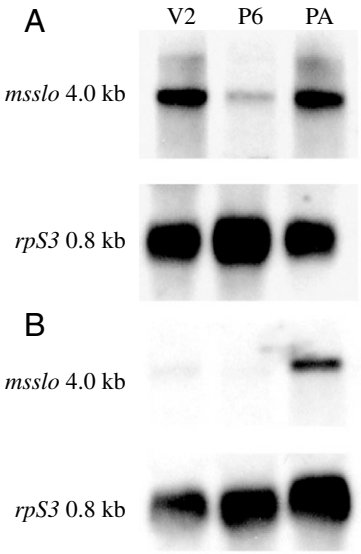

Fig. 6. Muscle-specific developmental changes in *msslo* expression occur in the heart and midgut. (A) In the heart, *msslo* mRNA expression appears to transiently decline in pupae from larval levels and then return to similar expression levels in pharate adults. (B) In contrast to the heart, midgut levels of *msslo* mRNA are highest in pharate adults. Total RNA (5 μg) of day 2 fifth instar larvae (V2), day 6 pupae (P6) and pharate adults (PA) was hybridized to the digoxigenin-labeled *msslo* 680 bp cDNA insert. For the loading control, the lower portion of the blots was hybridized to the digoxigenin-labeled *M. sexta* ribosomal protein S3 (rpS3) cDNA insert (Jiang et al., 1996). The sizes (kb) of the hybridizing RNAs are shown on the left.

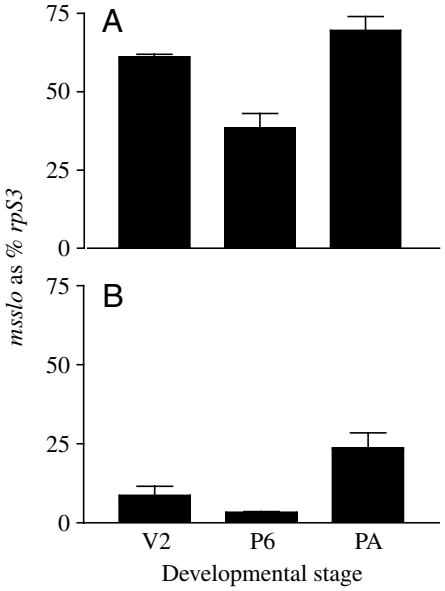

Fig. 7. Semi-quantitative analysis of *msslo* mRNA developmental profile in visceral muscle. Densitometric analysis of heart (A) and midgut (B) northern blots using GeneTools software confirmed muscle-specific developmental profiles. (A) In the heart, *msslo* transcript levels transiently decline during development whereas (B) in the midgut, levels are low until the adult stage. Semi-quantification of the chemiluminescence was done by comparing the intensity of the *msslo* mRNA band with that of the internal control, *rpS3*. Mean and S.E.M. are plotted (*N*=3). Abbreviations: day 2 fifth instar larvae (V2), day 6 after pupation (P6) and pharate adults (PA).

developmental regulation of expression levels can be used to tailor the channels to meet diverse cellular and physiological requirements. For example, inserts can modify single-channel properties such as calcium and voltage sensitivity, activation rate and conductance (Tseng-Crank et al., 1994; Xie and McCobb, 1998), subcellular distribution (Zarei et al., 2001), sensitivity to phosphorylation (Tian et al., 2001) and association with modulatory subunits (Benkusky et al., 2002; Ramanathan et al., 2000; Weiger et al., 2000). Regulation of BK channel expression levels during development contributes to channel density in the plasma membrane altering tissue excitability (Broadie and Bate, 1993; Martin-Caraballo and Dryer, 2002; Muller et al., 1998, 2000; Muller and Yool, 1998; Salkoff, 1985). We have isolated *msslo* mRNAs containing multiple inserts and quantified tissue-specific developmental changes in transcript levels. It is likely that msSlo may be regulated through similar mechanisms and participate in altering neuronal and muscular excitability and the circuits that control stage-specific behavior.

Steroid hormone modulation of ion channel gene expression

Steroid hormone status can dynamically regulate the excitability of vertebrate neurons, endocrine and muscle cells by affecting the expression levels, splicing or activity of BK channels. For example, adrenal steroids downregulate BK channel currents in hippocampal neurons (Kerr et al., 1989) and can mediate the inclusion of the stress axis regulated exon (STREX) in adrenal chromaffin cells (Shipston et al., 1996; Xie and McCobb, 1998; Tian et al., 1999). Fluctuations in estrogen and/or progesterone during pregnancy may influence alternative exon splicing in mouse uterine muscle, altering muscle contractility (Benkusky et al., 2000). The steroid hormone 20-hydroxyecdysone (20HE) mediates many of the metamorphic changes in the nervous and muscular systems that reorganize the caterpillar to form adult structures and behavior (Truman, 1992; Weeks and Levine, 1995). Steroid-mediated transcriptional control of ion channel gene expression could alter the electrical properties of larval neurons and muscles to tailor their electrical properties for new adult circuits and behavior. Developmental changes in potassium currents occur in *Manduca* leg (Hayashi and Levine, 1992; Grüenwald and Levine, 1998), flight motoneurons (Duch et al., 2000; Hayashi and Levine, 1992), antennal lobe neurons (Mercer and Hildebrand, 2002a,b) and glia (Lohr et al., 2001), but the ion channel genes or gene products producing these potassium currents are not known yet.

We have isolated and molecularly characterized the developmental expression profile for the only two voltage-gated potassium ion channel genes isolated from *Manduca sexta*: *msslo* reported here and *Manduca sexta ether à-go-go*

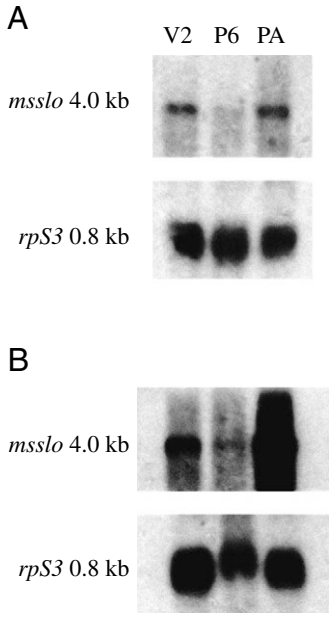

Fig. 8. Regional-specific expression of *msslo* in dorsal longitudinal muscles (DLM). (A) Message levels of *msslo* in the abdominal DLMs are similar in larvae and pharate adults and are higher than in early-stage pupae. (B) By contrast, transcript levels are dramatically increased in the adult thoracic DLM in comparison with larval *msslo* mRNA levels. These changes are temporally correlated to the switch in the contractile properties of the thoracic DLMs from tonic in larvae to phasic in adults. Total RNA (1 μg per lane) was isolated from abdominal and thoracic DLMs from day 2 fifth instar larvae (V2), day 6 pupae (P6) and pharate adults (PA) and hybridized to the digoxigenin-labeled *msslo* 680 bp cDNA insert or loading control *M. sexta* ribosomal protein S3 (rpS3) cDNA insert (Jiang et al., 1996). The sizes (kb) of the hybridizing RNAs are shown on the left.

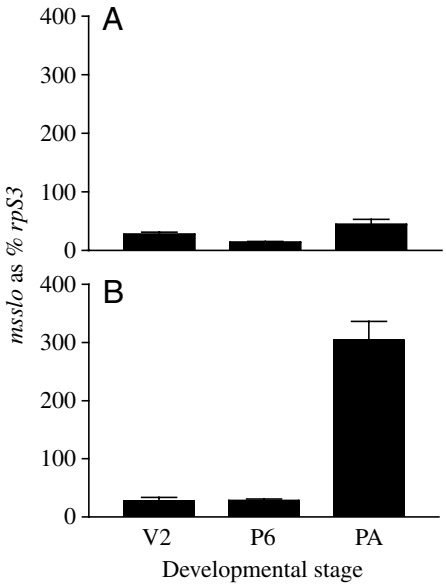

Fig. 9. Semi-quantitative analysis of *msslo* mRNA developmental profile in skeletal muscle confirms regional-specific expression patterns. Densitometric analysis using GeneTools software revealed (A) similar and low levels of *msslo* transcripts in larval and adult abdominal DLMs compared with early-stage pupae. (B) The dramatic increase in *msslo* expression in thoracic DLMs is in contrast to the abdominal pattern. Semi-quantification of the chemiluminescence was done by comparing the intensity of the *msslo* mRNA band with that of the internal control, *rpS3*. Mean and S.E.M. are plotted (*N*=5). Abbreviations: day 2 fifth instar larvae (V2), day 6 after pupation (P6) and pharate adults (PA).

or *mseag* (Keyser et al., 2003). The changes in expression levels of both genes are temporally correlated to the fluctuations in 20HE that mediate metamorphosis, suggesting that they may be regulatory targets of the ecdysteroids. The developmental profiles for each gene are distinctive and are tissue-specific. For example, when 20HE titers are increasing early in adult development, CNS levels of *mseag* transcripts are highest, while *msslo* transcripts are low. When steroid titers have declined prior to emergence, CNS *mseag* levels are low, while CNS *msslo* transcripts have slightly increased and flight muscle transcripts have dramatically increased. This differential developmental regulation for *msslo* and *mseag* may contribute to altering the cell's excitability to meet stage-specific needs.

As in vertebrates, steroid-mediated transcription control of *msslo* exon choice could be another mechanism to modify cell excitability during postembryonic development. Because the size of the alternate exons is relatively small (100 bp) in comparison with the 4 kb transcript, our northern blot analysis would not detect these changes. With single-cell RT-PCR of identified neurons, it will be feasible to investigate whether *msslo* exon selection is regulated at the cellular level, changes during development and if the exon choice is mediated by the ecdysteroids.

Are developmental changes in msslo *expression in visceral muscle related to stage-specific functions?*

In the *Drosophila* heart, four K^+ currents, including a Slo current, have been detected through mutational and pharmacological analysis (Johnson et al., 1998). The role that dSlo plays in maintaining heartbeat is critical, as both *slo* mutants and animals injected with the agent charybdotoxin, which blocks fast Ca^{2+}-gated K^+ channels, exhibit greatly diminished heartbeat and rhythmicity (Johnson et al., 1998). Given the importance of the Slo channel in the *Drosophila* heart, it is likely that the developmental regulation of *msslo* expression we observed in *Manduca* has stage-specific physiological relevance. For example, during metamorphosis, there is a switch in pacemaker regions (Davis et al., 2001; Slama, 2003). In larvae, peristaltic contractions of the heart are anterograde, while in the adult the heartbeat cycles between anterograde and retrograde contractions. This change in pacemaker regions may occur as a result of the heart becoming innervated during the metamorphic transition (Davis et al., 2001; Dulcis et al., 2001). Because dSlo channels are critical for pacemaker activity in *Drosophila* (Johnson et al., 1998), it is possible that developmental regulation of msSlo channel

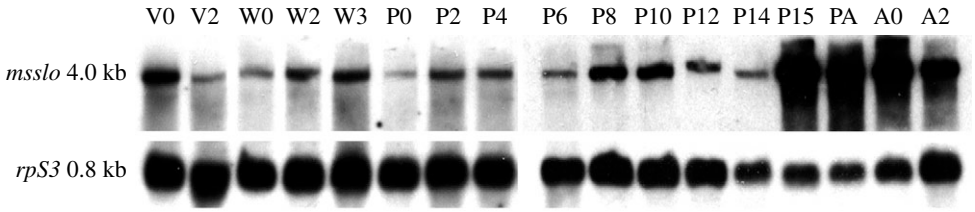

Fig. 10. Developmental profile of *msslo* expression in the thoracic dorsal longitudinal muscles (DLM). Small fluctuations in *msslo* mRNA levels occur during the transition from the feeding (V) to wandering (W) larval stages and from W to pupal ecdysis (P0). Transcript expression remains low in the early pupal stages (P1–P6), with a moderate increase occurring at pupal days 8–10. The massive upregulation of *msslo* expression occurs late in adult development on day 15 and appears to gradually decline 2 days after the adult emerges. Total RNA (1 μg per lane) was isolated from thoracic DLMs from days 0 and 2 fifth instar larvae (V0, V2), days 0, 2 and 3 of wandering larvae (W0, W2, W3), and days 0, 2, 4, 6, 8, 10, 12, 14 and 15 after pupation (P0, P2, P4, P6, P8, P10, P12, P14, P15), pharate adults (PA) and in adults just after they emerged and 2 days after emergence (A0, A2). The RNA was hybridized to the digoxigenin-labeled *msslo* 680 bp cDNA insert or loading control *M. sexta* ribosomal protein S3 (rpS3) cDNA insert (Jiang et al., 1996). The sizes (kb) of the hybridizing RNAs are shown on the left.

expression levels or their spatial distribution could contribute to the change in pacemaker localization.

The midgut is a primary site for nutrient and ionic regulation in insects. In the midgut of *Drosophila*, Slo expression is apically localized to interstitial cells within the copper cell region (Brenner and Atkinson, 1997). These cells are thought to be involved with potassium ion transport between the hemolymph and the gut lumen. An analogous role has been proposed for the goblet cells in the *Manduca* larval midgut (Cioffi, 1979). During postembryonic development, the levels of *msslo* transcript are barely detected in larvae and pupae and appear to be upregulated in the pharate adult stage. This upregulation of *msslo* may be related to differences in ion transport that accompany a dietary change from feeding on tobacco leaves to drinking nectar.

Upregulation of msslo *mRNA expression in the dorsal longitudinal muscles is correlated with key developmental events*

Electrophysiological analysis in *Drosophila* confirms the presence of mature Slo currents in the embryonic, larval body wall muscle and adult DLMs but not in pupal muscles (Elkins et al., 1986; Salkoff, 1983a,b, 1985; Singh and Wu, 1990). The lack of detectable currents in pupal muscles could be due to very low channel density since expression levels of the *dslo* mRNA in these muscles was thought to be much lower than in the CNS since they could only be detected by RT-PCR or reporter gene expression (Becker et al., 1995; Brenner et al., 1996). By contrast, *msslo* transcripts are easily detected in northern blot analysis in homologous muscles at all stages and at a higher level of expression than in the CNS. These results

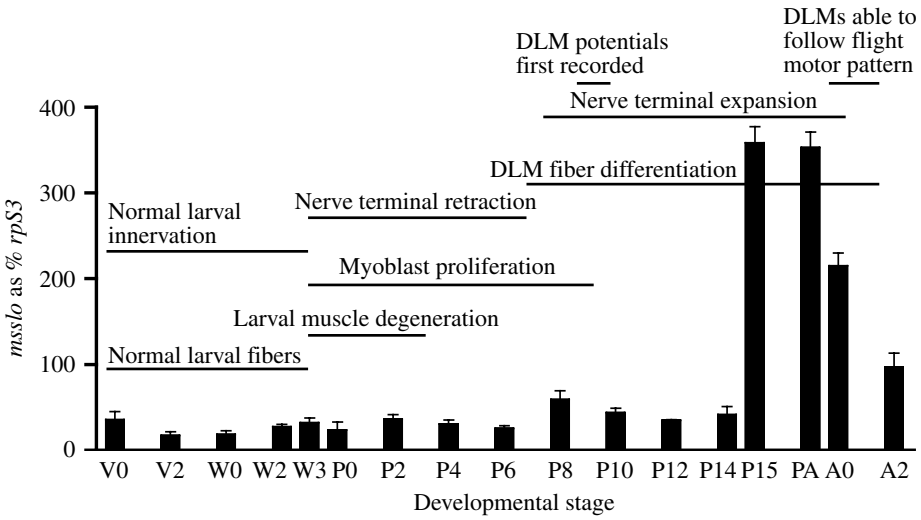

Fig. 11. Temporal correlation of changes in *msslo* expression with the development of the dorsal longitudinal flight muscle. The dramatic increase in *msslo* mRNA occurs late in adult development, preceding the muscle's competence to respond to the mature flight motor pattern. The slight increase in message at P8 precedes the first time DLM muscle potentials can be recorded and the onset of maturation of the flight motor pattern within the CNS. Semi-quantification of *msslo* mRNA was done by comparing its signal intensity to the signal intensity of the housekeeping gene *rpS3* using image analysis software (GeneTools). Mean and S.E.M. are plotted (*N*=5). Developmental events based on Kammer and Kinnamon (1979), Rheuben and Kammer (1980), Duch et al. (2000) and Duch and Levine (2002).

raise the possibility that functional msSlo currents may be present and participate in early as well as late neuromuscular development of the flight system.

The first increase in *msslo* transcript in the DLMs occurs during the wandering stage and is concurrent with the onset of degeneration of larval muscle fibers, retraction of the innervating nerve terminals and myoblast proliferation (Fig. 11) (Duch et al., 2000). BK channels can participate in programmed cell death in vascular smooth muscle (Krick et al., 2001), and potassium efflux is a major component in neuronal apoptosis (Yu, 2003; Yu et al., 1997). If the *msslo* channel is functional at this time, it could play a role in larval muscle fiber degeneration. Another possibility is that the increase in *msslo* mRNA and channel density may alter muscle properties to facilitate pupal ecdysis, which occurs just after the wandering stage.

The next increase in *msslo* message occurs approximately midway through adult development (P8–P10), temporally correlated with significant increases in muscle mass, differentiation, more extensive terminal innervation and the onset of muscle membrane excitability (Fig. 11) (Duch et al., 2000; Rheuben and Kammer, 1980). Nerve–muscle interactions are critical in the development of flight muscles in *Drosophila* (Fernandes and Keshishian, 1998) and in *Manduca sexta* (Bayline et al., 2001), so if msSlo currents are present in the developing DLMs, they may play an active role in these processes.

Upon adult emergence in *Drosophila*, the inward voltage-gated calcium current and the Slo current mature, with the latter supplanting the fast inactivating Shaker current as the repolarizing current (Salkoff, 1985). That the *msslo* mRNA upregulation precedes eclosion by at least 3 days suggests there is a build-up of gene products that precedes the appearance of the mature current, as is seen with voltage-gated Ca^{2+} channels in developing *Drosophila* flight muscle (Wei and Salkoff, 1986). Although specific currents have yet to be identified in *Manduca* flight muscle, there is a slight reduction in the duration of the adult flight muscle action potential as compared with the larval one (Rheuben and Kammer, 1980). Upregulation of *msslo* may contribute to the more rapid repolarization of the action potential through increased channel density. This change in electrical properties may be necessary for the performance of the high-frequency flight motor program (Kammer and Kinnamon, 1979). With the use of RNA interference (Feinberg and Hunter, 2003; Uhlirova et al., 2003) and electrophysiological analysis, we will be able to test whether developmental changes in *msslo* expression contribute to the remodeling of electrical properties of specific neurons and muscles and synaptic plasticity.

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