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Drosophila homologs of two mammalian intracellular Ca^{2+}-release channels: identification and expression patterns of the inositol 1,4,5-triphosphate and the ryanodine receptor genes

GAITI HASAN[1,2] and MICHAEL ROSBASH[1]

[1]*Howard Hughes Medical Institute and Department of Biology, Brandeis University, Waltham, MA 02254, USA*
[2]*Molecular Biology Unit, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India*

Summary

We have identified and cloned portions of two *Drosophila* genes homologous to two classes of mammalian intracellular Ca^{2+}-release channels, the ryanodine receptor and the inositol 1,4,5-triphosphate (IP3) receptor. The *Drosophila* ryanodine receptor gene (*dry*) encodes an approx. 15 kb mRNA. It is expressed in the mesoderm of early stage-9 embryos and subsequently in somatic muscles and their precursor cells. In adults, *dry* mRNA was detected in tubular muscles and at a lower level in neuronal tissues. Embryonic expression of the *Drosophila* IP3 receptor gene (*dip*) appears more dynamic and is associated with developing anterior sense organs. In adults, *dip* expression occurs in several tissues, and relatively high levels of *dip* mRNA in adult antennae suggest a role for this gene product during olfactory transduction.

Key words: ryanodine and IP3 receptors, mesodermal expression, sensory organ development, tubular muscles, antennal expression.

Introduction

The release of intracellular Ca^{2+} is an intermediate step in many cellular signaling processes (Berridge and Irvine, 1989; Tsein and Tsein, 1990). In vertebrates, two classes of proteins, the inositol 1,4,5-triphosphate (IP3) receptor and the ryanodine receptor, act as channels for the release of intracellular Ca^{2+}. The IP3 receptor causes release of intracellular Ca^{2+} in response to IP3, which is generated during signaling mechanisms that involve activation of phospholipase C (Majerus et al., 1985). This signal transduction pathway is used in processes as diverse as the responses to hormones, growth factors and neurotransmitters (Berridge and Irvine, 1984), as well as in various sensory systems such as olfaction (Reed, 1992), gustation (Hwang et al., 1990) and vision (Payne et al., 1988). It must also function in the central brain, the tissue from which it was initially purified and cloned (Furuichi et al., 1989; Mignery et al., 1990). Ryanodine receptor function is best understood in vertebrate skeletal muscle, in which this protein spans the junction between the T-tubules of the plasma membrane and the sarcoplasmic reticulum. It is required for the intracellular Ca^{2+}-release that occurs prior to muscle contraction, in response to nerve impulses delivered to the muscle plasma membrane (Catterall, 1991). A second form of the ryanodine receptor has been identified in cardiac muscle and more recently in vertebrate brain (McPherson et al., 1991), where its function is poorly understood. In addition, both the IP3 and ryanodine receptors have been postulated to function during Ca^{2+}-induced Ca^{2+}-release in neuronal and non-neuronal tissues requiring Ca^{2+} oscillations (Tsein and Tsein, 1990). The presence of these intracellular Ca^{2+} channels in such diverse tissues indicates that they are likely to be involved in many different cellular functions.

Drosophila offers an ideal system to examine the role of these proteins during cell signaling in development and in adult sensory and central brain processes. To this end, we have cloned and characterized two *Drosophila* genes that code for these two classes of intracellular Ca^{2+}-release channels. Our results suggest that both genes are well conserved between mammals and *Drosophila*. We have also analyzed the expression of these genes during development and in adults. Their expression patterns suggest that they function both during embryonic development and in adult tissues. Spatial distribution of the mRNAs for the *Drosophila* IP3 and ryanodine receptors indicates that both channels serve neuronal functions. In addition, the ryanodine receptor is likely to function at the neuromuscular junction.

Materials and methods

Molecular cloning by PCR

The primers used in PCR experiments for cloning of the *dry* gene

were designed to anneal to the cDNA sequence encoding the amino acids (T/K)CFICG and EEHNM respectively. The exact sequences were as follows: 5′ primer A(C/A)-(C/G/T/A)TG(C/T)TT(C/T)AT(C/T/A)TG-(C/T)GG; 3′ primer CAT(G/A)TT(G/A)TG(C/T)TC(C/T)TC. Total RNA from heads of Canton-S wild-type flies was extracted by standard procedures and treated with RNAase-free DNAaseI. Approximately 1 μg of this RNA was heated at 90°C for 5 minutes and added to a reverse transcription (RT) reaction containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 1 mM each dATP, dGTP, dCTP and dTTP, 40 units RNasin (Promega), 50 μM of the 3′ primer and 200 units of Super-RT reverse transcriptase (BRL) in a total volume of 20 μl. The RT reaction was incubated at 42°C for 60 minutes and at 95°C for 10 minutes. It was then added to 80 μl of the same reaction mix minus both RNasin and reverse transcriptase and containing 125 μM of each dNTP, 50 μM of the 5′ and 3′ primers and 2.5 units of Taq polymerase (Boehringer-Mannheim). After an initial 2 minutes at 94°C, amplification was allowed to proceed for 5 cycles with the following regime: 94°C, 1 minute; 42°C, 2 minutes; 72°C, 3 minutes; followed by 25 cycles of: 94°C, 1 minute; 50°C, 2 minutes; 72°C, 3 minutes. A 90 bp product from the amplification reaction was gel-purified and cloned into the *Sma*I site of the plasmid vector Bluescript KS from USB. The DNA sequence was determined from the double stranded plasmid using a Sequenase kit (USB).

For cloning of the *dip* gene essentially the same procedures were employed except that poly(A)$^+$ RNA from heads was used for the RT reaction. Primers were designed from the amino acids EHNMWHY and MTEQRKQ with the following sequences: 5′ primer - GA(G/A)CA-(C/T)AA(C/T)ATGTGGCA(C/T)TA; 3′ primer - TG(C/T)TT(C/G/T/A)C(G/T)(C/T)TG(C/T)TC-(C/G/T/A)GTCAT.

cDNA isolation and sequencing

The cloned PCR fragments for the *dry* and *dip* genes were used as probes to screen a head cDNA library in the vector λEXLX (Palazzolo et al., 1990). Larger cDNA clones obtained in this way were sequenced using sequential oligonucleotide primers from both strands. Analysis of the DNA sequence was performed with the GCG software package for VAX computers (Devereux et al., 1984).

Northern blots

The poly(A)$^+$ RNA from embryonic developmental stages, larvae and pupae was a kind gift of S. DeSimone. Poly(A)$^+$ RNA from adult heads and bodies was prepared by standard procedures. Approximately 2-5 μg were loaded per lane of a 0.75% formaldehyde agarose gel and subsequently transferred to a Hybond-N nylon membrane (Amersham). Blots were hybridized to ^{32}P-labelled cDNA fragments of the respective genes under standard conditions.

In situ hybridization to polytene chromosomes

Probes for in situ hybridization to polytene chromosomes were labelled by random priming with biotinylated dUTP (Enzo Diagnostics). The hybridization was visualized with streptavidin-conjugated horseradish peroxidase in a kit from Enzo Diagnostics.

In situ hybridization to whole-mount embryos

In situ hybridization to embryos was carried out with the Genius DNA labelling and detection kit (Boehringer Mannheim) according to the procedure of Tautz and Pfiefle (1989). Digoxigenin-labelled probes were made by random-primed labelling of cDNA fragments reduced to a size of approx. 300 bp. Random priming reactions were carried out at 15°C overnight and then at room temperature for 2 hours with an addition of 5 units of Klenow fragment.

In situ hybridization to adult frozen sections

Frozen sections of 10 μm were processed and hybridized to digoxigenin-labelled probes as described by Nighorn et al. (1991). Probes were labelled following the procedure described for embryo in situs.

Quantitative PCR (RT-QPCR)

Adult flies were snap-frozen in dry ice and shaken so as to break off their heads, antennae and legs. These tissues were separated by hand for subsequent steps. Eyes were purified by a published procedure (Matsumoto et al., 1982) and were a kind gift of L. Zwiebel. Total RNA was extracted from 50 heads, 50 antennae, 25 legs and 50 eyes using standard procedures and subjected to extensive DNAase treatment. The relative abundance of different transcripts was measured using a reverse-transcription-based competitive PCR assay (RT-QPCR; Gilliland et al., 1990). The assay involved the coamplification of the target RNA with a competitive RNA template (spike), thereby minimizing most of the variability normally associated with PCR. Known amounts of spike RNA were added to the total RNA samples, and control experiments showed that after coamplification the resulting ratio of target DNA product to spike DNA product was an accurate measure of the amount of target transcript. Primers were designed to generate a product of 197 bp for the *dry* mRNA, a product of 220 bp for the *dip* mRNA, and a product of 360 bp for the *RP49* mRNA. The spikes for *dip* and *dry* were constructed by deleting 30 and 40 bp, respectively, between the two primer sites. The spike for *RP49* was generated by using the corresponding piece of genomic DNA that contains an intron of 80 bp between the two primer sites and which had been cloned in the transcription vector pSP65. The other two DNAs (*dip* and *dry*) were cloned into a transcription vector (TA from Invitrogen) using the manufacturers instructions. All three spike DNA constructs were transcribed into RNA. The primers specific for each RNA were used for RT-QPCR performed with approximately equivalent amounts of RNA from each preparation. Approximately 0.5 ng of total RNA, together with an empirically determined optimal quantity of spike, was used for each reverse transcription reaction with M-MLV reverse transcriptase (BRL) and carried out according to conditions recommended by the suppliers. The 20 μl reactions contained 50 pmoles of the 3′ primer. After heat-killing, 10 μl of each reaction were added to 40 μl of a PCR mix containing the 5′ and 3′ primers at 1 μM each final concentration. PCR was carried out with Vent Polymerase (exo minus; New England Biolabs) using conditions specified by the suppliers. ^{35}S-dATP (0.25 μCi) was added to each reaction in order to visualize the PCR products with a Molecular Dynamics Phosphorimager. The PCR was performed on a Perkin-Elmer GeneAmp PCR System 9600 as follows: Each cycle consisted of 95°C, 5 seconds; 50°C, 5 seconds; 72°C, 45 seconds. 28 cycles were used for *RP49*, 31 for *dry* and 36 for *dip*. Control reactions for reverse transcription and for the PCR were performed in each case. There was no significant contamination with genomic DNA as there was no detectable signal without the addition of reverse transcriptase. Quantitation of the appropriate PCR products was performed with the phosphorimager and the ratio of target band to spike band calculated. The amount of RP49 mRNA in each RNA preparation was measured relative to spike, and the values obtained from the *dip* and *dry* were normalized relative to RP49 values.

Results

Isolation of the Drosophila *homologs for the ryanodine receptor and the IP3 receptor gene by PCRs with degenerate oligonucleotides*

The mammalian ryanodine and IP3 receptors share significant homology at their carboxy-terminal (C-terminal) ends (Furuichi et al., 1989). Degenerate oligonucleotides were designed from two closely spaced regions that are well conserved between the two mammalian proteins (see Materials and methods and Fig. 1). These oligonucleotides were used in reverse transcription and polymerase chain reactions (PCR - Saiki et al., 1985) of total head RNA from *Drosophila melanogaster*, and a fragment of approximately the right size (90 bp) was obtained and cloned. Ten independent clones derived from two PCRs were analyzed, and in all cases the sequences between the priming oligonucleotides were found to be identical. Conceptual translation of this nucleotide sequence yielded protein sequence information which suggested that the PCR amplified fragment was from a *Drosophila* ryanodine receptor gene (*dry*). This was confirmed by isolating larger clones from a head cDNA library using the 90 bp fragment as a hybridization probe. These clones coded for the C-terminal end of the protein, as expected from the positions of the primers used to isolate the original 90 bp PCR fragment. This is also evident on alignment of the *Drosophila* protein sequence with the mammalian protein. The rabbit cardiac muscle ryanodine receptor is composed of 4969 amino acids (Otsu et al., 1990), of which the 509 C-terminal amino acids share 61% identity and 79% similarity with the sequenced portion of the *Drosophila* ryanodine receptor gene, subsequently referred to as *dry* (Fig. 1).

Since none of the clones sequenced from the 90 bp fragment were derived from a *Drosophila* IP3 receptor gene, oligonucleotides from other regions of the mammalian IP3 receptor were designed for the purpose of cloning this gene. This was aided by the publication of regions of identity at the C terminus of the *Drosophila* and mouse IP3 receptor proteins (Miyawaki et al., 1991). PCRs using oligonucleotides specific for the IP3 receptor were performed with poly(A)$^+$ RNA from *Drosophila* heads, and a fragment of the expected 350 bp size was obtained. Cloning and sequencing of this fragment indicated that it codes for a protein similar to the rat IP3 receptor. Larger cDNA clones complementary to this fragment were isolated from a head cDNA library and sequenced.

A comparison of the IP3 receptor protein sequences from rat (ratIP3) and *Drosophila* (Dip) is shown in Fig. 1, and demonstrates that the two proteins share extensive homology throughout the sequenced region (73% similarity and 57% identity). This region corresponds to the C-terminal 460 amino acids of the rat IP3 receptor. The full length rat receptor protein consists of 2749 amino acids (Mignery et al., 1990).

The two *Drosophila* genes described here are also related to each other; like the mammalian ryanodine and IP3 receptors, they share significant homology near their C termini (Furuichi et al., 1989). A comparison of the 300 C-terminal amino acids of the cloned *Drosophila* ryanodine and IP3 receptor proteins shows that they are 62.6% similar and

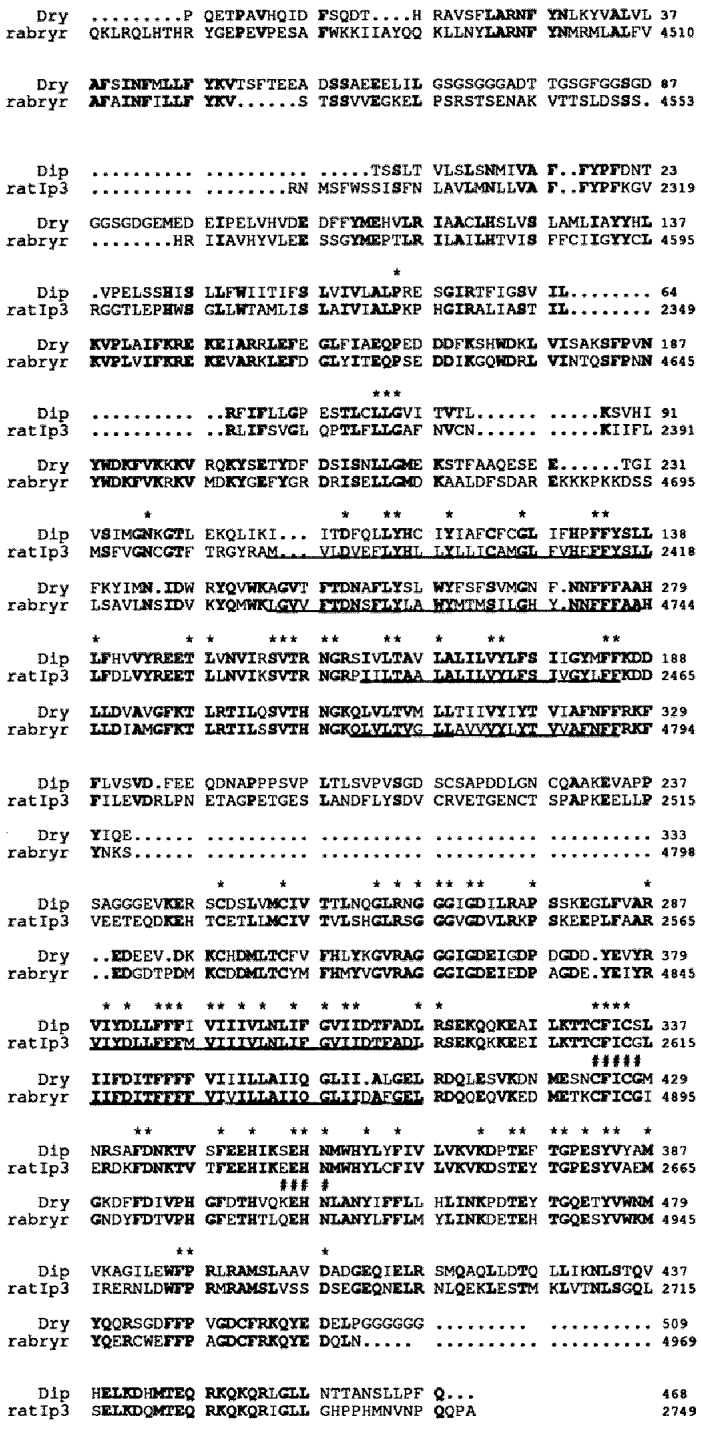

Fig. 1. Sequence alignment of the carboxy-terminal ends of the *Drosophila* ryanodine receptor (Dry) with the rabbit cardiac muscle ryanodine receptor (rabryr), and the *Drosophila* IP3 receptor (Dip) with the rat brain IP3 receptor (ratIp3). All four sequences have also been aligned. Bold letters indicate identical amino acids between two sequences of the same pair. Spaces indicated by dots have been introduced to maximize the alignments. Asterisks (*) indicate amino acids and underlines are putative membrane spanning domains that are conserved among all four proteins. Hashes (#) indicate the sequences from which primers were designed for the initial PCR.

33.6% identical (Fig. 1). The relationship between all four proteins is also shown in Fig. 1. Both ryanodine receptor genes appear equally related to the two IP3 receptor genes, indicating that an ancient duplication event probably gave rise to these two classes of intracellular Ca^{2+}-release channel genes. The regions of identity among all four proteins (depicted by asterisks) include the three putative membrane spanning domains, which have been underlined.

Chromosomal localization of dry *and* dip

The *dry* gene hybridizes to a single band on polytene chromosomes located near the centromeric end of chromosome 3L at band position 76C-D (data not shown). No known mutations with a neuromuscular phenotype have been found to map to this region. The *dry* gene also appears to be single copy by hybridization to Southern blots washed at high stringency. However, on low stringency blots we could detect in each case an additional band of hybridization, suggesting the presence of a related gene (data not shown). In a similar analysis with the *dip* gene, no differences were observed in the hybridization patterns of Southern blots hybridized and washed at either high or low stringency. The *dip* gene also hybridizes to a single site on polytene chromosomes at band position 42B1-2 on the right arm of the second chromosome (data not shown). No known mutations map to this region of the second chromosome.

Developmental expression of dry *and* dip *by northern analysis*

A characteristic feature of the ryanodine and IP3 receptor genes of mammals is the very large size of their mRNAs and encoded proteins. The rabbit cardiac ryanodine receptor is encoded by a 17 kb mRNA, while the mRNA for the rat IP3 receptor is 10 kb in length. From the size of the mRNAs seen for the two *Drosophila* genes (Fig. 2), it would appear that the *Drosophila* proteins are also very large. To address the question of which cellular processes are likely to involve these two Ca^{2+}-release channel genes, we initially examined their expression by northern blot analysis (Fig. 2). With the *dry* probe, a band of hybridization of approximately 15 kb is first seen in 12- to 18-hour embryos. This band is more intense in 18- to 24-hour embryos. The same levels of mRNA are seen in larvae and adults, but the band is apparently absent from mid-pupae. Although the *dry* mRNA band is visible both in RNA from adult heads and bodies, it is clearly of greater intensity in body RNA, suggesting that as in mammals this protein may be enriched in muscle tissue.

Two bands of hybridization, differing very slightly in size, are seen with the *dip* probe (Fig. 2). The smaller-sized band (approx. 9.7 kb) is seen only in early embryos of 0-6 hour. A larger band of approx. 10 kb is expressed in mid-late embryos. Unlike the ryanodine receptor mRNA levels, however, the *dip* mRNA levels do not increase in late embryos and a low level of expression is observed throughout development in larvae, mid-pupae and adults. Equivalent mRNA levels are seen in adult head and body RNAs. At present, we cannot say what accounts for the size difference between the two mRNAs seen for the IP3 receptor. Although they could be derived from two different genes, it is more likely that they are alternatively spliced mRNAs

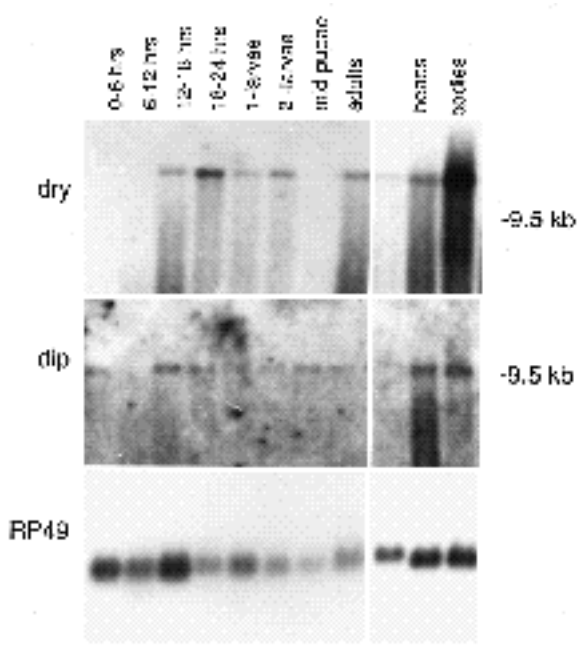

Fig. 2. Developmental profile of *dry* and *dip* transcripts. Poly(A)$^+$ RNA from various stages of embryonic development (first and second instar larvae, mid-pupae, adults, and adult heads and bodies) was electrophoresed and transferred to a nylon membrane. The same membrane was sequentially hybridized to probes from the *dry* gene, the *dip* gene and a ribosomal protein gene (*RP49*). Position of the 9.5 kb RNA marker on the gel has been indicated.

transcribed from the same gene, since we were unable to detect a second gene by Southern blotting (see above). In mammals, IP3 receptor transcripts are known to be alternatively spliced in a tissue specific manner (Danoff et al., 1991).

Expression of the dry *gene in embryos*

The northern analysis presented in Fig. 2 suggests that both the ryanodine and IP3 receptor genes are expressed during embryonic development. Further temporal and spatial localization of their RNA expression was addressed by in situ hybridization. Embryonic expression of the *dry* gene is first detectable in the mesodermal layer of early stage-9 embryos, at approximately 5 hours of development (Fig. 3A; m). This is earlier than predicted by the developmental northern blot (Fig. 2) and could be due to a difference in the sensitivity of the two methods. More enhanced expression is seen in late stage-9 embryos, when segmental indentations of the mesoderm, characteristic of this stage, are clearly visible (Fig. 3B; ind). At stage 10, when the mesoderm undergoes its third post-blastodermal mitosis, expression of the *dry* gene becomes restricted to the more external mesodermal cell layers, i.e. the somatopleura, which gives rise to the somatic musculature (Fig. 3C; sp). The procephalic musculature is also seen to be expressing the *dry* gene at stage 10 (Fig. 3C; pm). Thereafter, expression is maintained in each segment, in progenitors of the cephalic and somatic muscles (Fig. 3D,E; pm, sm), where it is localized during the final stages of embryonic development (stage 16; Fig. 3F; sm, cm). The resolu-

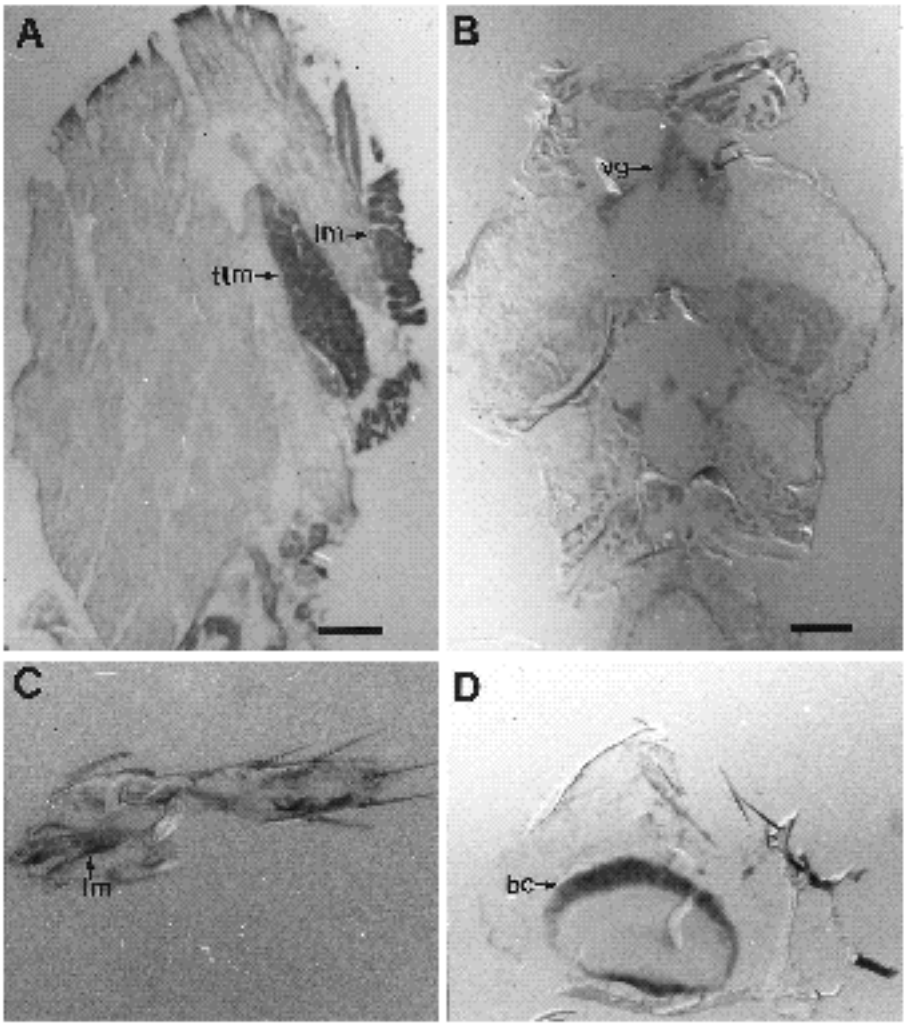

Fig. 5. Adult expression of *dry* in sections of the thorax, legs and head. Sections were hybridized to digoxigenin-labelled probes. (A) Sagittal section at a mid-lateral position through the thorax. Anterior is to the top and the dorsal side is towards the left. Hybridization is to the tergotrochanteral muscle (ttm) and some of the leg muscles (lm). (B) Horizontal section through the thorax including the ventral ganglion. Hybridization to the cortical regions of the ventral ganglion (vg) has been indicated. Anterior is towards the top. (C) Sagittal section through a part of a leg showing hybridization to leg muscles (lm). (D) Sagittal section through a lateral part of the adult head with hybridization to the cortical region of the central brain (bc). The orientation is the same as in Fig. 5A. Scale bars, 80 μm.

tion of the staining method prevents us from defining precisely which somatic/cephalic muscles (and their progenitors) are positive for *dry* expression, although the extent of the expression suggests that *dry* mRNA is present in all the somatic muscles. The pattern of expression is striking in that the RNA appears in the mesoderm prior to the start of muscle differentiation and is subsequently localized to progenitors of the somatic muscles and to the muscles themselves. There is also a notable lack of detectable expression in the visceral muscles (Fig. 3E; vm).

Embryonic expression of the dip *gene*

As seen by northern analysis (Fig. 2), *dip* transcripts are present in 0- to 6-hour early embryos. However, we were unable to detect this expression by in situ hybridization, possibly because it is low and may not be localized. The first distinct hybridization is seen at approximately 10.5 hours (in early stage-13 embryos) as a segmental pattern in cells of the lateral epidermis lying along the intersegmental furrows and in posterior epidermal cells (data not shown). In late stage-13 embryos, this expression intensifies and extends to the head region within cells of the gnathal buds, which lie ventral to the stomodeal opening (Fig. 4A; gb). The spatiotemporal appearance of these cells in the head region suggests that they are likely to be progenitors of anterior sense organs (Hartenstein, 1988). In stage-14 embryos, the anterior expression extends dorsally to cells in the clypeolabrum while the lateral epidermal expression decreases to barely detectable levels (Fig. 4B; cl). By stage 15, all detectable expression of the *dip* gene is localized to the anterior head region, presumably in primordia of anterior sense organs (Fig. 4C; pl, cl, gb). As development progresses (stage 16), some of these sense organs can be identified as two pairs of dorsal structures which lie along the pharynx walls (Fig. 4D,E; do) and a more lateroventral pair, which is likely to be the labial organ (Fig. 4D,E; lo). Finally, at stage 17 only the labial organ expresses this gene (Fig. 4F; lo).

Expression of dry *and* dip *in adults*

Expression of the *dry* and *dip* genes in structures of the adult head and thorax was investigated by two methods. In situ hybridization to frozen cryostat sections revealed that the *dry* gene is expressed maximally in tubular muscles of the thorax, namely, the 'jump' muscle or the tergotrochanteral muscle and the leg muscles (Fig. 5A-C; ttm, lm). In addition, expression is also seen in the cortex of the ventral ganglion and the brain (Fig. 5B,D; vg, bc). Some expression was also observed in muscles of the head and proboscis (data not shown). However, there is no dis-

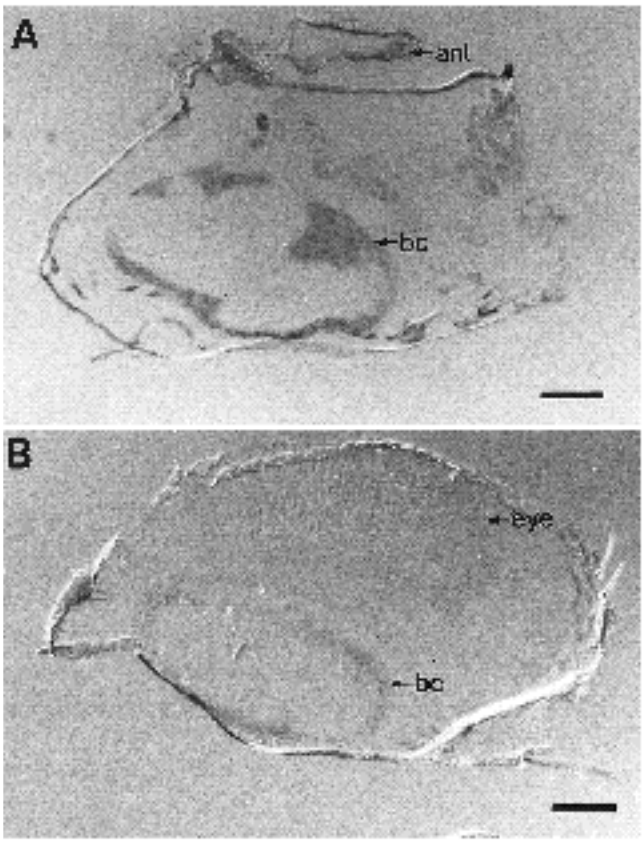

Fig. 6. Expression of the *dip* gene in sections of the adult head. Hybridization to digoxigenin-labelled probes was as described in the legend to Fig. 4. Orientation of the sections is as stated for Fig. 5A. (A) Sagittal section with hybridization to the third segment of the antenna (ant) and to the brain cortex (bc). (B) Sagittal section more lateral to that in A. The compound eye has been indicated with an arrow. Hybridization to the brain cortex (bc) is detectable. Scale bars, 60 μm.

cernible hybridization to any of the fibrillar or indirect flight muscles. These observations are significant, since the tubular and fibrillar thoracic muscles are known to differ in their ultrastructure and biochemistry of contraction (Crossley, 1978).

Expression of the *dip* gene in adults occurs in the cortex of the central brain and the antennae (Fig. 6A; bc, ant). In sections from the same animal, no hybridization was detectable to any region of the eye (Fig. 6B). This was surprising since there is genetic evidence linking IP3 with the visual transduction pathway in *Drosophila* (Bloomquist et al., 1988). We therefore used a second method, quantitative PCR (RT-QPCR), to compare the abundance of *dry* and *dip*, as well as the control gene *RP49* (encoding a *Drosophila* ribosomal protein), in a few relevant adult structures (heads, antennae, legs and eyes).

The RT-QPCR involves the addition to the PCR reaction of a known amount of synthetic RNA (the spike) that can be amplified by reverse transcriptase and PCR in the same reaction and with the same primers as the transcript of interest. The sequence between the primers is also identical to the tissue-derived transcript except for a small deletion or insertion that allows resolution of the two amplified DNA fragments by gel electrophoresis. The ratio of the two bands, when between approximately 0.2 and 5.0, estimates the amount of transcript in the RNA preparation. More importantly, the ratio of the two bands, compared between RNA derived from two tissues (i.e., the ratio of the ratios), measures the relative abundance of the transcript of interest between the two tissues (see Materials and methods). For example, the *dip* transcript is twice as abundant in RNA from heads as in RNA from legs, as the ratio of the *dip* RNA band to its spike band in RNA from heads is twice that of RNA from legs (Fig. 7A).

A summary of experiments of this nature shows that the *dip* transcript is detectable in all 4 tissues examined (Fig. 7B), suggesting that the *dip* gene is expressed in eyes, as well as in heads, legs, and antennae. The most striking quantitative feature is the four-fold difference in abundance between RNA from antennae and RNA from heads or eyes as well as an even greater eight-fold difference in abundance between antennae and legs. The data are consistent with the in situ hybridization experiments that detected intense signals in antennae. The same approach shows that the *dry* transcript is also present in eyes, heads, legs, and antennae (Fig. 7C). In this case there is an approximately four-fold difference in abundance between RNA from legs and RNA from heads, eyes, or antennae (which are all approximately the same), also consistent with the in situ hybridization experiments shown above. Antennae and legs were purified on two separate occasions and RNA from both preparations gave essentially the same results, i.e., maximal abundance for *dry* RNA in legs and for *dip* RNA in antennae.

Discussion

Sequence analysis presented here for a portion of two *Drosophila* Ca^{2+}-release channel proteins indicates a high level of conservation with the corresponding mammalian proteins. For both mammalian proteins, the C-terminal end forms the membrane spanning domain required for tetramerization and assembly into a functional Ca^{2+} channel (Mignery and Sudhof, 1990; Catterall, 1991); it seems likely that the same structural organization occurs in *Drosophila*. Based on mRNA size, the remaining non-sequenced portions of these *Drosophila* genes appear comparable to the corresponding regions of the mammalian genes, suggesting that they are likely to encode very large cytoplasmic domains, like those shown to regulate the opening and closing of the mammalian channels (Mignery and Sudhof, 1990; Caterall, 1991). Although further sequence analysis is required to determine the extent to which the cytoplasmic domains are conserved, partial information for this region of the *dip* gene has been published and is consistent with a high level of conservation throughout most of the cytoplasmic domain (Miyawaki et al., 1991). The characterization of complete cDNA clones will be required to address the possibility of multiple *dry* or *dip* receptor proteins, generated perhaps by differential splicing or from multiple genes (see below).

The pattern of expression seen for the *dry* gene during embryonic development raises some interesting possibilities regarding the function of this gene in muscle develop-

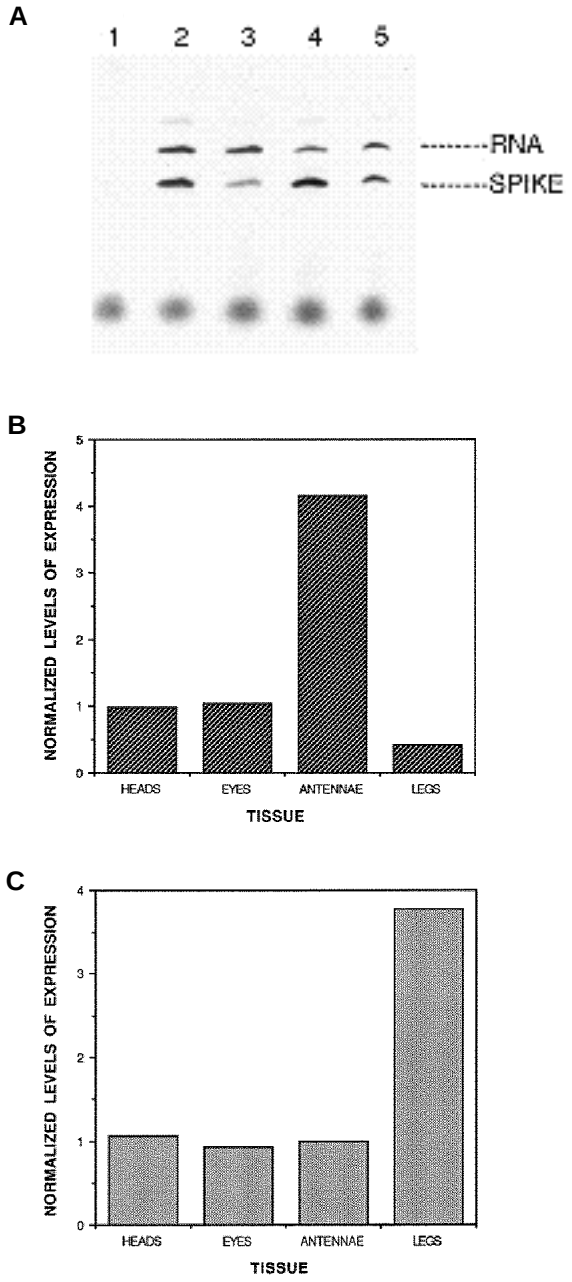

Fig. 7. RT-QPCR detection of the *dry* and *dip* transcripts in RNA from heads, eyes, antennae, and legs. (A) Total RNA from heads and legs was assayed for the *dry* transcript after the addition of the *dry* spike RNA. RNA was prepared and the reactions carried out as described in Materials and methods. Lane 1, no RNA; lane 2, leg RNA plus 100 fg (femtograms) of spike; lane 3, leg RNA plus 33 fg of spike; lane 4, head RNA plus 100 fg of spike; lane 5, head RNA plus 33 fg of spike. The *dry*-derived band and the spike-derived band are indicated. (B) Normalized levels of *dip* expression in the four tissues. (C) Normalized levels of *dry* expression in the four tissues.

ment. Its expression in the mesoderm starts well before the expression of other muscle-specific genes such as the myosin heavy chain (Michelson et al., 1990). In fact, the appearance of *dry* mRNA appears to be synchronous with that of a muscle determining gene *nau*, which is the myoD homolog in *Drosophila* (Michelson et al., 1990). One reason for this early expression may be that the Dry protein is required for mesodermal development, perhaps in cell-signaling processes that precede commitment to the somatic muscle lineage. This idea is supported by the finding that expression of the ryanodine receptor also occurs in very early differentiating muscle cells in a vertebrate tissue culture model (Airey et al., 1991). It is also of interest that *dry* expression is detectable only in somatic muscles of late-stage embryos and in tubular muscles of the adult. Both of these muscle types are rich in sarcoplasmic reticulum (SR), while the larval visceral muscles and the adult fibrillar muscles have a very scanty SR (Crossley, 1978). There is thus a correlation between muscles that detectably express *dry* and those with an extensive SR, suggesting that the Dry protein is located in *Drosophila* muscle SR and therefore functions as the excitation-contraction coupling protein of the neuromuscular junction, as in the case of vertebrate muscles (Catterall, 1991).

The correlation between the amount of SR and the level of *dry* RNA raises the possibility that *dry* is being expressed in other muscles at levels commensurate with their low levels of SR. Alternatively, as in mammals, there may be other ryanodine receptor genes in *Drosophila*, and it is possible that these are expressed differentially in physiologically different muscle types (Otsu et al., 1990). This possibility is supported by the presence of additional bands of hybridization on a low-stringency Southern blot with the *dry* probe (data not shown).

Expression of *dry* in adult neuronal tissues, observed by both in situ hybridization and the PCR method, suggests that, as in vertebrates, this channel also serves as yet poorly understood neuronal functions (McPherson et al., 1991). It should be possible to elucidate these functions in *Drosophila* by obtaining and analyzing mutants in this gene, as well as by localizing the protein within the brain.

Expression of the *Drosophila* IP3 receptor gene suggests that it functions during sensory transduction in both larvae and adults. Initial embryonic expression of *dip* in lateral and posterior epidermal cells occurs at about the same time that epidermal cells undergo mitosis and move inward to form sensory precursors. However, expression appears more epidermal than subepidermal and is also fairly widespread, indicating that it may not be restricted to or even occurring within sensory precursor cells. Since this expression is also transient, it is difficult to speculate on its role. Expression of *dip* in the embryonic head region can be followed more readily within some of the presumptive sensory precursor cells and the anterior sense organs. The functions of the three pairs of sense organs, within which it is detected at stage 16, are not well understood, though one pair of dorsal structures, seen in Fig. 4E, may be the equivalent of the ‘stemmata’ described in other fly species. The stemmata are light-sensing larval organs that have also been described for *Drosophila* (Steller et al., 1987). This expression pattern does not indicate whether the IP3 receptor is required in these cells for their development, as suggested by the transient expression of its mRNA, or for their sensory functions, or both. We have begun to address this question by looking for the presence of the Dip protein in late embryos and larvae using anti-Dip antibodies. The results to date have been inconclusive (data not shown).

Spatial expression of the *dip* gene in adult *Drosophila* has been analyzed by the usual procedure of in situ hybridization as well as by PCR with RNA isolated from dissected tissues. The latter approach was taken in part to verify that little or no expression is detectable in eyes. Contrary to expectation, the RT-QPCR assay gave rise to similar *dip* RNA levels in eyes and in heads. We cannot completely rule out the possibility that the eyes were contaminated with a small amount of brain tissue or that the PCR product was derived from genomic DNA (but see Materials and methods). It is also possible that another IP3 receptor gene exists in *Drosophila* and that eye mRNA derived from this gene gave rise to the PCR signal; as noted above, attempts to identify such a gene by low stringency Southern blots have been unsuccessful (data not shown). More likely, in our view, is the possibility that the in situ hybridization to eye tissue is relatively insensitive, perhaps because of some peculiarity of this mRNA's localization. The presence of *dip* gene expression in eyes is expected from the important role of phospholipase C in visual phototransduction in *Drosophila* (e.g., Yoshioka et al., 1985) and is also supported by our recent finding that antibodies to the Dip protein stain regions of the photoreceptor cells as well as cortical regions of the brain and antennae (data not shown).

Expression of *dip* in the adult antennae suggests that the IP3 receptor could be required during olfaction in *Drosophila*. A role for IP3 as the second messenger during olfactory sensory transduction has been suggested by various experiments in both vertebrates and invertebrates (Breer et al., 1990; Reed, 1992). Interestingly, the IP3 receptor may be involved primarily in the development and/or function of sensory structures in larvae, since its RNA is not detected in either the brain or ventral nerve cord, even at very late stages of embryonic development (Fig. 4E,F). In contrast, adults express *dip* RNA in the central brain and ventral ganglion (data not shown), as well as in sensory organs like antennae, legs and eyes. This difference in expression may reflect the greater functional complexity of the adult brain as compared to the larval brain. The use of antibodies and mutants should help resolve these issues, especially those related to olfaction.

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A recent publication (*Journal of Biological Chemistry*, **267**, 16613-16619; August 15, 1992) reports the molecular cloning and characterization of the *Drosophila* IP3 receptor gene. The two sets of results are mostly in agreement, with the exception of the localization of the gene by in situ hybridization to polytene chromosomes. As a consequence, we repeated this experiment and found that our original assignment (42B1-B2) was incorrect. The correct position (83A5-A9) is as reported in the *J. Biol. Chem.* publication.

Fig. 3. Expression of *dry* mRNA during embryonic development. Figures show the hybridization pattern of a digoxigenin-labelled *dry* cDNA probe to wild-type embryos at various stages of development. A-D are lateral views with the dorsal side uppermost. E and F are mid-dorsal and dorsal views, respectively. In all cases, anterior is to the left. (A) Early stage-9 embryo. Arrow indicates faint hybridization to the mesodermal layer (m). (B) Late stage-9 embryo. Arrow indicates segmental indentations of the mesoderm (ind). (C) Stage-10 embryo with hybridization to the procephalic mesoderm (pm) and the somatopleural layer of the mesoderm (sp). (D) Late stage-12 embryo with hybridization to segmentally located somatic muscle precursor cells (sm) and precursors of the cephalic muscles (pm). (E) Stage-14 embryo. Pattern of hybridization is similar to that in the embryo shown in D. No hybridization is detectable among cells of the visceral mesoderm (vm). (F) Stage-16 embryo in which all hybridization is localized to somatic (sm) and cephalic muscles (cm). Scale bar, 40 μm. Embryo stages have been defined according to Wieschaus and Nusslein-Volhard (1986). Further analysis of stages was according to Campos-Ortega and Hartenstein (1985).

Fig. 4. Expression of *dip* mRNA during embryonic development. Wild-type embryos were hybridized to a digoxigenin-labelled *dip* cDNA probe. A-D and F are lateral views. E shows the anterior end of the same embryo at three different levels of focus going from ventral to dorsal and left to right. Lateral orientations and the scale are as in Fig. 3. (A) Late stage-13 embryo with hybridization to the gnathal buds (gb) in the head region. Segmental expression in lateral epidermal cells (le) has been indicated in one segment. (B) Stage-14 embryo. Anterior expression extends dorsally to cells in the clypeolabrum (cl). (C) Stage-15 embryo with expression localized to the head region in the procephalic lobe (pl), clypeolabrum (cl) and gnathal buds (gb). (D) Stage-16 embryo expressing *dip* in specific differentiating anterior sense organs in the dorsal region of the head (do) and more ventrally in the labial organ (lo). (E) Horizontal view of the head region of a stage-16 embryo showing, from left to right, the labial organ (ventral focus) and two dorsal organs (mid-dorsal and dorsal foci). Arrows indicate the pair of sense organs in focus. (F) Stage-17 embryo with hybridization detectable only to the labial organ (lo).