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Insect calcium channels

Molecular cloning of an α_1-subunit from housefly (*Musca domestica*) muscle

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Abstract

The complete amino acid sequence of an invertebrate calcium channel α_1-subunit from housefly (*Musca domestica*) larvae (designated Mdlα_1) has been deduced by cDNA cloning and sequence analysis. Mdlα_1 shares higher percent sequence identity with 1,4-dihydropyridine (DHP)-sensitive L-type than with DHP-insensitive calcium channels. As shown by whole mount in situ hybridization and immunostaining Mdlα_1 is predominantly expressed in the larval body wall musculature.

Key words: Calcium channel; cDNA cloning; Hybridization (in situ); Body wall muscle; Phylogenetic tree

1. Introduction

Intracellular free Ca^{2+} acts as a second messenger for a variety of physiological processes. In electrically excitable cells Ca^{2+} entry is mediated by different types (L-, N-, P- and T-type) of voltage-activated calcium channels, which are distinguished in vertebrates by biophysical and pharmacological criteria (for a review see [1]). These channels exist as hetero-oligomeric complexes of several subunits (α_1, α_2-δ, β and γ-subunits), which have been cloned and sequenced from various tissues [2–5]. α_1-Subunits form the channel pore and are structurally homologous to the channel-forming subunits of voltage-activated sodium and potassium channels. Phylogenetic analyses of α_1-subunits from different types of vertebrate calcium channels suggest that they evolved from a common ancestor gene, which diverged into at least two main subfamilies [1]. One subfamily, classified as L-type channels according to electrophysiological criteria [6], is sensitive to calcium channel drugs, like 1,4-dihydropyridines (DHP), phenylalkylamines (PAA) and benzothiazepines (reviewed in [7]). All other calcium channel types are not DHP-sensitive but can be distinguished by toxins (for review see [8]).

Much less is known about the structure and pharmacological sensitivity of voltage-gated calcium channels in insects, which are separated from vertebrates by about 600 million years in evolution [9]. In arthropods different voltage-gated calcium channels play a key role for muscle contraction: presynaptic channels control the release of excitatory transmitter (mainly glutamate) [10,11] at the neuromuscular junction, whereas calcium channels in the plasma membrane of muscle cells mediate the influx of calcium ions for contraction and participate in the propagation of the action potential [12]. The structural characterization of invertebrate calcium channels is rudimentary [13–15] and none of their subunits have yet been cloned.

Here we report for the first time the cloning of an invertebrate calcium channel α_1-subunit from *Musca domestica* larvae (designated Mdlα_1) that is preferentially expressed in body wall muscle. The evolutionary relationship of Mdlα_1 compared to the so far cloned vertebrate calcium channels has been studied by calculating a phylogenetic tree.

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Abbreviations: DHP, dihydropyridine; DIG, digoxigenin-11-dUTP; Mdlα_1, *Musca domestica* larvae α_1-subunit; PAA, phenylalkylamines; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

2. Materials and methods

2.1 Fly culture

Musca domestica (wild type) were bred in a glass container over moistened peat at 25°C and fed with full-fat curd. For RNA isolation

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the latest larval stages were selected. For whole mount in situ hybridization and antibody staining, eggs were collected from layers of a mixture of curd, bran and yeast, which were exposed to the fly culture for 17 hours.

2.2. Cloning of Mdlα_1 cDNAs

An oligo(dT)-primed, size-selected (>0.5 kilobase pairs (kb)) cDNA library was constructed in phage λgt10 using poly(A)$^+$ RNA. Screening with a carp (*Cyprinus carpio*) skeletal muscle cDNA clone fragment (nucleotides (nts) 4123 to 4745) [16] which is highly conserved among all known calcium channel α_1-subunits, yielded 2 positive clones, λ-Mm4 (nts 3185 to 6035) and λ-Mm1 (nts 2860 to 5849). Rescreening with λ-Mm1 as a probe yielded 18 additional clones including the 6154 base pair (bp) clone λ-Mm10. A 123 bp in-frame deletion (nts 730 to 852) resulting in a loss of the IS5 transmembrane region (see Fig. 1) and 21 adjacent amino acid residues was corrected with a poly chain reaction (PCR) amplified *Eco*RV–*Nsi*I fragment (nts 621 to 1030) yielding λ-Mm10$^+$. The PCR-derived nucleotide sequence was verified by sequencing both strands of 3 individual PCR clones derived from 2 independent PCR experiments. 5′- and 3′-peripheral regions (nts 621 to 729; resp. nts 853 to 1030) of the PCR amplified fragment were identical to the corresponding sequence of λ-Mm10. Sequencing of the cDNA was carried out on both strands by the dideoxy chain-termination method [17] using standard and λ-Mm10$^+$-specific sequencing primers.

2.3. Whole mount in situ hybridization and immunostaining

The λ-Mm10$^+$ *Sac*I–*Sph*I restriction enzyme (RE) fragment (nts 122–4293) was random labeled with digoxigenin-11-dUTP (DIG) (Boehringer Mannheim protocols). A PCR-generated *Musca* actin probe (nts 61 to 1067) (numbering is according to *Drosophila* actin clone λ-DmA1 [18]) was used as a positive muscle control, while the *Eco*RI-linearized and DIG-labeled bacterial plasmid pBR328 was used as a negative control. Whole mount in situ hybridization was carried out as previously described [19] with slight modifications adapted for *Musca* embryos [20]. Immunostaining with antibody anti-MCP$_{385\ 401}$ (raised against the synthetic Mdlα_1 specific peptide MCP$_{385\ 401}$, corresponding to residues 385–401 of the Mdlα_1 sequence (see Fig. 1)) in whole mounts was according to standard protocols described for *Drosophila* [21]. The specificity of the antibody reaction was tested in parallel experiments by preblocking anti-MCP$_{385\ 401}$ with 1 μM of the corresponding peptide MCP$_{385-401}$.

3. Results and discussion

Fig. 1 shows the primary structure of the *Musca domestica* muscle calcium channel α_1-subunit (Mdlα_1) deduced from the PCR corrected 6277 base pair (bp) clone λ-Mm10$^+$ (for cloning procedures see section 2) aligned to the rabbit skeletal muscle (RSkm) [2]. The nucleotide sequence surrounding the first ATG-codon after the last in-frame nonsense codon, TGA (nucleotides −69 to −67) of the 5′-untranslated region represents a favoured sequence flanking a translation initiation codon [22] and was therefore counted as the first methionine. The Mdlα_1 protein is composed of 1687 amino acids with a calculated relative molecular mass (M_r) of 193,865. Hydropathy profile analysis [23] of the deduced amino acid sequence revealed a motif typical for the pore-forming subunits of voltage-dependent sodium and calcium channels [24] with four hydrophobic repeats (I–IV) each consisting of six putative transmembrane segments (S1–S6) (Fig. 1). Mdlα_1 contains five potential cAMP-dependent protein kinase phosphorylation sites [25] (threonine residue (Thr) 753 and serine residues (Ser) 1416, 1436, 1560, 1579) (Fig. 1) assigned to the cytoplasmic side. Like in rabbit skeletal muscle [26], one of these phosphorylation sites (Thr-753) is positioned in the intracellular loop between IIS6 and IIIS1, which is involved in skeletal muscle-type excitation–contraction coupling (ECC) [27]. All other phosphorylation sites are positioned in the carboxy-terminal tail. Only one of them, Ser-1560, is conserved in rabbit skeletal muscle [2] (see Fig. 1) and rabbit heart [28]. As expected for a calcium channel α_1-subunit, conserved glutamate residues believed to form a Ca^{2+} binding site (selectivity filter), [29] were found in the putative pore-forming regions in all four domains (Glu-285, Glu-617, Glu-1005, Glu-1311).

All 20 positive clones isolated from the *Musca domestica* larvae library encoded the same Mdlα_1-subunit as revealed by restriction enzyme mapping and partial sequencing (not shown). As shown in Fig. 2 blot hybridization analysis of *Musca domestica* larvae mRNA using different cDNA fragments (covering the entire α_1-subunit clone λ-Mm10$^+$) as specific probes allowed the detection of the Mdlα_1 mRNA as a single hybridization signal at 7.0 ± 0.3 kb (means ± S.D., n = 3) under low stringency conditions. This 7.0 kb transcript was also found at lower densities in mRNA isolated from adult *Musca domestica* heads (not shown). Therefore we conclude that homologous transcripts of different size are either absent or occur at much lower abundance.

In addition to the 7.0 kb transcript a highly abundant 1.8 kb mRNA transcript was identified in mRNA from larvae and adult heads. It only hybridized with a cDNA fragment encoding the carboxy-terminal 254 amino acids and a fragment corresponding to the 3′-untranslated region (see Fig. 2). This mRNA species must therefore carry high homology to the 3′-end of clone λ-Mm10$^+$ comprising a portion of the open reading frame (ORF) (carboxy-terminal tail) and the noncoding region. No attempts were made to further characterize this 1.8 kb transcript.

Fig. 3 shows an immunoblot analysis of *Musca domestica* larvae membranes with the the affinity purified sequence-directed antibody anti-MCP$_{385-401}$. The antibody is directed against residues 385–401 (see Fig. 1) in the intracellular loop between domains I and II. As this region is highly variable between the known vertebrate α_1-sequences this antibody should be Mdlα_1-selective. Anti-MCP$_{385-401}$ specifically recognized a 176 ± 7 kDa (means ± S.D., n = 8) polypeptide which corresponds to the full length or at least to a major portion of the Mdlα_1 polypeptide (calculated molecular mass = 194 kDa).

The tissue specific expression of the Mdlα_1 transcript was investigated by whole mount in situ hybridization and whole mount immunohistochemistry carried out in fully differentiated embryos at a late developmental stage (14–17 hours of age). Fig. 4 (panel a and b) shows the specific staining of Mdlα_1 and actin mRNA revealing a typical segmental organization. Stripes that laterally line the body segments coincide with the segmental distribu-

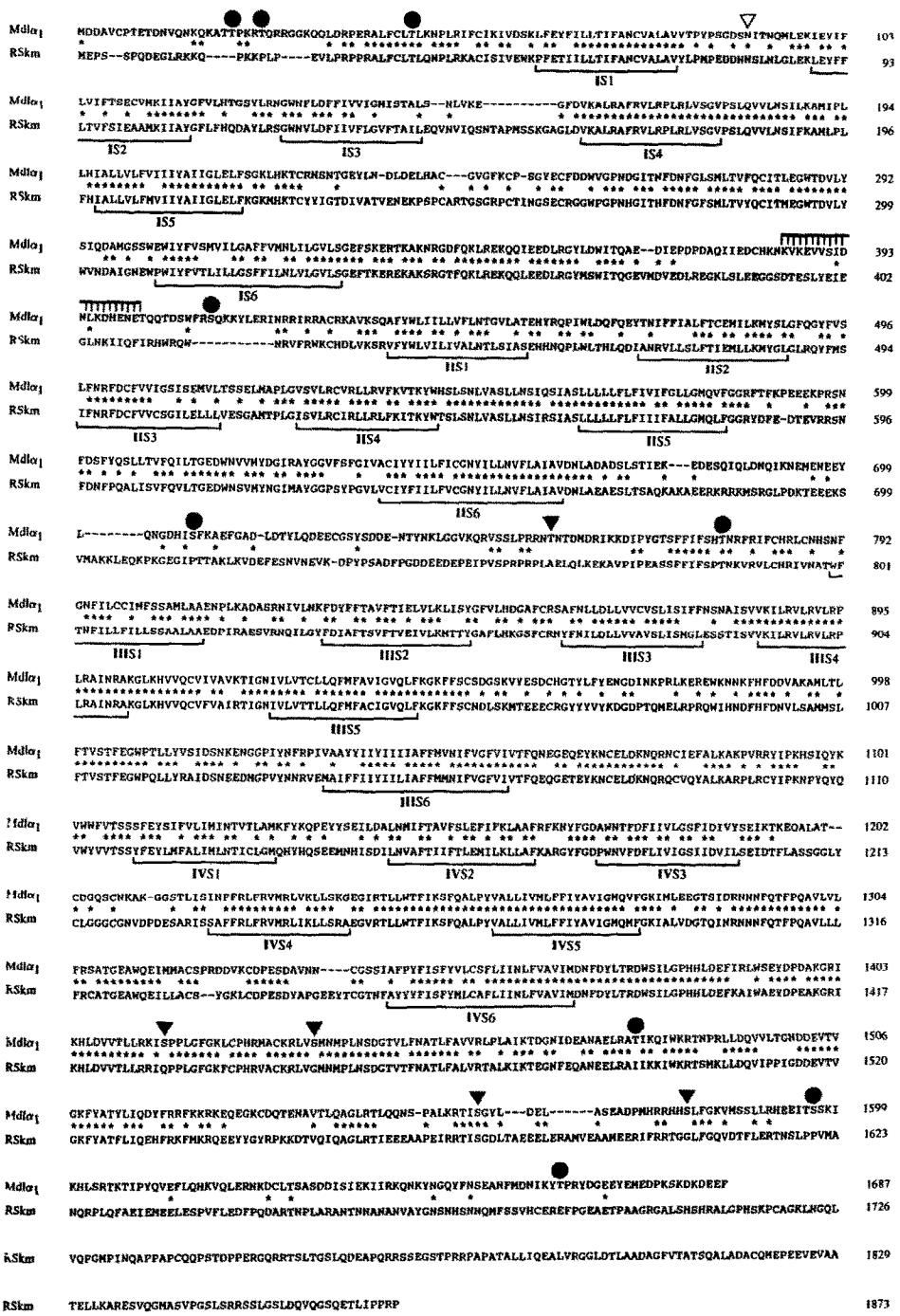

Fig. 1. Alignment of the amino acid sequences of calcium channel α_1-subunits from *Musca domestica* (Mdlα_1) and rabbit skeletal muscle (RSkm) [2] using the single letter code. Among vertebrate calcium channels RSkm has the highest overall amino acid sequence identity (51%) to Mdlα_1 (see Fig. 5A). Numbers of amino acid residues, starting with the initiating methionine, are given on the right hand side of each line. Asterisks indicate identical amino acid positions. Predicted transmembrane segments S1 to S6 in each of the four repeats I to IV are marked. Consensus sites for potential N-glycosylation (open arrowhead), cAMP-dependent phosphorylation (filled arrowheads) and protein kinase C phosphorylation (filled circles) [35] are indicated. The Mdlα_1 specific antibody anti-MCP$_{385-401}$ was raised against a synthetic peptide corresponding to residues 385–401.

tion of the late embryonic and larval somatic musculature [30]. This staining pattern resembles that described for other mRNAs preferentially expressed in the somatic musculature (like e.g. the glutamate receptor in *Drosophila* larvae, [11]). This pattern was never observed in control experiments using ultrabithorax (Ubx) cDNA, which gave the expected neuronal-type staining pattern (not shown) as previously described for *Musca domestica* embryos [20]. Only weak, diffuse background staining was observed when *Eco*RI-linearized plasmid pBR328

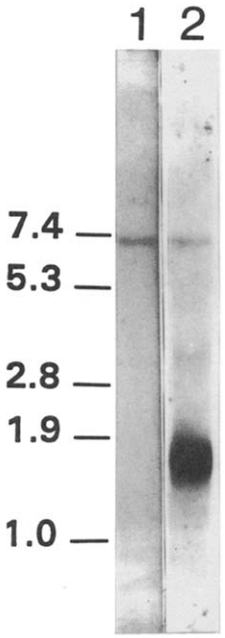

Fig. 2. Blot hybridization analysis of *Musca domestica* larvae mRNA. Blotted poly(A)$^+$ RNAs (4 μg per lane) were probed with digoxigenin-11-dUTP (DIG) labeled λ-Mm10$^+$ cDNA restriction (RE) fragments following a random priming procedure (Boehringer Mannheim). After hybridization (42°C; formamide concentration 30% (v/v)) blots were washed at low stringency (0.2 × SSC/0.1% (w/v) sodium dodecyl sulfate (SDS) at room temperature) and bands were detected according to the DIG-protocols (Boehringer Mannheim). A RNA ladder (Pharmacia) was used as a size marker (in kilobases). DIG-labeled probes were nts 1879–3641 (lane 1) or nts 4294–5057 (lane 2). Like fragment nts 1879–3641 the fragments nts −186–121, nts 122–1283, nts 1284–1878 and nts 3642–4293 only recognized the 7.0 kb band. Probes from fragments nts 4294–5057 as well as nts 5058–6091 (not shown) hybridized with an additional 1.8 kb band (lane 2).

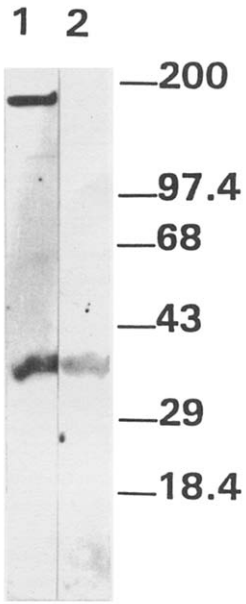

Fig. 3. Immunoblot analysis of *Musca domestica* larvae membranes. Membrane preparation was carried out in a slightly modified way according to [36]. Mdlα_1 was immunostained with the affinity purified sequence-directed antibody anti-MCP$_{385\ 401}$ (lane 1). Blot processing was performed as described elsewhere [16]. Non specific staining (lane 2) was determined by pre-blocking affinity purified anti-MCP$_{385\ 401}$ with 300 nM of the antigenic peptide. Peptide synthesis [37], coupling to bovine serum albumin, antibody generation and affinity purification of antisera were carried out as described [38,39].

was used as the labeled probe (not shown). Specific staining was absent in the anterior region of the embryo, where the cerebral/suboesophageal ganglion is located and in the ventral longitudinal axis along the ventral nerve cord. To confirm the presence of the Mdlα_1 polypeptide in the somatic musculature we carried out whole mount immunostaining using the sequence-directed antibody anti-MCP$_{385-401}$. As shown in Fig. 4c the distribution of immunoreactivity was undistinguishable from the in situ hybridization pattern. The segmental staining of the body wall muscles was completely absent after preblocking of the antibody with 1 μM of the synthetic peptide MCP$_{385-401}$ (not shown).

The abundance of Mdlα_1 in adult *Musca domestica* was studied by using semiquantitative PCR [31]. The highest expression was found in thorax, less in heads and much less in abdomen (not shown). Obviously, the preferential expression of Mdlα_1 mRNA in the thoracal segments is also due to the large mass of flight muscles in this preparation. This is in accord with observations in *Drosophila melanogaster* where calcium channel currents of adult and larval muscle cells showed identical electro-

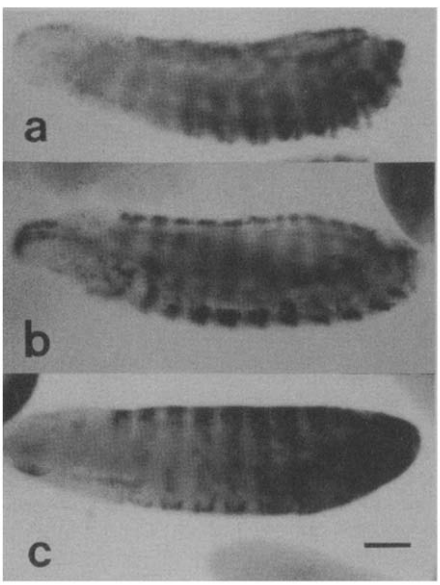

Fig. 4. Whole-mount in situ hybridization and immunostaining of *Musca domestica* embryos at a late, fully differentiated stage. The orientation is anterior-left and dorsal-up. Whole mount in situ hybridization (a,b) was carried out using DIG-labeled double stranded cDNA coding for actin (see section 2) as a positive muscle probe (a) or a fragment of clone λ-Mm10$^+$ (nts 122–4293) as a specific probe for Mdlα_1 transcription (b). Immunostaining of Mdlα_1 with affinity purified anti-peptide antibody anti-MCP$_{385-401}$ is shown in (c). Bar = 100 μm.

A

	Rb(skm){CaCh1/L}	Cp(skm)	Rb(c){CaCh2/L}	Hum(n){CaCh3/L}	Rb(n){CaCh4/P}	Hum(n){CaCh5/N}	Rat(n){CaCh6/T}	Mdl(m)
Rb(skm){CaCh1/L}	**	74	73	73	46	47	46	63
Cp(skm)	65	**	67	66	44	45	44	58
Rb(c){CaCh2/L}	56	51	**	82	47	49	47	65
Hum(n){CaCh3/L}	55	50	64	**	48	48	46	64
Rb(n){CaCh4/P}	31	30	31	31	**	78	75	47
Hum(n){CaCh5/N}	34	32	34	33	56	**	73	48
Rat(n){CaCh6/T}	34	32	32	32	50	53	**	47
Mdl(m)	51	48	47	47	29	32	33	**

B

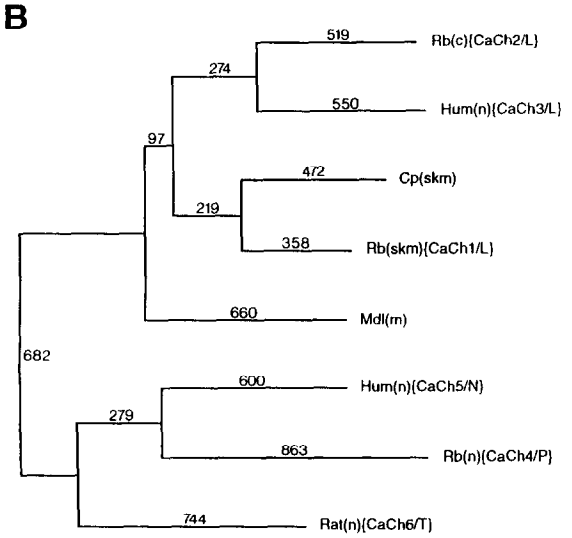

Fig. 5. Similarity matrix and phylogenetic tree comparing Mdlα_1 and α_1-subunits of representative calcium channel subfamilies of vertebrates. Channels are designed as follows: name of the species (Rb, rabbit; Cp, carp; Rat, rat; Hum, human), followed by the organ where expressed (skm, skeletal muscle; c, cardiac; n, neuronal; m, body wall muscle). The nomenclature (CaCh1–CaCh6) is according to [40]. Letters L,P,N and T are indicating the different channel subtypes. Rb(skm){CaCh1/L}, [2]; Cp(skm), [16]; Rb(c){CaCh2/L}, [28] (= RbC); Hum(n){CaCh3/L}, [41] (= α_{1D}); Rb(n){CaCh4/P}, [42] (= B-I); Hum(n){CaCh5/N}, [43] (= α_{1B}); Rat(n){CaCh6/T}, [44] (= rbE-II). (A) Percentages of amino acid identity of calcium channel pairs as determined by the MULTALIN program (version 4; Cherwell scientific). Each gap is counted as the 21st amino acid. Lower left corner of the diagonal: overall amino acid identity. Upper right corner: percentage identity within the totality of two conserved regions (positions 39–678 and 764–1563 (Mdlα_1 counting)). (B) Single most parsimonious tree obtained with PAUP (Version 3.0rβ [33]), calculated from the aligned sequences according to panel A (lower left corner). PAUP options: Exhaustive search, midpoint rooting, random addition of taxa, 100 replications. Tree length, 6220; consistency index, 0.93 [45]; consistency index excluding uninformative characters, 0.89; rescaled consistency index 0.74 [46]. Branch lengths are listed above the branches. Bootstrap analysis was performed (100 replications) [34] resulting in bootstrap scores of 100% in all branches.

physiological properties [32]. Taken together these data suggest that the cloned cDNA encodes a calcium channel α_1-subunit, that is predominantly expressed in *Musca domestica* muscle where it could represent the pore-forming subunit of a muscle calcium channel participating in ECC.

Sequence comparision (one gap counted as one amino acid replacement) of Mdlα_1 with other calcium channel α_1-subunits (Fig. 5A) revealed a higher sequence identity to L-type calcium channels (47% to 51%) compared to the N- and putative P- and T-types (29% to 33%). To examine the evolutionary relationships between Mdlα_1 and representatives of different types of vertebrate calcium channels a phylogenetic tree was constructed using parsimony analysis [33] and bootstrap procedure [34] as depicted in Fig. 5B. This analysis indicates that Mdlα_1 is grouped to the 1,4-dihydropyridine (DHP)-sensitive L-type subfamily forming the most ancestral split within. The second structural subfamily is represented in a major branch, formed by the DHP-insensitive N-, P- and T-type calcium channel α_1-subunits.

Using the whole-cell patch clamp technic we identified slow inward calcium currents in embryonic *Musca domestica* muscle cells from primary cultures (Hering et al., manuscript in preparation). Current kinetics resembled L-type calcium currents in mammalian smooth and heart muscle but were insensitive to micromolar concentrations of the potent phenylalkylamine (PAA) calcium channel blocker (±)-desmethoxyverapamil as well as the DHP channel activator (−)-BayK8644 [6]. To test the electrophysiological and pharmacological properties of Mdlα_1 its heterologous expression in *Xenopus* oocytes will be required. Despite considerable effort (including extensive modifications of the 5′- and 3′-regions of clone λ-Mm10$^+$) the functional expression of Mdlα_1 has so far not been successful in our laboratory. Further efforts will be necessary including the coexpression of a *Musca domestica* β-subunit (Grabner et al., manuscript in preparation) in mammalian cell lines.

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References

[1] Tsien, R.W., Ellinor, P.T. and Horne, W.A. (1991) Trends Pharmacol. Sci. 12, 349–354.
[2] Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. and Numa, S. (1987) Nature 328, 313–318.
[3] Ellis, S.B., Williams, M.E., Ways, N.R., Brenner, R., Sharp, A.H., Leung, A.T., Campbell, K.P., McKenna, E., Koch, W.J., Hui, A., Schwartz, A. and Harpold, M.M. (1988) Science 241, 1661–1664.

[4] Ruth, P., Roehrkasten, A., Biel, M., Bosse, E., Regulla, S., Meyer, H.E., Flockerzi, V. and Hofmann, F. (1989) Science 245, 1115–1118.
[5] Jay, S.D., Ellis, S.B., McCue, A.F., Williams, M.E., Vedvick, T.S., Harpold, M.M. and Campbell, K.P. (1990) Science 248, 490–492
[6] Hess, P. (1990) Annu. Rev. Neurosci. 13, 337–356.
[7] Glossmann, H. and Striessnig, J. (1990) Rev. Physiol. Biochem. Pharmacol. 114, 1–105.
[8] Striessnig, J. and Glossmann, H. (1992) Handb. Exp. Pharmacol. 102, 775–805.
[9] Whittington, H.B. and Morris, S.C. (1985) Phil. Trans. Royal Soc. 311, 1–92.
[10] Washio, H.M. and Inouye, S.T. (1978) J. Exp. Biol. 75, 101–112.
[11] Schuster, Ch., Ultsch, A., Schloss, P., Cox, J.A., Schmitt, B. and Betz, H. (1991) Science 254, 112–114.
[12] Ashcroft, F.M. and Stanfield, P.R. (1982) J. Physiol. 323, 93–115.
[13] Pauron, D., Qar, J., Barhanin, J., Fournier, D., Cuany, A., Pralavorio, M., Berge, J-B. and Lazdunski, M. (1987) Biochemistry 26, 6311–6315.
[14] Greenberg, R.M., Striessnig, J., Koza, A., Devay, P., Glossmann, H. and Hall, L.M. (1989) Insect Biochem. 19, 309–322.
[15] Pelzer, S., Barhanin, J., Pauron, D., Trautwein, W., Lazdunski, M. and Pelzer, D. (1989) EMBO J. 8, 2365–2371.
[16] Grabner, M., Friedrich, K., Knaus, H.G., Striessnig, J., Scheffauer, F., Staudinger, R., Koch, W.J., Schwartz, A and Glossmann, H. (1991) Proc. Natl. Acad. Sci. USA 88, 727–731.
[17] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[18] Fyrberg, E.A., Bond, B.J., Hershey, N.D., Mixter, K.S., and Davidson, N. (1981) Cell 24, 107–116.
[19] Tautz, D. and Pfeifle, C. (1989) Chromosoma 98, 81–85.
[20] Sommer, R. and Tautz, D. (1991) Development 113, 419–430.
[21] McDonald, P.M and Struhl, G. (1986) Nature 324, 537–545.
[22] Kozak, M. (1989) J. Cell. Biol. 108, 229–241.
[23] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[24] Durell, S.R. and Guy, H.R. (1992) Biophys. J. 62, 238–250.
[25] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[26] Roehrkasten, A., Meyer, H.E., Nastainczyk, W., Sieber, M. and Hofmann, F. (1988) J. Biol. Chem. 263, 15325–15329.
[27] Tanabe, T., Beam, K.G., Adams, B.A., Niidome, T. and Numa, S. (1990) Nature 346, 567–569.
[28] Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S. and Numa, S. (1989) Nature 340, 230–233.
[29] Heinemann, S.H., Terlau, H., Stühmer, W., Imoto, K. and Numa, S. (1992) Nature 356, 441–443.
[30] Campos-Ortega, J.A. and Hartenstein, V. (1985) The Embryonic Development of *Drosophila melanogaster*, Springer Verlag, Berlin.
[31] Bremer, S., Hoof, T., Wilke, M., Busche, R., Scholte, B., Riordan, J.R., Maass, G. and Tümmler, B. (1992) Eur. J. Biochem. 206, 137–149.
[32] Gho, M. and Mallart, A. (1986) Pflügers Arch. 407, 526–533.
[33] Swofford, D.L. (1992) Phylogenetic Analysis Using Parsimony (Paup), Version 3.0s. Illinois Natural History Survey, Champaign.
[34] Felsenstein, J. (1985) Evolution 39, 783–791.
[35] Kishimoto, A., Nishiyama, K., Nakanishi, H., Uratsuji, Y., Nomura, H., Takeyama, Y. and Nishizuka, Y. (1985) J. Biol. Chem. 260, 12492–12499.
[36] Striessnig, J. and Glossmann, H. (1991) Methods Neurosci. 4, 210–229.
[37] Knaus, H.G., Scheffauer, F., Romanin, C., Schindler, H.G. and Glossmann, H. (1990) J. Biol. Chem. 265, 11156–11166.
[38] De Jongh, K.S., Merrick, D.K. and Catterall, W.A. (1989) Proc Natl. Acad. Sci. USA 86, 8585–8589.
[39] Striessnig, J., Glossmann, H. and Catterall, W.A. (1990) Proc. Natl. Acad. Sci. USA 87, 9108–9112.
[40] Perez-Reyes, E., Wei, X., Castellano, A. and Birnbaumer, L. (1990) J. Biol. Chem. 265, 20430–20436.
[41] Williams, M.E., Feldman, D.H., McCue, A.F., Brenner, F., Velicelebi, G., Ellis, S.B. and Harpold, M.M. (1992) Neuron 8, 71–84.
[42] Mori, Y., Friedrich, T., Kim, M-S., Mikami, A., Nakai, J., Ruth, P., Bosse, E., Hofmann, F., Flockerzi, V., Furuichi, T., Mikoshiba, K., Imoto, K., Tanabe, T and Numa, S. (1991) Nature 350, 398–402.
[43] Williams, M.E., Brust, P.F., Feldman, D.H., Saraswathi, P., Simerson, S., Maroufi, A., McCue, A.F., Velicelebi, G., Ellis, S.B. and Harpold, M.M. (1992) Science 257, 389–395.
[44] Soong, T.W., Stea, A., Hodson, C.D., Dubel, S.J., Vincent, S R. and Snuch, T.P. (1993) Science 260, 1133–1136.
[45] Kluge, A.G. and Farris, J.S. (1969) Syst. Zool. 18, 1–32.
[46] Farris, J.S. (1989) Cladistics 5, 417–419.