The Mammalian Degenerin MDEG, an Amiloride-sensitive Cation Channel Activated by Mutations Causing Neurodegeneration in Caenorhabditis elegans*

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MATERIALS AND METHODS

Cloning of the cDNAs—A fragment of the expressed sequence tag (GenBank™ accession Z45660) was amplified by PCR and used to screen a rat brain cDNA library (Stratagene). A clone of 2.6 kilobases was sequenced on both strands. The human cDNA was obtained by PCR with Pwo Polymerase (Boehringer) on human brain cDNA using a primer positioned at base 56 of the rat clone (CAGCTCTCAGGAT-ACT) and a degenerate primer flanking the stop codon (TCARCANG-DATYTCYTNCAG) and sequenced on both strands. For both species, an open reading frame of 1536 bases was preceded by stop codons in all three frames.

Primary Cultures of Neurons and Glial Cells—Primary cultures of rat hippocampal neurons (embryonic day 17–18, 5 days in culture) and of hippocampal astrocytes (3-4-day-old rats, 3 weeks in culture) were prepared as described (18). Glial cells from adult rat brains were prepared and cultured as described (19).

RNA Isolation and Northern Blots—Human multitissue Northern blots containing about 2 μg of poly(A)+ RNA per lane (normalized for identical β-actin expression) were purchased from Clontech. For the blots with RNAs from rat, total RNA was isolated as described (20), 10 μg (Fig. 2C) or 20 μg (Fig. 2B) of RNA in each lane were separated on 1% agarose/formaldehyde gels and transferred onto nylon membranes. The probes were random prime labeled and corresponded to bases 1 to 1308 for the human probe and to bases 217 to 1363 for the rat probe (positions refer to the nucleic acid sequences submitted to GenBank™). The blots were hybridized overnight at 65°C in 5× SSC, 10 μg·ml−1 Denhardt’s solution, 0.1% SDS, 100 μg·ml−1 herring sperm DNA, washed with 0.1× SSC, 0.1% SDS at 70°C and subsequently exposed to Kodak X-Omat AR film for 3 to 5 days at –70°C. The sizes of the mRNAs were calculated relative to the markers on the commercial blot and relative to the mobility of the ribosomal RNAs for the rat mRNAs. For the blot with rat RNA, hybridization with a glyceroldehyde-3-phosphate dehydrogenase probe gave similar signals for all lanes of each panel in Fig. 2.

Construction of Expression Vectors and Mutagenesis—Noncoding sequences were removed by PCR amplification with the primers AGAAT-TCCGCGCGCACCAGT and ATCTGAGTCAAGGCAATCCT, and the EcoRl/Xhol-digested PCR product was subduced into the pBSK-SP6-Globin vector (15). Mutants of the rat clone were prepared as described (21) and sequenced. For expression in mammalian cells, the cDNAs were excised from the pBSK-SP6-Globin vector with EcoRl/Xhol and subcloned into the EcoRl/Sall-digested PCI expression vector (Promega).

Expression and Electrophysiological Analysis—For expression in Xenopus oocytes, cRNA was synthesized from the NotI-digested vector using a kit from Stratagene. Xenopus oocytes were injected with 5 pg of cRNA and used 1–3 days after injection. For mutants that were inactive at this concentration, 5 ng of cRNA were injected to confirm their inactivity. HEK293 cells were transfected with the MDEG-PCI constructs using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s protocol and used for electrophysiology after 8–20 h. Xenopus oocytes and HEK293 cells were maintained in the presence of 100 μM amiloride prior to electrophysiological analysis. Oocyte injection, microelectrode voltage clamp, and patch clamp recordings were essentially carried out as described (22, 23). The bath solution for outside-out patches and the pipettes for cell-attached patches contained 140 mM NaCl (or LiCl), 1 mM MgCl2, 1 mM CaCl2, 10 mM Hepes (pH 7.4). For outside-out patch and whole-cell recordings, pipettes contained 140 mM KCl, 2 mM MgCl2, 5 mM EGTA, 10 mM Hepes (pH 7.4). Data were filtered at 100 Hz and analyzed using Biopatch software (Biologic). All expression studies were performed with the clone from rat.

Computer Analysis—The MACAW program (NCBI) was used for the multiple sequence alignment and the schematic presentation of homologies. The phylogenetic tree was calculated with the GCG software (Genetic Computer Group, University of Wisconsin) using Kimura correction for multiple substitutions and the UPGMA option. The Blast network server (NCBI) was used for all data base searches.

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1 The abbreviations used are: PCR, polymerase chain reaction; αNaCh, βNaCh, γNaCh, δNaCh, amiloride-sensitive Na+ channel α, β, γ, and δ subunits, respectively.
RESULTS AND DISCUSSION

To identify possible mammalian degenerins, we compared the sequences of deg-1, mec-4, and mec-10 with the EST (expressed sequence tags) data base and found one matching sequence from brain. We used this partial sequence to clone the mammalian degenerin homologue (MDEG) from human and rat brain. The cDNAs from both species code for proteins of 512 amino acids (Fig. 1) that have all the hallmarks of the amiloride-sensitive Na\(^+\) channel/degenerin family. Two hydrophobic regions flank cysteine-rich domains that were shown to be extracellular for the epithelial Na\(^+\) channel (24). The homology with the other members of this ion channel family is rather low (20–29% identity). Despite the evolutionary distance between the species, phylogenetic analysis places MDEG closer to the degenerins of \(C.\) \(elegans\) and to a recently cloned molluscan amiloride-sensitive FRMF-amide-gated neuronal Na\(^+\) channel (25), than to known mammalian Na\(^+\) channel subunits (Fig. 1C).

The MDEG mRNAs of 4.2 and 2.9 kilobases are abundant in brain but were not detectable in any of the other tissues examined (Fig. 2A). MDEG appears to be specific for neurons. It is well expressed in hippocampal neurons and absent in glial cells (Fig. 2B). The mRNA appears just before birth, reaches maximal levels after birth, then declines slightly until adulthood (Fig. 2C).

MDEG did not induce detectable channel activity after expression in \(Xenopus\) oocytes or \(HEK293\) cells. However, MDEG is activated by the same mutations that cause gain of function in the \(C.\) \(elegans\) degenerins and neurodegeneration (5–8).

Replacement of Gly430 (marked with a skull and crossbones in Fig. 1A) by amino acids bulkier than Ser activated the MDEG channel. Remarkably, the cutoff for activation was identical with that reported for mec-4 (6) (Table I). All gain of function mutants discriminated poorly between Na\(^+\), K\(^+\), and Li\(^+\) (\(P_{Na}\)/\(P_{K}\) = 2.8 to 5.6, \(P_{Na}/P_{Li}\) = 2.5 to 5.5, Table II, Fig. 3C) and were inhibited by relatively high concentrations (\(K_{0.5} = 1.6–16\ \mu M\)) of amiloride and derivatives (Table II, Fig. 3B) in a voltage-dependent manner (for MDEG G430V, \(K_{0.5}\) for amiloride = 65 \(\mu M\) at −40 mV and 0.4 \(\mu M\) at −100 mV). The gain of function
mutants was not permeable for Ca\(^{2+}\), since no amiloride-sensitive Ca\(^{2+}\) current could be detected on Xenopus oocyte outside-out patches with 110 mM Ca\(^{2+}\) in the bath solution. The open probability was voltage-dependent and decreased with hyperpolarization (Fig. 3G). The voltage dependence was not due to a block of the channel by extracellular Ca\(^{2+}\) or Mg\(^{2+}\), since it was also found in the absence of those ions. MDEG channel activity was not restricted to Xenopus oocytes. HEK293 fibroblasts transfected with MDEG G430F expressed a channel identical with that found after expression in oocytes (Fig. 3D).

It seems unlikely that amino acid 430 lines the ionic pore, because the channel pore properties (selectivity, conductivity) were not altered much by the introduction of a positive charge (Lys) in this position (Table II). The activation of MDEG by bulky amino acids is probably due to steric hindrance. In the model presented in Fig. 4B, the MDEG sequence flanking Gly\(^{430}\) would be part of an inhibitory domain and channel opening would be caused either by steric constraints (for the gain of function mutants) or by activation by as yet unidentified mechanisms (for the wild type channel).

The MDEG channel is inhibited by mutations that inactivate the C. elegans degenerin deg-1 (26) and mec-4 (7). Replacement of the conserved Ser\(^{443}\) by Phe in MDEG G430F results in a completely inactive channel. No amiloride-sensitive current could be detected in oocytes injected with 5 ng of MDEG G430F/S443F cRNA (n = 4, not shown).

Constitutively active MDEG kills oocytes and mammalian cells. Xenopus oocytes injected with either gain-of-function MDEG mutant start to mature and die (not shown). HEK293 cells transfected with MDEG G430F swell and die (Fig. 4), a mode of cell death also reported for the degenerin-induced neurodegeneration in C. elegans (5, 6).

Human and rat MDEG differ only in five amino acids, suggesting a high evolutionary pressure and an important role in neuronal function. The phylogenetic neighbors and the structure of MDEG (Fig. 1) provide some indications about the possible physiological role of this ion channel. The degenerins mec-4 and mec-10 are required for mechanotransduction (6, 8), and it has been suggested that they could be part of a mechanosensitive channel (7–9, 26, 27). In contrast, the degenerin deg-1 is not involved in mechanotransduction (5). MDEG is expressed in hippocampal neurons where no Na\(^{+}\)-permeable mechanosensitive ion channel has been reported yet. We also failed to detect any activation of MDEG by stretch. We favor the hypothesis that MDEG is a ligand-gated channel because: (i) the closest homologue of MDEG is the FMRF-amide-gated channel from Helix (25), (ii) most of the MDEG channel protein is located extracellularly in the currently accepted structural model for this type of proteins (24), suggesting regulation by extracellular signals, (iii) a similar topology has also been proposed for another ligand-gated ion channel, the ionotropic purinergic receptor P2x (28).

### Table I

Activity of MDEG Gly\(^{430}\) mutants and neurodegeneration caused by the corresponding mec-4 Ala\(^{442}\) mutants in C. elegans.

| Amino acid | MDEG activity | Neurodegeneration in C. elegans with mec-4 |
|------------|---------------|------------------------------------------|
| Gly        | −             | −                                        |
| Ser        | +/−           | +/−                                      |
| Cys        | +/−           | +/−                                      |
| Thr        | +             | +                                        |
| Val        | +             | +                                        |
| Phe        | +             | +                                        |
| Lys        | +             | +                                        |

Data for mec-4 are from Ref. 6. For MDEG and MDEG mutants, the whole-cell amiloride (100 μM)-sensitive current was recorded in Xenopus oocytes injected with cRNA. The signs indicate: (+), activity of MDEG, neurodegeneration in C. elegans with the corresponding mec-4 mutants; (+/−), partial activity of MDEG (<2% of the activity obtained with mutants labeled with (+)), abnormal touch sensitivity of C. elegans; (−), inactivity of MDEG, no neurodegeneration with mec-4.

### Table II

Electrophysiological properties and pharmacology of MDEG Gly\(^{430}\) mutants expressed in Xenopus oocytes.

Selectivity ratios were measured on multichannel outside-out patches in bi-ionic conditions. Single-channel conductances were from cell-attached patch mean i-V relationships measured between −100 mV and 0 mV with 140 mM Na\(^{+}\) or Li\(^{+}\) in the pipette solution. K\(_{0.5}\) values for amiloride and benzamil were calculated from dose-response curves like that shown in Fig. 3B.

| Amino acid | Selectivity ± S.E. (n) | Conductance K\(_{0.5}\) at −70 mV |
|------------|-------------------------|-------------------------------|
|            | Na\(^{+}\)/K\(^{+}\) | Li\(^{+}\)/K\(^{+}\) | Na\(^{+}\) | Li\(^{+}\) | Amiloride | Benzamil |
| Cys        | 4.4 ± 0.7 (6)          | 4.5 ± 0.7 (7)               | 1.3 | 1.1 |
| Thr        | 5.6 ± 0.4 (10)         | 5.5 ± 0.3 (10)              | 1.7 | 1.1 |
| Val        | 4.8 ± 0.4 (21)         | 4.9 ± 0.4 (11)              | 3.5 | 4.4 |
| Phe        | 3.0 ± 0.4 (11)         | 4.0 ± 0.4 (8)               | 1.7 | 1.1 |
| Lys        | 2.8 ± 0.1 (8)          | 2.5 ± 0.1 (5)               | 1.7 | 1.1 |
Caused either by mutation of the channel, as shown here and for the ced-9/bcl2 (32) connection. Preventing MDEG
of cation channels (5–8, 26) failed to activate MDEG, but other neuropeptides or neurotransmitters might be the
physiological activators of this novel neuronal ion channel.

So far, C. elegans has proved a valuable animal model for
studying neuronal development and neuronal death. Pathways
controlling programmed cell death in C. elegans have their
counterpart in vertebrates (e.g. the ced-3/ICE (31) and the
ced-9/bcl2 (32) connection). On one hand, gain of function of
the putative degenerin channels causes degenerative death of
neurons in C. elegans, and, on the other hand, excessive activation
of cation channels (e.g. the glutamate-gated channels) is in-
volved in human neurodegeneration (33). Preventing MDEG
in mammals and the degenerins in
involved in human neurodegeneration (33). Preventing MDEG in
involved in human neurodegeneration (33).

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