THE TRANSPORTER-LIKE PROTEIN INEBRIATED MEDIATES HYPEROSMOTIC STIMULI THROUGH INTRACELLULAR SIGNALING

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Summary

We cloned the inebriated homologue MasIne from Manduca sexta and expressed it in Xenopus laevis oocytes. MasIne is homologous to neurotransmitter transporters but no transport was observed with a number of putative substrates. Oocytes expressing MasIne respond to hyperosmotic stimulation by releasing intracellular Ca2+, as revealed by activation of the endogenous Ca2+-activated Cl− current. This Ca2+ release requires the N-terminal 108 amino acid residues of MasIne and occurs via the inositol trisphosphate pathway. Fusion of the N terminus to the rat γ-aminobutyric acid transporter (rGAT1) also renders rGAT1 responsive to hyperosmotic stimulation.

Immunohistochemical analyses show that MasIne and Drosophila Ine have similar tissue distribution patterns, suggesting functional identity. Inebriated is expressed in tissues and cells actively involved in K+ transport, which suggests that it may have a role in ion transport, particularly of K+. We propose that stimulation of MasIne releases intracellular Ca2+ in native tissues, activating Ca2+-dependent K+ channels, and leading to K+ transport.

Key words: Manduca sexta, inebriated, Ca2+-activated Cl− current, inositol trisphosphate, permeable phospholipase C.

Introduction

The Na+/Cl−-dependent neurotransmitter transporter (NTT) superfamily consists of proteins that transport neurotransmitters [γ-aminobutyric acid (GABA), serotonin, dopamine, norepinephrine, epinephrine, glycine, proline], osmolytes (taurine, betaine) and metabolites (creatine, alanine, taurine). Neurotransmitter transporters are expressed in the synaptic region and terminate neurotransmission by active reuptake of neurotransmitters.

Drosophila inebriated (Ine) and RosA are ‘orphan transporters’ with no known function. These two gene products have the same putative topology as members of the NTT superfamily but share only 36–41% amino acid identity with them (Soehnge et al., 1996; Burg et al., 1996). Drosophila ine mutants show no behavioral defects. At the neuromuscular junction of these mutants, brief high-frequency nerve stimulation in the presence of quinidine, a K+ channel inhibitor, evokes abnormal repetitive neuronal firing and an increase in transmitter release, thus increasing the excitatory junction potential amplitude. Double mutants of ine and shaker, a K+ channel, or ine and hyperkinetic (HK), a beta subunit of shaker, exhibit a ‘down-turned wings and indented thorax’ phenotype with behavioral defects (uncoordination and flightlessness) (Stern and Ganetzky, 1992). This phenotype is also observed in shaker mutants carrying either an additional mutation in the ether-a-go-go (eag) K+ channel or overexpressing a Na+ channel gene, para (Ganetzky and Wu, 1983; Stern et al., 1990). The shaker:eag double-mutant phenotype is apparently caused by the elimination of two types of K+ channel that are partially redundant. The observation that the shaker:ine double mutant also gave a similar phenotype led Stern and Ganetzky (1992) to suggest that Ine also modulates ion conductance, especially of K+. However, elucidation of the ine sequence showed that it encodes a protein related to the NTT superfamily. Thus, Soehnge et al. (1996) proposed that Ine is either a GABA or glutamate transporter because these neurotransmitters are common in insect neuromuscular junctions.

In an effort to identify neurotransmitter transporters in the tobacco hornworm Manduca sexta, we cloned a cDNA that encodes a protein, MasIne, which has 55% amino acid identity with Ine and RosA proteins from Drosophila. MasIne and Ine have similar tissue distributions, suggesting a functional identity between the two proteins. We also demonstrate that, in Xenopus oocytes, MasIne responds to hyperosmotic...
stimulation by activating the phospholipase C (PLC) and inositol trisphosphate (InsP₃) signaling cascade, which in turn activates Ca²⁺-activated Cl⁻ currents in these oocytes. PLC activation is mediated by the N terminus of MasIne. The ability of a transporter protein to activate intracellular signaling suggests that these proteins may have more diverse functions than previously believed.

**Materials and methods**

**Isolation of MasIne cDNA**

Total RNA was isolated from 3-day-old eggs of *Manduca sexta* and from the brain and ventral nerve cord of 2- to 4-day-old fifth-instar larvae as described by Mbungu et al. (1995). Reverse transcription/polymerase chain reaction (RT-PCR) was performed using degenerate primers complementary to conserved sequences of mammalian dopamine, GABA, serotonin and norepinephrine transporters. The forward primer was 5'-CGGAATTCTTG(C/G)CAAT(C/T)/G/A/ITT-GG/(A/G)GGTT/(C/T)CCNTA, and the reverse primer was 5'-GCAACGTTGGAAGAT/(T/C)/(G/A)TIG(1/1)GG/(G/A)/GIIIGICGC/(G/A)/CTA/C(T/G)/CCCA. Products of 0.7 kb were obtained, and these were cloned and sequenced.

Isolation of full-length MasIne cDNA was achieved by PCR screening as described by Ross and Gill (1996) using an embryonic size-selected cDNA library in pSPORT1 (Mbungu et al., 1995). A MasIne-specific primer, 5'-GGTACCGGTC-CGGAATTCCGGG, and a pSPORT1-vector-specific primer were used. Both strands of the cDNA clone were sequenced by dideoxy chain termination using Sequenase 2.0 (Amersham).

**Antibody generation**

Secondary structure analysis predicted that a putative extracellular loop between TM3 and TM4 (amino acid residues 277–320) and the C terminus (amino acid residues 685–725) would be highly antigenic regions. These regions were PCR-amplified using specific primers and Taq DNA polymerase (Life Science Technologies). The PCR products were then cloned in tandem into pQE30 (Qiagen) and sequenced to ensure that no mutations were introduced during PCR. TheHis-tag fusion peptide was expressed and then purified by Ni-NTA affinity chromatography followed by a 30 % ethanol series. To expose MasIne, the sections were boiled for 20 min. The supernatants (8 ml) were mixed with 1.5 μg of the affinity-purified MasIne-specific IgG and incubated at 4 °C overnight. Protein A–Sepharose CL-4B gel (50 % v/v; 15 μl) was added, and the mixture was incubated for a further 4 h at 4 °C. After a brief centrifugation, the pellet was washed in the following three solutions: Tween 20/urea buffer (100 mmol l⁻¹ Tris-HCl, pH 7.5, 200 mmol l⁻¹ NaCl, 2 mol l⁻¹ urea, 0.5 % Tween 20), Tween 20 buffer (50 mmol l⁻¹ Tris-HCl, pH 7.5, 150 mmol l⁻¹ NaCl, 0.5 % Tween 20, 1 mol l⁻¹ EDTA) and TBS buffer (the Tween 20 buffer without Tween). Following these washes, the Sepharose gel was resuspended in 2× SDS sample buffer and separated on a 10 % gel using SDS–PAGE. The proteins were then transferred electrophoretically (0.5 A for 1.5 h) to an Immobilon P membrane (Millipore). Western blot analysis was performed using MasIne-specific IgG as the primary antibody and goat anti-rabbit IgG conjugated with alkaline phosphatase as the secondary antibody. The membrane was then stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma). For experiments using [³⁵S]methionine-labeled oocytes, the membranes were exposed to intensifying screens (Kodak).

**Immunohistochemistry**

Tissues and oocytes were fixed overnight in alcoholic Bouin’s fixative, dehydrated in stages through 70–100 % ethanol and 30–100 % xylene, and infiltrated and embedded in paraffin wax. Sections (7–10 μm) on silane-coated slides were dried at 40 °C, washed with xylene and rehydrated in a graded ethanol series. To expose MasIne, the sections were boiled (twice, 10 min each) in 100 mmol l⁻¹ sodium citrate (pH 6.0), washed with phosphate-buffered saline (PBS) and blocked in PBS containing 2 % normal goat serum (NGS) (blocking buffer) for 1 h. The sections were incubated overnight in a humidified chamber at 4 °C with affinity-purified MasIne-specific IgG in blocking buffer.

For fluorescence immunohistochemistry, goat anti-rabbit Cy3-conjugated IgG (Jackson Immuno Research) was used as the secondary antibody. Following five washes in PBS with 0.1 % Triton X-100, the secondary antibody (diluted 1:200 in PBS) was added and incubated at room temperature (26 °C) for 60 min. The slides were then washed sequentially with the Tween 20/urea, Tween 20 and TBS buffers described above. The sections were mounted with mounting medium (Vector) and analyzed using fluorescence microscopy.

For alkaline phosphatase staining, the secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (Jackson Immuno Research), was diluted 1:200 in blocking buffer. After a 5 h incubation at room temperature, the slides were washed twice with PBS, once with PBS containing
0.05% Triton-X100, and then twice with PBS. They were then incubated for 20 min in substrate solution (20 µl of naphthol AS-MX phosphate, 35 mg ml\(^{-1}\) in dimethyl formamide, and 10 ml of filtered Fast Red Violet LB salt, 0.2 mg ml\(^{-1}\) in 0.1 mol l\(^{-1}\) Tris-Cl, pH 8.5, prepared and mixed just before use). Color development, monitored by light microscopy, was stopped with Tris-Cl buffer, pH 8.5. The slides were sealed in 2 parts 10% gelatin:1 part glycerol (Zitnan et al., 1999) and kept at –20°C until visualized by light microscopy.

**MasIne expression in Xenopus oocytes**

The MasIne open reading frame (ORF) was cloned into PBS KS+ AMV-PA50 (pAMV) (Nowak et al., 1998) containing an alfalfa mosaic virus RNA 5'-untranslated region (UTR), which enhances protein expression (Jobling and Gehrke, 1987). A 98 bp bovine preprolactin 3'-UTR, which stabilizes cRNA in *Xenopus* oocytes (Vassilakos et al., 1995), was synthesized by PCR and cloned upstream of the poly(A) tail. Two MasIne constructs using either base pair 135 or base pair 459 as the start site and base pair 2318 as the stop site were made using PCR (Expand High Fidelity PCR System, Boehringer Mannheim). The second amino acid residue of MasIne-135 was intentionally changed from asparagine to aspartic acid to introduce an Nco I site. To obtain the wild-type aspartic acid residue, a back mutation was made. DNA sequencing verified the final constructs.

The 108-rGAT1 construct was made by amplifying the AMV 5'-UTR and 135-458 bp of MasIne and cloning it in-frame with the rest of the wild-type rGAT (rat GABA transporter) into pAMV. Both ΔMasIne-135 and ΔMasIne-459 deletion constructs were made by AccI/BamHI restriction digestion, which deleted the fragment between base pairs 819 and 2285 for MasIne-135 and the corresponding region of MasIne-459. The restricted ends were blunt-ended with T4 DNA polymerase and religated. This deletion causes no reading frame shift. Thus, ΔMasIne-135 has only the N-terminal 228 residues and ΔMasIne-459 has only the N-terminal 120 residues, respectively, and the C-terminal 10 amino acid residues; TM3–TM12 and most of the C terminus are deleted.

A sample of cRNA was obtained in vitro using the mMessage mMachine (Ambion) transcription kit. For expression, stage V or VI oocytes were injected with approximately 32 ng of MasIne cRNA. Oocytes were incubated at 18°C in either complete Barth’s medium or in ND96 with 2% horse serum. MasIne-135- and MasIne-459-expressing oocytes were assayed after at least 96 h of incubation, and 108-rGAT1, ΔMasIne-135 and ΔMasIne-459 were assayed between 30 and 60 h of incubation. Ligand uptake assays were performed as described by Guastella et al. (1990). Expression of MasIne-135 and MasIne-459 was analyzed by incubating the injected oocytes (80–100 each) in medium containing [³⁵S]methionine (37 MBq ml\(^{-1}\)) for 30 h (pulse) followed by incubation with medium containing cold methionine for an additional 42 h (chase); these oocytes were used for immunoprecipitation and western blot analysis.

**Electrophysiology**

The oocytes were voltage-clamped using a Dagan TEV 200 or a GeneClamp 500 (Axon Instruments, Inc.) voltage-clamp amplifier. Agar bridges (3 mol l\(^{-1}\) KCl) were used as reference electrodes. The microelectrodes were filled with 3 mol l\(^{-1}\) KCl and had resistances of 1–2 MΩ. A DigiData 1200 (Axon Instruments, Inc.) A/D converter and pCLAMP 6.0 or 7.0 (Axon Instruments, Inc.) were used to record the data digitally. Analysis was performed using the Origin software (Microcal).

Oocytes in the recording chamber were exposed either to isotonic solution (100 mol l\(^{-1}\) Tris-Cl, 1 mmol l\(^{-1}\) MgCl₂ and 5 mmol l\(^{-1}\) Hepes, pH 7.4; 214 mosmol l\(^{-1}\)) or to hyperosmotic solutions (Tris-Cl isotonic solution plus different concentrations of sucrose). Oocytes expressing wild-type MasIne were first exposed to isotonic solution and then to hyperosmotic solution. For the ion-selectivity experiments, Cl⁻ was replaced by gluconate and Tris⁺ was replaced by Na⁺, Li⁺ or K⁺.

To test for ligand-independent leakage currents, the membrane potential was held at −60 mV while oocytes were superfused with Tris-Cl isotonic solution and a background current was recorded. The solution was then changed either to ND96 or LiCl Ringer (96 mmol l\(^{-1}\) NaCl or 96 mmol l\(^{-1}\) LiCl, 2 mmol l\(^{-1}\) KCl, 1 mmol l\(^{-1}\) MgCl₂, 5 mmol l\(^{-1}\) Hepes, pH 7.5) for 2–3 min, and the leakage current was recorded. To measure the ligand-independent Na⁺-coupled transient current, we used the I\(_{\text{Cl}}\) voltage-step protocol (see below). Both rGAT1 and rSERT were used as positive controls.

To record Ca²⁺-activated Cl⁻ currents, two different voltage-step protocols were applied to oocytes. To characterize the outwardly rectifying Cl⁻ currents and to test for ligand-independent Na⁺-coupled transient current, a protocol named ‘I\(_{\text{Cl}}\) voltage-step’ was used. The membrane potential was held at −40 mV for 30 ms, then stepped to test potentials between +100 mV and −140 mV in −30 mV increments for 195 ms and finally stepped back to −40 mV for 60 ms (Hartzell, 1996; Mager et al., 1993). To characterize inward Cl⁻ currents, we used the ‘I\(_{\text{Cl}}\) voltage-step’ protocol. The voltage was held at −35 mV for 50 ms, stepped to +20 mV for 900 ms, then stepped to test potentials between +80 mV and −140 mV in −20 mV increments for 900 ms, and finally stepped back to −35 mV for 100 ms (Hartzell, 1996).

Stock solutions of nimulnic acid, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetra-acetic acid acetoxyethyl ester (BAPTA-AM), 1-(6-[[17β]-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino[hexyl]1H-pyrole-2,5-dione (U73122) and 1-(6-[[17β]-3-methoxyestra-1,3,5(10)-tri-en-17-yl]amino[hexyl]-2,5-pyridoline-dione (U73343) were prepared in dimethylsulfoxide (DMSO), while LaCl₃, heparin, guanosine-5′-O-(2-thiodiphosphate) triethylth (GDP-β-S) and K₃BAPTA (the potassium salt of BAPTA) were dissolved in water or buffer.
Results

Cloning and sequence analysis of MasIne

The 4956 bp full-length MasIne cDNA has two potential start codons, at base pairs 135 and 459, that fit the consensus Kozak sequence (Kozak, 1987) and a stop codon at base pair 2318. Thus, MasIne has two potential open reading frames encoding polypeptides of either 727 or 619 amino acid residues. TMPred analysis (http://www.ch.embnet.org/software/TMPRED_form.html) suggests that MasIne has 12 transmembrane domains, as observed in other Na+/Cl̅-dependent neurotransmitter transporters, and a large extracellular loop between TM3 and TM4. Of the 109 amino acid residues conserved in most other neurotransmitter transporters, 91 are present in MasIne. This protein has 55% amino acid identity with Drosophila Ine and RosA (Fig. 1), but only 37% identity with Manduca GAT and 30–42% identity with other neurotransmitter transporters. Dendogram analyses show that the Drosophila and Manduca inebriated proteins are divergent from the other neurotransmitter transporters (data not shown), suggesting that these proteins have a function distinct from that of other neurotransmitter transporters.

Tissue distribution of inebriated proteins in Manduca and Drosophila

Two major broad bands at 58–80 and 150–205 kDa were observed in immunoprecipitations of M. sexta fifth-instar day 1 midgut and day 1 whole larvae (Fig. 2). The 58–80 kDa bands are of the size expected for the unglycosylated MasIne monomer. The 170–200 kDa broad band is probably the glycosylated and oligomerized MasIne. Broad bands are expected because MasIne is a membrane protein, and two alternatively spliced homologues, Ine and RosA, have been identified in Drosophila. Similar broad bands were also observed in oocytes expressing MasIne (see Fig. 5D). These results show that the IgG for MasIne is specific for this protein.

In the central nervous system of Manduca sexta larvae, high levels of MasIne expression were observed in the glial cells, neuropil and axons in the connective and segmental nerves. No expression was detected in the neuronal perikarya (Fig. 3A,B). The preimmune serum showed no immunoreactivity with the brain (Fig. 3C), optic lobes (Fig. 4C) or any other tissue. In the larval midgut, MasIne expression was observed in the basolateral membrane of goblet cells (Fig. 3D,E). In larval Malpighian tubules, MasIne was expressed in the basolateral membrane of the proximal region and weakly expressed in the middle region (Fig. 3F). MasIne was also expressed in the basolateral membrane of the epithelial cells of the ileum, the rectum and the outer Malpighian tubules of the cryptonephridial system (Fig. 3G,H). In muscles, MasIne expression was detected in the T-tubules but not the myofibrils (Fig. 3I). In adult Manduca sexta optic lobes, MasIne was expressed in retinula cells (cell body and rhabdomere), optic nerve axons, lamina and projections to the medulla (Fig. 4A).

Part of the fusion peptide used to generate the antibodies, amino acid residues 277–320 of MasIne, has 63% identity with Ine. We therefore tested the ability of the MasIne antibody to detect Ine expression in Drosophila melanogaster. The MasIne-specific IgG detected the Ine protein in adult Drosophila melanogaster in the optic lobes, muscle system and axon bundles. In the optic lobes, Ine expression was observed in the retinula, lamina and axonal projections to the medulla (Fig. 4B) with the same pattern as that observed in Manduca sexta. Ine was also expressed in neurons and axon bundle of the antennal chordotonal organs of Drosophila melanogaster (Fig. 4D), and in the T-tubules of the leg and dorsal longitudinal flight muscles (Fig. 4E). The MasIne antibody also detected the Ine protein in the foregut (esophagus), midgut (various regions) and anal plate of Drosophila melanogaster larvae (Fig. 4F–I), as previously reported in the embryo using in situ hybridization (Soehnge et al., 1996). The similarity in the tissue distribution of MasIne in Manduca sexta and Ine in Drosophila melanogaster strongly suggests that the two proteins are orthologs.

Functional characterization

To elucidate its function, MasIne was expressed in Xenopus oocytes using the two potential start codons, base pairs 135 and 459. Both MasIne-135 and MasIne-459 were expressed at high levels in the plasma membrane of Xenopus oocytes (Fig. 5A–C). Immunoprecipitation using specific antibodies showed that MasIne-135 and MasIne-459 were expressed in Xenopus oocytes injected with the corresponding cRNA (Fig. 5D). The expected molecular mass of MasIne-135 is 81 kDa and that for MasIne-459 is 69 kDa. After a 30 h incubation in medium containing [35S]methionine (pulse), a major band at 80–90kDa was observed in MasIne-135-expressing oocytes, whereas MasIne-459 appeared as a major band at 58–66kDa. Following an additional 42 h expression in cold medium (chase), MasIne-135-expressing oocytes had major broad bands at 100–150 and >205 kDa, while MasIne-459-expressing oocytes showed bands at 84–116 and >205 kDa, suggesting glycosylation and oligomerization of the proteins.

To determine whether MasIne could transport a substrate, oocytes expressing the protein were used in uptake assays, as previously described by Mbungu et al. (1995). No uptake was observed with either construct using radiolabeled dopamine, serotonin, norepinephrine, octopamine, histamine, tyramine, glycine, GABA, glutamate, proline, lysine, phenylalanine, leucine, choline, taurine or creatine (data not shown). To determine whether the expressed MasIne was functional, we tested for ligand-independent leakage currents. MasIne-135-expressing oocytes displayed a higher level of Na⁺, Li⁺ and K⁺ leakage current than did MasIne-459-expressing or water-injected oocytes (Fig. 6; Table 1). In addition, MasIne-135-expressing oocytes showed significantly higher background currents in Tris-Cl isotonic solution than did control oocytes (Table 1). The positive controls, rGAT1 and rSERT, also showed ligand-independent leakage currents to Na⁺ and Li⁺ but not to K⁺ (Mager et al., 1994, 1996; data not shown). K⁺ leakage currents have also been observed with Manduca KAAT1 and human DAT (Bossi et al., 1999;
Fig. 1. A comparison of the MasIne amino acid sequence and alignment with those of Drosophila RosA (dRosA) and Ine (dIne), Manduca GAT1 (MasGAT) and Drosophila SERT (dSERT). Matching residues are marked in black. The full-length MasIne cDNA (GenBank accession AF032873) is 4956 bp in length and has an open reading frame that starts at position 135 and ends at position 2318. MasIne-135 contains 727 amino acid residues with a deduced molecular mass of 81 kDa. An alternative start site is at residue 459, which would give a protein of 619 amino acid residues with a molecular mass of 69 kDa. MasIne has 55% identity with RosA and Ine, but only 30–42% identity with other neurotransmitter transporters.
Sonders et al., 1997). Thus, MasIne-135 generates a transporter-like leakage current. 

MasIne-135-expressing oocytes did not show substrate-independent Na\(^+\)-coupled transient currents (Fig. 6D), however, nor did MasIne-459-expressing oocytes, although these were observed with rGAT1 (Fig. 6E), rSERT (data not shown) and KAAT1 (Bossi et al., 1999; Mager et al., 1993, 1994). This lack of a transient current suggests either that MasIne-135 does not bind Na\(^+\) or that the bound Na\(^+\) does not move within the electric field during voltage steps. These data show that MasIne-135 was functionally expressed in Xenopus oocytes and that it has unique conducting properties compared with other neurotransmitter transporters.

### Sensitivity to hyperosmotic stimulation

During two-electrode voltage-clamp assays, we observed that MasIne-135-expressing oocytes were sensitive to hyperosmotic stimulation (400 mosmol l\(^{-1}\)), displaying Ca\(^{2+}\)-activated Cl\(^-\) currents (I\(_{\text{Cl(Ca)}}\)) with kinetics similar to those observed in InsP\(_3\)-injected oocytes (Hartzell, 1996; Kuruma and Hartzell, 1999). These oocytes responded to hyperosmotic stimulation within 6–15 s with outwardly rectifying Cl\(^-\) currents, which peaked at 40–60 s (Fig. 7A,B), suggesting that I\(_{\text{Cl-1}}\) was induced. With a longer hyperosmotic exposure, the strong outwardly rectifying current decreased, and a significant inward steady current appeared (I\(_{\text{Cl-2}}\)), reaching its peak in 3–5 min (Fig. 7C). The time course of MasIne-135-mediated current (Fig. 7F) is similar to that observed in InsP\(_3\)-injected oocytes (Hartzell, 1996; Kuruma and Hartzell, 1999). MasIne-459-expressing oocytes and water-injected oocytes showed a small and gradual response to the 400 mosmol l\(^{-1}\) stimulation (Fig. 7F). When water-injected oocytes were stimulated by 725 mosmol l\(^{-1}\) (isotonic solution plus 500 mmol l\(^{-1}\) sucrose), we were able to measure a BAPTA-sensitive Cl\(^-\) current that started to increase after more than 80 s (data not shown). However, some batches of oocytes, when stimulated at 725 mosmol l\(^{-1}\), and using the same voltage-step protocol, developed steady inward and outward Cl\(^-\) currents controlled by unknown mechanisms. Our data represent only those batches of oocytes that did not show such currents.

The rapid response to 400 mosmol l\(^{-1}\) solutions was not observed in MasIne-459- (Fig. 7D,E), rGAT1- or rSERT-expressing oocytes (Fig. 7G) indicating that the MasIne-135-induced Cl\(^-\) currents were specific. Tail current measurements (data not shown) and the current/voltage (I/V) relationship (Fig. 7G) gave a reversal potential for the osmotically induced

### Table 1. Leakage current measurement on MasIne-expressing oocytes

<table>
<thead>
<tr>
<th></th>
<th>Current (nA)</th>
<th>Tris-Cl</th>
<th>NaCl</th>
<th>KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>MasIne-135</td>
<td>–59.4±7.2 (19)</td>
<td>–201.0±22.2 (13)</td>
<td>–284.2±38.5 (8)</td>
<td></td>
</tr>
<tr>
<td>MasIne-459</td>
<td>–34.2±5.6 (8)</td>
<td>–83.7±20.5 (5)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>H(_2)O- or non-injected</td>
<td>–27.0±6.7 (18)</td>
<td>–103.0±28.7 (8)</td>
<td>–166.8±32.1 (5)</td>
<td></td>
</tr>
</tbody>
</table>

All leakage currents, in nA, were taken from data in the I\(_{\text{Cl-1}}\) voltage step protocol (see Materials and methods) at the –80 mV step. The currents are averages of the recording between 160 and 190 ms after the step. Tris-Cl represents the Tris-Cl-based isotonic solution, while NaCl and KCl represent the corresponding substituted isotonic solutions. Values are means ± s.e.m. (N).
Fig. 3. Tissue distribution of MasIne in Manduca sexta. (A) In the third-instar larval brain, MasIne was highly and uniformly expressed throughout the neuropil (Np) and the glial cell layer (GL) and its processes (GP). (B) MasIne expression in the first abdominal ganglion of a third-instar Manduca sexta larva. High levels of expression were observed in the glial cell layer (GL) and its processes (GP), the axons in the neuropil (Np), the intersegmental connectives (IC) and the segmental nerve (SN). (C) The preimmune serum did not interact with the third-instar larval brain. (D,E) MasIne expression is restricted to the basolateral membrane of midgut goblet cells (Gb); He, hemolymph side of the midgut; L, lumen side of the midgut. (F) High levels of MasIne expression were observed in the proximal region (pMT), but very low levels of expression were observed in the middle region (mMT) of the Malpighian tubule. (G) Iliac epithelial cells (Ie) showed high MasIne levels, but MasIne was not expressed in the sphincter (Sp) region. (H) MasIne was expressed in the basolateral membrane of epithelial cells of the rectum (Re) and the outer flat Malpighian tubules (MT), but very low levels of expression were observed in the round inner Malpighian tubules in the cryptonephridial system (CN). (I) In the leg muscle (M) of third-instar Manduca sexta, MasIne was expressed on T-tubules (T). Scale bars: A,B,C,F,H, 100 μm; E,I, 20 μm; G, 200 μm.
current at $-19\text{mV}$, which is close to the Cl$^-$ equilibrium potential ($E_{\text{Cl}}$). In addition, substitution of extracellular Cl$^-$ with gluconate abolished this induced outward current, suggesting activation of a Cl$^-$ conductance (Fig. 7H). When
Tris + was replaced with Na + or K + , there was no change in the reversal potential (Fig. 7H). To characterize the osmotically induced currents, we chose to use Tris-Cl for subsequent experiments.

**Pharmacological characterization of MasIne-135-induced currents**

Niflumic acid and NPPB blocked the osmotically induced conductance (Table 2), while, La 3+ , a stimulator of Ca 2+ -activated Cl − channels (Ackerman et al., 1994), potentiated it (Table 2). Incubation with BAPTA-AM also completely blocked this current (Table 2). Extracellular Ca 2+ did not affect it, since the addition of 5 mmol l −1 EGTA to the Ca 2+ -free superfusion solution did not affect the amplitude of the response (Table 2). Thus, hyperosmotic stimulation of MasIne-135 apparently causes intracellular Ca 2+ release, thereby activating I Cl(Ca) in *Xenopus* oocytes (Ackerman et al., 1994; Hartzell, 1996; Kuruma and Hartzell, 1999).

Since the kinetics of the currents observed here are similar to those observed in InsP 3 -injected oocytes, we proposed that the InsP 3 signaling cascade is involved in the MasIne-135-induced currents.

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**Table 2. Effect of pharmacological agents on Ca 2+ -activated Cl − currents in MasIne-135-expressing oocytes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage activity 1</th>
</tr>
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<tbody>
<tr>
<td>Untreated (N=15)</td>
<td>100±5.1</td>
</tr>
<tr>
<td>Niflumic acid, 1 mmol l −1 (N=5)</td>
<td>17.3±8.6</td>
</tr>
<tr>
<td>NPPB, 100 μmol l −1 (N=5)</td>
<td>41.2±9.5</td>
</tr>
<tr>
<td>Lanthanum chloride, 4 mmol l −1(N=5)</td>
<td>294.3±5.1</td>
</tr>
<tr>
<td>BAPTA-AM, 50 μmol l −1 (N=8)²</td>
<td>8.7±1.4</td>
</tr>
<tr>
<td>EGTA, 0.6 mmol injected (N=4)³</td>
<td>9.2±3.8</td>
</tr>
<tr>
<td>EGTA, 5 mmol l −1 (N=6)</td>
<td>102.2±4.5</td>
</tr>
<tr>
<td>Heparin, 0.6 μg injected (N=6)</td>
<td>4.9±8.7</td>
</tr>
<tr>
<td>U73122, 2 μmol l −1 (N=6)⁴</td>
<td>32.0±8.1</td>
</tr>
<tr>
<td>U73343, 2 μmol l −1 (N=6)⁴</td>
<td>110.3±10.4</td>
</tr>
<tr>
<td>GDP-b-S, 0.2 mmol injected (N=5)³</td>
<td>119.3±28</td>
</tr>
<tr>
<td>MasIne-459-expressing oocytes, untreated (N=19)</td>
<td>11.7±8.4</td>
</tr>
<tr>
<td>Water-injected oocytes, untreated (N=12)</td>
<td>5.2±8.0</td>
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1Percentage activity was normalized to currents observed in untreated MasIne-135-expressing oocytes exposed to 400 mosmol l −1 hyperosmotic stimulation, and the maximum outward current during a voltage step from −40 to +100 mV was measured. Oocytes were exposed to the agent in the superfusion solution unless indicated otherwise.

2Incubated for 5 h before recording.

3Incubated for 15 min after injection before recording.

4Incubated for 15 min prior to superfusion.

Values are means ± S.E.M. with the control value taken as 100%.
mediated current. To test this hypothesis, oocytes were injected with heparin, an InsP3 receptor inhibitor, or were incubated with U73122, a permeable phospholipase C (PLC) inhibitor. These compounds inhibited the osmotic MasIne response (Table 2). However, the inactive analogue of U73122, U73343, did not affect the response, showing that the action of U73122 is specific. Injection of GDP-β-S, a G-protein inhibitor, had no effect on the MasIne-135-mediated current (Table 2). Thus, activated MasIne-135 stimulates the PLC/InsP3 signaling cascade but not through a G-protein pathway.

**Function of the MasIne-135 N terminus**

Since oocytes expressing MasIne-135 responded to hyperosmotic stimuli, resulting in intracellular Ca\(^{2+}\) release, but oocytes expressing MasIne-459 did not, we hypothesized that the N-terminal 108 amino acid residues played a crucial role. To test this hypothesis, we fused the N-terminal 108 amino acid residues of MasIne-135 with rGAT1 which, on its own, did not respond to hyperosmotic stimuli. Oocytes expressing this fusion construct, 108-rGAT1, spontaneously gave rise to a combination of both outwardly rectifying Cl\(^{-}\) and inward steady leakage currents even without hyperosmotic stimulation (Fig. 8A). Both currents were also observed in MasIne-135-expressing oocytes after hyperosmotic stimuli of more than 3 min (Fig. 7C). In addition, both the outward and inward currents were inhibited by BAPTA-AM; however, BAPTA-insensitive steady Cl\(^{-}\) currents were also observed (Fig. 8B). These data suggest that 108-rGAT1 mediated three currents, ICl-1 and ICl-2, both sensitive to BAPTA, and a BAPTA-insensitive leakage Cl\(^{-}\) current (Hartzell, 1996) (Fig. 8C). Our interpretation of these data is that 108-rGAT1 activated a constant release of intracellular Ca\(^{2+}\) and, as a consequence, ICl-1, ICl-2 and leakage currents were activated. The currents observed with
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108-rGAT1 were not seen in oocytes expressing wild-type rGAT1 (Fig. 8C), suggesting that the MasIne N terminus was modulating these effects.

GABA transport by 108-rGAT1 was approximately 10% of that of rGAT1 but approximately 10 times that of uninjected oocytes (92±9 fmol oocyte⁻¹ 30 min⁻¹ for 108-rGAT1, 870±58 fmol oocyte⁻¹ 30 min⁻¹ for rGAT1 and 9±2 fmol oocyte⁻¹ 30 min⁻¹ for uninjected oocytes; means ±
Since the N terminus activated spontaneous Ca$^{2+}$-activated currents in 108-rGAT1-expressing oocytes but not in MasIne-135-expressing oocytes, we hypothesized that other domains of MasIne-135 interact with the N terminus, thereby...
regulating MasIne function. To test this hypothesis, MasIne deletion constructs were made to express only the N terminus. In these constructs, the 10 C-terminal putative transmembrane regions of both MasIne-135 and MasIne-459 were deleted giving \( \text{D} \text{MasIne-135} \) and \( \text{D} \text{MasIne-459} \), respectively. When expressed in oocytes, \( \text{D} \text{MasIne-135} \), but not \( \text{D} \text{MasIne-459} \), activated the spontaneous outward and inward Cl\(^-\) currents without hyperosmotic stimulation (Figs 9A,C, 10A–C). Injection of K\(_b\)BAPTA significantly reduced the outward Cl\(^-\) current, while the strong inwardly rectifying Cl\(^-\) current was only slightly affected, suggesting that a large proportion of the inward Cl\(^-\) current was Ca\(^{2+}\)-independent (Fig. 9B,C). We believe that this BAPTA-insensitive inward Cl\(^-\) current differs from I\(_{\text{Cl-2}}\) (Hartzell, 1996), as discussed below. Treatment of these oocytes with U73122, but not U73343, inhibited approximately 45% of both inward and outward currents (Fig. 9D,E). Heparin treatment also inhibited approximately 40% of the inward current (Fig. 10D). These results demonstrate that \( \text{D} \text{MasIne-135} \) spontaneously activates the InsP\(_3\) signaling cascade and support our hypothesis that other domains of MasIne interact with its N terminus and regulate its function negatively.

Our results suggest that \( \text{D} \text{MasIne-135} \), like 108-rGAT1, spontaneously activated I\(_{\text{Cl-1}}\), I\(_{\text{Cl-2}}\) and steady leakage currents through the InsP\(_3\) signaling pathway. In addition, \( \text{D} \text{MasIne-135} \) also mediated an endogenous, hyperpolarization-activated Cl\(^-\) current (I\(_{\text{Cl}(h)}\)) (Figs 9B,C, 10A) (Kowdley et al., 1994; Tokimasa and North, 1996). I\(_{\text{Cl}(h)}\) was probably dependent on
InsP₃ signaling because it was sensitive to heparin (Fig. 10D) and U73122 (Fig. 9D,E). However, as reported by Parker and Miledi (1988), I_{Cl(h)} was not activated by Ca²⁺ because it was insensitive to BAPTA (Fig. 9C). We do not know why I_{Cl(h)} was observed only in ΔMasIne-135-expressing oocytes rather than 108-rGAT1- or MasIne-135-expressing oocytes. Oocytes expressing 108-rGAT1 and ΔMasIne-135 died within 72 h, in contrast to the 8–10 day survival time of MasIne-135-expressing oocytes, and the resting membrane potential of these oocytes was close to E_{Cl}, suggesting a constant release of intracellular Ca²⁺.

Discussion

We have cloned a _Manduca sexta_ homologue of the inebriated gene from _Drosophila melanogaster_. MasIne and _Drosophila_ Ine and RosA are highly homologous to the neurotransmitter transporter family of proteins. However, phylogenetic analysis (data not shown) shows that the inebriated proteins are divergent from other neurotransmitter transporters, suggesting that they have a common, yet distinct, function from that of the other transporters. We were able to express MasIne in the plasma membrane of _Xenopus_ oocytes, but none of the ligands and/or substrates tested was transported.

A lack of transport could also result either because we failed to identify the correct substrate or because an additional protein may be required for transport. For example, LAT and γ⁺LAT amino acid transporters require an additional membrane-spanning protein, CD98, for amino acid uptake (Mastroberardino et al., 1998; Kanai et al., 1998; Torrents et al., 1998; Nakamura et al., 1999). However, our data show that inebriated is not a GABA or glutamate transporter, as proposed by Soehnge et al. (1996). Indeed, these transporters have been cloned and are distinct proteins (Mbugu et al., 1995; Donly et al., 1997).

Nevertheless, oocytes expressing MasIne displayed ligand-independent leakage conductances to alkali ions, suggesting that MasIne shares this property with other transporters. However, Na⁺-coupled, ligand-independent transient currents, which are often seen with other neurotransmitter transporters (Mager et al., 1993, 1994; Bossi et al., 1999), were not observed.

Although ligand transport was not observed in _Xenopus_ oocytes with the substrates analyzed, MasIne responded to hyperosmotic stimulation by modulating the activation of outward Cl⁻ currents. Using pharmacological agents, we showed that these Cl⁻ currents were dependent on the release of intracellular Ca²⁺ through a PLC and InsP₃ signaling pathway. Moreover, the current observed, I_{Cl(Ca)}, displayed a voltage- and time-dependence similar to those induced by InsP₃ injection of oocytes (Hartzell, 1996). This hyperosmotic-sensitive Cl⁻ current was observed in _Xenopus_ oocytes when the full-length MasIne protein, MasIne-135, was expressed. No Cl⁻ currents were observed with expression of MasIne-459 or with the GABA and serotonin transporters during hyperosmotic stimulation.

Expression of a fusion protein consisting of the MasIne-135 N terminus with rGAT1 resulted in spontaneous activation of I_{Cl(Ca)}, even without hyperosmotic stimulation. These currents were also observed when the N terminus alone was expressed in oocytes without osmotic stimulation. These data suggest that the N-terminal 108 amino acid residues of MasIne modulate PLC activation.

Since the N terminus spontaneously activated the endogenous currents, the data indicate that, when the full-length protein is expressed, the N terminus interacts with other MasIne domains and, therefore, is unable to activate these currents. Hyperosmotic stimulation releases this N terminus, leading to PLC activation. In contrast, both 108-rGAT1 and ΔMasIne-135 lack this ‘negative regulator’ so that spontaneous activation of PLC occurs. Moreover, water-injected oocytes can also respond to hyperosmotic stimulation, but this response is significantly delayed (by more than 1 min) and at occurs much higher osmolalities (approximately 300 mosmol l⁻¹ greater than those evoking equal responses in MasIne-expressing oocytes). This observation indicates that oocytes contain an endogenous hyperosmotic-responsive system that activates I_{Cl(Ca)}.

Although none of the compounds analyzed was transported, we speculate that MasIne is a transporter. The transport of an unknown ligand, which could be an osmolyte, may cause activation of the PLC/InsP₃ cascade. This activation, dependent on the MasIne N terminus, is apparently not mediated through a G-protein, since GDP-β-S had no effect on the response observed. Furthermore, since this N terminus has no homology with either Gα or protein tyrosine kinases, both known PLC activators, it appears that PLC activation is mediated through a novel mechanism. Activation of the PLC/InsP₃ cascade leads to increases in intracellular Ca²⁺ concentrations, which can then stimulate Ca²⁺-activated channels, including those that transport K⁺.

The inebriated protein is expressed in nearly identical tissue patterns in the nervous and muscular systems of both _Manduca sexta_ and _Drosophila melanogaster_. However, differences in the expression patterns are observed in the gut, perhaps reflecting dietary differences between the two insect species. _Manduca sexta_ larvae feed on a diet high in K⁺, which is used for nutrient transport in the midgut. To maintain homeostasis, K⁺ in the hemolymph is transported into the midgut by a coupled K⁺/2H⁺ transport system (Harvey, 1992; Wieczorek et al., 1999). In the midgut, the V-ATPase in goblet cell apical membranes pumps H⁺ from the cytoplasm into the goblet cell cavity. The protons are exchanged with K⁺ by a K⁺/2H⁺ transport system (Harvey, 1992; Wieczorek et al., 1999). In the midgut, the V-ATPase in goblet cell apical membranes pumps H⁺ from the cytoplasm into the goblet cell cavity. The protons are exchanged with K⁺ by a K⁺/2H⁺ exchanger, resulting in net K⁺ transport from the goblet cell into the midgut. Both passive and active K⁺ transport processes are present in the goblet cell basolateral membrane (Chao et al., 1990; Moffett et al., 1982; Moffett and Lewis, 1990; Zeiske et al., 1986), indicating that several mechanisms of K⁺ transport exist in this membrane. We speculate that MasIne, localized entirely in the basolateral membrane of goblet cells, may play a role in K⁺ transport from the hemolymph into the goblet cells.
In lepidopteran insects, such as Manduca sexta, the Malpighian tubules, ileum, rectum and cryptonephridial system, a sac-like structure that packs Malpighian tubules tightly with the rectal epithelium, are all involved in the maintenance of salt and water balance in the hemolymph (Ramsay, 1976). In these tissues, MasIne is expressed at high levels only in regions involved in ion reabsorption (Phillips, 1981; Ramsay, 1976). The middle region of Malpighian tubules is not specialized for ion reabsorption, and MasIne expression in this region is very low. The glial cell layer and its processes form a blood–brain barrier around the neurons and neuropil, which constantly secretes Na⁺ and absorbs K⁺ (Schofield, 1989; Treherne and Schofield, 1981), so that high hemolymph K⁺ levels do not affect neuronal function. Potentially, MasIne in glial cells may regulate K⁺ absorption. MasIne expression in the axonal plasma membrane suggests that it could also modulate neuronal excitability by stimulating Ca²⁺-sensitive channels that cause membrane repolarization (Stern and Ganetzky, 1992).

This is the first report demonstrating the involvement of the PLC/InsP₃ signaling cascade in a member of the Na⁺/Cl⁻-dependent neurotransmitter transporter family. The mechanisms by which these processes are modulated are not known, and additional studies are needed to define the mechanisms involved more fully.

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References


