The Drosophila inebriated-Encoded Neurotransmitter/Osmolyte Transporter: Dual Roles in the Control of Neuronal Excitability and the Osmotic Stress Response

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ABSTRACT

Water reabsorption by organs such as the mammalian kidney and insect Malpighian tubule/hindgut requires a region of hypertonicity within the organ. To balance the high extracellular osmolarity, cells within these regions accumulate small organic molecules called osmolytes. These osmolytes can accumulate to a high level without toxic effects on cellular processes. Here we provide evidence consistent with the possibility that the two protein isoforms encoded by the inebriated (ine) gene, which are members of the Na+/Cl−-dependent neurotransmitter/osmolyte transporter family, perform osmolyte transport within the Malpighian tubule and hindgut. We show that ine mutants lacking both isoforms are hypersensitive to osmotic stress, which we assayed by maintaining flies on media containing NaCl, KCl, or sorbitol, and that this hypersensitivity is completely rescued by high-level ectopic expression of the ine-RB isoform. We provide evidence that this hypersensitivity represents a role for ine that is distinct from the increased neuronal excitability phenotype of ine mutants. Finally, we show that each ine genotype exhibits a “threshold” [NaCl]: long-term maintenance on NaCl-containing media above, but not below, the threshold causes lethality. Furthermore, this threshold value increases with the amount of ine activity. These data suggest that ine mutations confer osmotic stress sensitivity by preventing osmolyte accumulation within the Malpighian tubule and hindgut.

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osmolytes accumulated and the mechanism of accumulation can vary among species. For example, cells of the mammalian kidney epithelium respond to osmotic stress by accumulating osmolytes such as betaine (a derivative of glycine in which the three hydrogens of the \( \text{NH}_3^+ \) are replaced with methyl groups), which is accomplished by transport from the extracellular medium, and sorbitol, which is accomplished by \textit{de novo} synthesis (Uchida et al. 1989, 1993). In contrast, the yeast \textit{Saccharomyces cerevisiae} responds to osmotic stress by accumulating the osmolyte glycerol, which is accomplished by \textit{de novo} synthesis. In each case, however, hypertonic stress increases osmolyte accumulation at least in part by transcriptional induction of the genes enabling osmolyte accumulation (Meikle et al. 1988; Smardo et al. 1992; Uchida et al. 1993; Yamauchi et al. 1993; Ferraris et al. 1994). Thus, the hypertonic stress-induced accumulation of betaine within kidney cells and glycerol within yeast requires the transcriptional induction of the gene encoding the GABA/betaine transporter (BGT1) and the genes encoding the yeast glycerol synthetic enzymes, respectively. The mechanism(s) by which hypertonic stress triggers these transcriptional responses is unclear. Mechanical forces on the cell membrane caused by the hypertonicity-induced cell volume changes or the increases in intracellular \([\text{Na}^+]\) and \([\text{K}^+]\) have each been implicated (Uchida et al. 1989; Smardo et al. 1992). In addition, in both yeast and mammalian kidney cells, initiation of this response requires activation of the \textit{HOG/p38} family of mitogen-activated protein (MAP) kinases, perhaps other MAP kinases, and a cyclosporin-sensitive molecule (Brewster et al. 1993; Sheikh-Hamad et al. 1998).

In Drosophila, it was previously shown that the \textit{inedbrated} (\textit{ine}) gene, which affects neuronal excitability, encodes two protein isoforms that share certain similarities to BGT1 (Burg et al. 1996; Soehnge et al. 1996). In particular, both BGT1 and the \textit{Ine} proteins are members of the \textit{Na}^+/Cl\textit{\textsuperscript{-}}-dependent neurotransmitter/osmolyte transporter family (reviewed by Amara and Kuhar 1993). Members of this family of proteins share sequence similarity, a common structure of 12 transmembrane domains, intracellular domains at the N and C termini, and a 60- to 70-amino-acid-long extracellular loop between the third and fourth transmembrane domains. Substrates of members of this family include a variety of small-molecule neurotransmitters, metabolites such as creatine, and osmolytes such as betaine or taurine. In addition, the \textit{ine} isoforms and BGT1 are each expressed both in the central nervous system and in organs that perform water reabsorption: the Malpighian tubule/hindgut and kidney for \textit{ine} and BGT1, respectively (Borden et al. 1992; Rasola et al. 1993; Soehnge et al. 1996). Although the Drosophila protein encoded by the predicted gene CG 1732 (Flybase curation FBr0105495) has greater homology to BGT1 than \textit{Ine} (52\% identity for CG 1732 vs. 40\% identity for \textit{Ine}), the similar expression patterns for BGT1 and \textit{Ine} raised the possibility that the \textit{Ine} transporter might similarly perform dual roles: neurotransmitter reuptake in the central nervous system and osmolyte transport in the Malpighian tubule/hindgut. If so, then \textit{ine} mutants would be expected to be defective in osmolyte accumulation and thus should be more sensitive than wild type to maintenance on a hypertonic medium.

Here we show that \textit{ine} mutants are hypersensitive to maintenance on media containing elevated \([\text{NaCl}],[\text{KCl}],\) and \([\text{sorbitol}]\) and provide evidence that this hypersensitivity represents a role for \textit{ine} that is distinct from its role in the control of neuronal excitability. Furthermore, we show that both wild-type and \textit{ine} mutants exhibit a “threshold” response to osmotic stress: For each genotype, maintenance on media containing an \([\text{NaCl}]\) above this threshold causes inviability. We also assess the relative contributions to the osmotic stress response of each of the two isoforms of \textit{ine}. We find that each protein isoform, when independently overexpressed with the \textit{GAL}4 system (Brand and Perrimon 1993), can rescue the osmotic stress response defect of \textit{ine} mutants, suggesting that the two isoforms have similar functions. However, \textit{Ine-P2} alone, when expressed from its normal chromosomal position, is sufficient for only a small degree of resistance to osmotic stress. These results demonstrate a role for the \textit{ine}–encoded transporters in the osmotic stress response and introduce this response to genetic analysis in Drosophila.

**MATERIALS AND METHODS**

**Fly stocks:** Isolation of \textit{ine\textsuperscript{1}}, \textit{ine\textsuperscript{2}}, and \textit{ine\textsuperscript{3}} was described previously (Stern and Ganetzky 1992; Soehnge et al. 1996). The wild-type parent chromosome of each \textit{ine} mutant carries the eye color mutation \textit{bw} and was isogenized prior to use. Flies bearing the yeast \textit{GAL}4 gene driven transcriptionally by the heat-shock promoter (called \textit{hs-GAL}4) were kindly provided by Dan Kalderon. The \textit{Sh\textsuperscript{28}} and \textit{eag\textsuperscript{2}} mutants were provided by Bob Kreber and Barry Ganetzky. Flies bearing the duplication \textit{Dp (1;4) f\textsuperscript{r} f\textsuperscript{t}}, in which cytological region 14A-16A from the \(X\) chromosome was appended to the fourth chromosome, were also provided by Barry Ganetzky. This duplication includes the sodium channel gene \textit{para} and is thus called \textit{Dp para}. This duplication is maintained by complementation of the \(X\) chromosome deficiency \textit{Df(1)1\textsuperscript{iso}}.

**In situ hybridization:** To study the localization of each \textit{ine} isoform independently, we amplified by PCR DNA sequences at the 5′ end that are unique to each isoform: the first exon of \textit{ine-RB} and the first three exons of \textit{ine-RA}. These PCR products were subcloned into the pcDNA1 vector (Invitrogen, San Diego). T7 and Sp6 RNA polymerases were used to synthesize both antisense and sense RNA probes, which were labeled with digoxigenin. Hybridizations \textit{in situ} to whole-mount developing embryos were performed as described previously (Soehnge et al. 1996).

**Identification of the \textit{ine\textsuperscript{3}} mutation:** Previous experiments failed to identify the \textit{ine\textsuperscript{3}} mutation within the coding region of the \textit{ine-RA} transcript. Therefore, we sequenced the three exons specific for \textit{ine-RA} from genomic DNA from both \textit{ine\textsuperscript{3}} and \textit{iso bw} (isogenic \textit{ine\textsuperscript{3}} fly line). Four pairs of PCR primers (sequences listed below) were designed to amplify four fragments of genomic DNA that completely cover exons I, II, and
III of ine. PCR products amplified from the ine" mutant and iso
base were sequenced directly and compared with the BESTFIT
program of the GCG package.

The primers used were the following: ine2F1, CACTCCGA
CATGCTAAGT; ine2R1, CACACGGTGATTGGCATCA; ine2F2,
CTCCGCCATTTGGGAAGG; ine2R2, CTGAAGAGCTGAGCTG;
in2F3, CACCAACTGAGACTTAC; ine2R3, GCAACACAT
AAGTGTC; ine2F4, GGCTGAGTACTGAGTAC; and ine2R4,
CAGCATGAGGCTGAAGTC.

Construction of transgenic flies carrying UAS-ine-RB: The
full-length ine-RB cDNA was introduced into the EcoRI site of
the pUAST vector (Brand and Dörmund 1995) and injected into
yrw67c23 embryos for P-element-mediated germline transforma-
tion as described previously (Spradling 1986). One trans-
formant carrying UAS-ine-RB on the third chromosome was
obtained.

Viability assays on hypertonic media: Flies were grown in
uncrowded half-pint bottles and collected for only the first 4
days following the initial eclosions. Following etherization,
flies were aliquoted into groups of 20, placed into vials, and
allowed 1 day for recovery from etherization. Then flies were
transferred into vials containing instant medium (Carolina)
prepared according to the manufacturer's instructions, except
that the appropriate salt solution was used in place of water.
Fly viability was determined 4 days later by visual inspection.

For the threshold viability assays shown in Figure 5, all fly manipu-
lations and assays were conducted at 18° and 70% humidity,
and fly viability on salt media was determined every day for
10 days. For all other fly viability experiments, fly manipula-
tions and assays were conducted at room temperature and
ambient humidity.

RESULTS

Molecular and functional properties of inebriated: Mutations in
ine confer a number of nervous system phenotypes, including increased motor neuron excitability,
oscillations of the light-induced photoreceptor poten-
tial, and increased perineurial glial thickness in combi-
nation with mutations in pushover (push) and NF1 (Wu
and Wong 1977; Stern and Ganetzky 1992; Burg et
al. 1996; Yager et al. 2001). Two distinct ine cDNAs were
previously identified and sequenced (Burg et al. 1996;
Soehnge et al. 1996). As shown in Figure 1, A and B,
both cDNAs encode identical sequences in the region of
the protein homologous to neurotransmitter trans-
porters; however, the longer cDNA (ine RA) encodes a
943-amino-acid protein called Ine-P1, which contains
an N-terminal intracellular domain that is ~500 amino
acids longer than the shorter cDNA (ine RB, which en-
codes Ine-P2). Figure 1B shows the ine gene organiza-
tion. These two isoforms most likely arise from the use of
different promoters.

The extremely long N-terminal intracellular domain
observed in Ine-P1 is not commonly observed in members
of this transporter family. This observation raised the
possibility that this extended intracellular domain
reflected an additional Ine activity distinct from neuro-
transmitter transport. If so, then Ine-P1 and Ine-P2 might
perform distinct functions in Drosophila and thus might
exhibit different expression patterns. To test this possi-
bility, we constructed probes for in situ hybridization
that were specific for either the ine RA or the ine RB
cDNAs. As shown in Figure 1C, the embryonic expres-
sion patterns of the two cDNAs are virtually indistin-
guishable, suggesting that the two cDNAs function in
the same cells.

An osmotic stress-sensitive phenotype of ine mutants:
Two observations raised the possibility that ine might be required for osmolyte transport and thus for the Drosoph-
ila osmotic stress response. First, both forms of ine are
expressed robustly in fluid reabsorption tissues such as
the Malpighian tubule, hindgut, and anal plate (Soehnge
et al. 1996; Figure 1C), which together comprise the
invertebrate analog of the kidney. Second, transport of
the osmolytes betaine, taurine, and β-alanine into cells in
the mammalian renal medulla is accomplished
by transporters such as BG1 that are members of the
same transporter family as ine (Burg 1995). These obser-
vations raised the possibility that the Ine transporter
might function to transport osmolytes as well.

If Ine performs osmolyte transport in the Malpighian
tubules and hindgut, then ine mutants, which would be
defective in such transport, would be expected to be
more sensitive to osmotic stress than wild-type flies. To
test this possibility, we maintained three independently
isolated ine mutants and wild-type flies on media con-
taining various [NaCl] (Figure 2A). We found that ine
mutants exhibited viability similar to wild type when
maintained for 4 days on 0 m or 0.1 m [NaCl]. However,
in e and ine mutants exhibited significantly greater
lethality than wild-type or ine mutants when maintained
for 4 days on 0.2 m [NaCl]. Furthermore, whereas ~90%
of wild-type flies could survive maintenance on 0.4 m
[NaCl], ine and ine mutants exhibited essentially complete
viability on this [NaCl], and ine mutants exhibited
only slight viability. The abdomens of both wild-type and
ine mutants became progressively thinner during their
maintenance on lethal, but not sublethal, [NaCl]
(data not shown). This observation is consistent with
the possibility of desiccation, which could have contrib-
uted to the observed lethality.

To confirm that this reduced viability reflected in-
creased sensitivity to a hypertonic medium, rather than
increased sensitivity specific to NaCl, we tested the sensi-
tivity of ine mutants to elevated [KCl] and [sorbitol]. We
found that ine mutants displayed increased sensitivity to
both, although the sensitivity of both wild-type and ine
mutants to sorbitol was considerably less than the sensi-
tivity to NaCl and KCl (Figure 2, B and C). This signifi-
cantly reduced sensitivity to sorbitol compared to NaCl
and KCl suggests that the observed lethality in NaCl
and KCl might not arise solely from desiccation. One
possibility is that some of the NaCl and KCl provided
to the flies might accumulate intracellularly and contrib-
ute to lethality. Alternatively, the reduced sensitivity to
sorbitol might result from some ability of sorbitol to
cross the cell membrane, which would give sorbitol a
partial osmoprotective effect. As with NaCl, ine mutants
We found that neither of the isozymes is specifically sensitive to hypertonic stress, we compared the overexpression of Ine-P2 is sufficient for a normal osmotic stress response demonstrates that Ine-P1 is required for most of, but not all of, the osmotic stress response. Ine-P2 alone is sufficient for a small amount of osmotic stress response.

Specific elimination of Ine-P1 in ine2 mutants: The phenotype of ine1 and ine2 mutants most likely represents the null phenotype: ine1 is a deletion mutation that removes most of the open reading frame, and ine2 mutants produce undetectable levels of mRNA from either of the ine isoforms (SOEHNGE et al. 1996), although the ine1 sequence change was not identified. The observation that ine2 mutants survive significantly better than ine1 and ine2 mutants on media containing 0.2 M NaCl or 0.2 M KCl suggested that the ine2 mutation does not completely eliminate Ine activity. To identify the ine2 mutation, we compared the sequence of ine in the ine2 mutant and in the isogenic wild-type strain. We found that ine2 is a nonsense mutation in codon 125 of the Ine-P1 isoform (see Figure 1). This mutation is expected to leave Ine-P2 unaffected. The observation that the ine2 mutant retains partial activity for the osmotic stress response demonstrates that Ine-P1 is required for most of, but not all of, the osmotic stress response. Ine-P2 alone is sufficient for a small amount of osmotic stress response.

Overexpression of Ine-P2 is sufficient for a normal osmotic stress response: An additional way to assess the sensitivity of ine mutants to NaCl, even in the absence of heat shock (not shown). In addition, we constructed flies carrying ine-RB under transcriptional control of the heat-shock promoter (constructed and kindly supplied by M. Burg and W. Pak; BURG et al. 1996) completely rescued the increased sensitivity of ine mutants to NaCl, even in the absence of heat shock (not shown). In contrast, ine mutants expressing either the hs-GAL4 line or the UAS-ine-RB line alone exhibited slightly better survival than ine1 and ine2 mutants when maintained on media containing 0.2 M [KCl], although the difference was less extreme than the difference observed on NaCl-containing media.

To test the possibility that ine mutants might be hypersensitive to any environmental stress, rather than specifically sensitive to hypertonic stress, we compared the sensitivity of ine flies and wild type to two types of heat-shock stresses: long-term maintenance at a temperature of 34°C and 3-hr heat shocks at 37°C during long-term maintenance at room temperature. We found that ine flies displayed the same viability as wild type to these stresses (not shown).

Figure 1.—Organization and expression of ine. (A) The putative membrane topology of the two Ine protein isoforms. The two isoforms are identical except that Ine-P1 (encoded by ine-RA) contains an N-terminal intracellular region that is ~300 amino acids longer than the corresponding region in Ine-P2, encoded by ine-RB. This 300-amino-acid extension, unlike the region common to both proteins, has no similarity to other transporters. Potential sites of N-linked glycosylation and phosphorylation are indicated. (B) Map of the ine region showing the exons of the two ine isoforms and the location of two identified mutations. The ine1 mutation is a nonsense mutation at codon 126 of ine-RA, whereas ine2 is a deletion that removes most of the open reading frame common to both transcripts and begins at codon 293 of ine-RA. Although ine1 has not been localized, it was previously shown by Northern blot analysis that transcripts of both ine isoforms are undetectable in the ine1 mutant (SOEHNGE et al. 1996). (C) Whole-mount in situ hybridization using ine-RB- and ine-RA-specific probes. Both isoforms exhibit very similar expression patterns in these developing embryos. Expression is found in the hindgut (hg), Malpighian tubules (mt), and the central nervous system (CNS) along the ventral midline and brain lobes (bl). (Top) Lateral view. (Top) Dorsal view.
to rescue is a result of its overexpression by the Gal4 system, although this overexpression has not been demonstrated.

Independent roles for *ine* in the control of neuronal excitability and the osmotic stress response: The osmotic stress-sensitive and neuronal excitability phenotypes of *ine* could reflect independent roles for *ine* in these two processes. In this view, *ine* expression in the central nervous system (CNS) controls neuronal excitability, whereas *ine* expression in the Malpighian tubes and hindgut is required for a proper osmotic stress response. However, it is also possible that these two types of defects reflect a common underlying cause. For example, it is possible that the behavioral changes caused by hyperexcitability in *ine* mutants in some way reduce the ability of the fly to survive a hypertonic environment (for example, by altering feeding behavior).

To distinguish between these possibilities, we tested other mutants with neuronal excitability defects similar to *ine* for osmotic stress sensitivity. Flies tested were mutant for either Shaker (*Sh*) or *ether a go-go (*eag*), which encode distinct potassium channel α-subunits, or flies carrying a duplication of the *paralytic* (*para*) gene (which we term *Dp para*), which encodes a sodium channel. Each genotype confers a motor neuron hyperexcitability phenotype that is similar to the phenotype of *ine* mutants (*Jan et al. 1977; Ganetzky and Wu 1983; Stern et al. 1990; Stern and Ganetzky 1992*). The osmotic stress-sensitive phenotypes of *eag* mutants and flies bearing *Dp para* were of particular interest, because *ine* mutations, *eag* mutations, and *Dp para* interact with *Sh* mutations in an identical manner: *eag* Sh and *Sh; ine* double mutants, and *Sh* mutants carrying *Dp para*, each show a characteristic “downturned wings and indented thorax” phenotype (*Stern et al. 1990; Stern and Ganetzky 1992*), which is not exhibited by any of the single mutants described above. The notion that the osmotic stress-sensitive defect of *ine* mutants is causally related to the neuronal hyperexcitability defect would be supported by observing a similar osmotic stress-sensitive defect in *eag* Sh, or *Dp para* flies.

As shown in Figure 4, none of the other excitability mutants tested exhibited a sensitivity to NaCl comparable to *ine* mutants. These results indicate that neuronal hyperexcitability by itself fails to confer sensitivity to NaCl and thus that neuronal excitability and osmotic stress response are separable processes. These results are consistent with the hypothesis that the *ine* phenotype of osmotic stress sensitivity reflects loss of osmolyte transport in the hindgut and Malpighian tubule, rather than a secondary consequence of a neuronal excitability defect. This possibility is supported by the observation that flies in which *ine* expression is driven only in neurons by the *elav-GAL4* construct exhibit rescue of the “downturned wings” phenotype of *Sh; ine* double mutants, but fail to exhibit any rescue of the osmotic stress-sensitive phenotype (not shown).

**Wild-type flies and *ine* mutants exhibit different thresholds for maintenance on NaCl:** The data shown in Figures 2–4 above demonstrate that *ine* mutants, but not
wild-type flies, die following maintenance on media containing 0.2 or 0.4 m NaCl. However, because these data represent viability at only a single time point, no information on mortality kinetics is contained. We compared the rate of death of ine1, ine2, and wild-type flies on media containing varying [NaCl]. We found that each genotype exhibited a “threshold” [NaCl]: Flies maintained on media containing [NaCl] below the threshold exhibited very little lethality, even after 9 days of maintenance on the hypertonic medium. However, flies maintained on media containing [NaCl] above the threshold died quickly (death typically began within 3–5 days following addition to the hypertonic media) and continuously until, after 9 days upon NaCl-containing media, <10% of the flies remained alive. The [NaCl] at which this threshold response occurred depended on the allele present at ine. As shown in Figure 5, wild-type flies exhibited an [NaCl] viability threshold between 0.5 and 0.6 m [NaCl]. In contrast, ine2 mutant flies exhibited a [NaCl] viability threshold between 0.15 and 0.2 m [NaCl]. Finally, ine1 mutants exhibited a threshold concentration between 0.2 and 0.25 m [NaCl], which is intermediate between wild type and ine2 and mutants. Thus, there is a close correlation between the strength of the mutant allele at ine and the sensitivity of the fly to osmotic stress. This observation suggests that threshold [NaCl] is determined, at least in part, by the amount of osmolyte accumulation that can be performed in the Malpighian tubule and hindgut.

**DISCUSSION**

**A role in osmolyte accumulation for the ine-encoded transporter:** Osmolyte accumulation and neurotransmitter reuptake can each be performed by members of the Na+/Cl−-dependent family of neurotransmitter/ osmolyte transporters (Borden et al. 1995; Rasola et al. 1995). In fact, one member of this transporter family, the mammalian betaine/GABA transporter BGT1, appears to have a dual function. BGT1 is expressed in both the kidney and the brain: In the brain, this transporter presumably performs reuptake of GABA following release into synapses, whereas in the kidney this transporter presumably enables the accumulation of the osmolyte betaine. The previous observation that the Drosophila ine-encoded transporter is expressed in the Malpighian tubule and hindgut as well as in certain cells within the central nervous system raised the possibility that this transporter might also perform such a dual function.

In this study, we provide functional evidence that supports this possibility. We show that ine mutants are more sensitive than wild type to maintenance on a hypertonic medium, that this hypersensitivity apparently occurs independently of the role of ine in the control of neuronal excitability, and that levels of ine transporter define a threshold [NaCl] above which the flies cannot survive for prolonged periods. We also show that the Ine short form, Ine-P2, can confer some osmotic stress resistance in the absence of Ine-P1, but overexpression is required to confer full resistance. We propose that the osmotic stress-sensitive phenotype of ine mutants results from an inability to accumulate osmolytes within the Malpighian tubule and hindgut.

**Relative roles of the two ine isoforms in the osmotic stress response:** Two ine isoforms have been identified, a short form (Ine-P2) and a long form (Ine-P1). The N-terminal intracellular domain of Ine-P1 is ~300 amino acids longer than the N-terminal intracellular domain of Ine-P2; the two isoforms are otherwise identical. The extremely long N-terminal domain of Ine-P1 is unusual among members of this transporter family, and it is unclear what function, if any, this extended domain might confer upon ine-P1 activity. Previous work on the rat GABA transporter suggested that both the N- and C-terminal intracellular domains were dispensable for transport activity (Mabjeesh and Kanner 1992), although a more recent report on studies on the norepinephrine transporter suggested that the C terminus might be required for activity (Liu et al. 1998). It is possible that this domain produces a signal independent of transport activity, such as signal transduction in response to substrate binding (for example, see Alonso et al. 1999). Alternatively, the extended domain might affect transporter localization or activity. Finally, the extended domain might have no discernible effect on protein function.

We have addressed this question by assaying the osmotic stress-sensitive phenotype of mutants expressing or overexpressing Ine-P2 alone. We found that ine2 mutants, which express only Ine-P2, survive osmotic stress slightly, but significantly, better than ine null mutants.
These results argue against the possibility that Ine-P1 has a novel function, not shared with Ine-P2, that is required for the osmotic stress response. Rather, these results suggest that Ine-P2 can perform all functions required for osmotic stress response that Ine-P1 can perform. The requirement for Ine-P2 overexpression for complete phenotypic rescue could be the result of insufficient translation, insufficient transport activity, or incorrect protein transport or localization. Because a mutation that selectively removes Ine-P2 is not available, we have no way of monitoring the relative ability of each isoform to rescue the osmotic stress phenotype. However, recent experiments on the relative ability of Ine isoforms to rescue the ine mutant neuronal phenotypes have suggested that although each isoform is active in the absence of the other, Ine-P1 is more active than Ine-P2 (Y. Huang and M. Stern, unpublished results; M. Burg and W. Pak, personal communication).

A role for Ine in response to hyperosmolarity has been suggested independently by Chiu et al. (2000). These investigators cloned the inebriated homolog from Manduca sexta, called Masine, and reported two isoforms: a short form and a long form containing an additional 108 amino acids at the N terminus. Injection of the long form, but not the short form, into Xenopus oocytes elicited a hyperosmolarity-induced Cl current, which was attributed to a phospholipase C-mediated activation of a Ca2+ flux. Furthermore, the additional 108 amino acids in MasIne-long was sufficient to confer a similar Cl current when appended to the GABA transporter GAT1. Thus, Chiu et al. (2000) also suggest a role for Ine in response to hyperosmolarity, although the mechanism that they suggest (induction of a Ca2+-activated K channel) is quite different from the mechanism of osmolyte accumulation that we propose. One possibility is that Ine and Mas-Ine utilize different mechanisms to respond to hyperosmolarity. The observation that the 108-amino-acid domain of MasIne-long shares only 9 amino acids with Ine-P1 is consistent with this possibility. Alternatively, Ine and Mas-Ine might each use both mechanisms to respond to hyperosmolarity. Further research will be required to distinguish between these possibilities.

A possible mechanism for organismal lethality in response to hypertonic stress: When cells are placed in a hypertonic media, an extremely rapid loss of water is followed by influx of Na+ and K+. This influx of ions causes the passive return of water to the cell, thus enabling cell volume to be recovered. However, this influx also increases the intracellular [Na+] and [K+] with detrimental consequences to the activity of essential cellular functions. To accommodate to osmotic stress, cells then replace the intracellular Na+ and K+ with nonperturbing osmolytes such as betaine, sorbitol, inositol, or glycerol. This replacement thus enables a restoration of normal cell volume and normal intracellular [Na+] and [K+]. We propose that the Ine transporter
plays an important role in this replacement by enabling the transport of an osmolyte, the substrate for the Ine transporter, which has not yet been identified. Thus, in *ine* mutants this replacement fails to occur properly, and, following osmotic stress, the elevated Na$^+$ and K$^+$ levels within hindgut epithelial cells persist for the duration of the exposure to osmotic stress. This long-term exposure could kill these epithelial cells by either necrotic or apoptotic mechanisms, as suggested by Sheikh-Hamad et al. (1998).

Although *ine* mutants are considerably more sensitive to osmotic stress than wild type, even *ine* null mutants can survive exposure to [NaCl] of $\sim$0.15 M. This low-level viability could reflect *ine*-independent osmolyte accumulation mechanisms, which would enable some capacity for Na$^+$ replacement even in the absence of *ine*. Alternatively, it is possible that *ine* null mutants are completely defective in osmolyte accumulation and the viability of *ine* mutants in low [NaCl] reflects the ability of hindgut epithelial cells to survive elevated intracellular [Na$^+$] for prolonged periods.

**Ability to accumulate osmolytes generates a [NaCl] viability threshold:** The mechanism for osmotic stress-induced lethality proposed above is supported by the observation that flies exhibit a sharp [NaCl] survival threshold. In particular, flies maintained on media containing any [NaCl] below the threshold concentration were able to survive prolonged maintenance on medium containing NaCl. In contrast, flies maintained on media containing any [NaCl] above the threshold were unable to survive: these flies showed essentially complete lethality after 6–9 days, depending on the [NaCl] of the media. The [NaCl] at which this viability threshold occurred was controlled by the genotype at *ine*: Mutants null for *ine* exhibited a viability threshold between 0.15 and 0.2 M [NaCl], whereas *ine$^+$* mutants, in which only Ine-P2 is expected to be produced, exhibited a viability threshold between 0.2 and 0.25 M [NaCl], and wild-type flies, in which both Ine isoforms are expected to be present, exhibited a viability threshold between 0.5 and 0.6 M [NaCl]. We speculate that this viability threshold reflects the maximum amount of osmolyte accumulation that is possible for flies of each genotype. When flies are exposed to [NaCl] above this maximum level, residual Na$^+$ accumulates intracellularly, and death results by mechanisms described above.

**Signaling pathways regulating the osmotic stress response:** From studies in yeast, and in canine kidney cells grown in culture, some of the signaling pathways required for induction of the osmotic stress response have been identified. In each system, osmotic stress activates the HOG/p38 MAP kinase system, and this activation is required for the transcriptional induction of the genes encoding osmolyte-accumulating enzymes (glycerol synthetic enzymes and the betaine transporter for the yeast and canine kidney cells, respectively; Brewster et al. 1993; Sheikh-Hamad et al. 1998). In addition, a role for calcineurin in tolerance to NaCl was identified in yeast (Garrett-Engele et al. 1995), whereas osmotic stress response in kidney cells requires a cyclosporine-sensitive molecule (D. Sheikh-Hamad, personal communication). The signaling pathways and mechanisms by which Drosophila respond to osmotic stress remain to be identified. However, the identification of a role for Ine in this process opens the way for this phenomenon to be studied with genetic methods.

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**LITERATURE CITED**


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