

**BETAINE TRANSPORT IN GIANT FIBER LOBES OF THE SQUID
*LOLLIGUNCULA BREVIS***

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ABSTRACT: Although it is known that cephalopods and other marine molluscs accumulate organic osmolytes to prevent cellular water loss in the high salinity of seawater, very few studies on molluscs, and no studies on cephalopods have been performed to characterize the underlying transport processes. We used [³H] betaine to quantify the uptake of betaine into giant fiber lobe motor neurons of the squid *Lolliguncula brevis*. We found that betaine uptake was Na⁺ and Cl⁻ dependent with a specific uptake rate of 7.7 fmoles [³H] betaine/μg protein/min. In addition, we found that 100 μM niflumic acid blocked 85% of betaine uptake. As expected for an osmoregulatory transporter, exposure to hypotonic seawater inhibited betaine uptake by 95%. These data indicate that squids use the betaine transporter to accumulate betaine, and support previous electrophysiological recordings of a betaine-induced Na⁺-dependent Cl⁻ current that is thought to be linked to the betaine transporter.

INTRODUCTION

The brief squid, *Lolliguncula brevis*, is one of few species of squids that are euryhaline or able to tolerate wide changes in salinity. *L. brevis* is an osmoconformer and adjusts its blood osmolality to values slightly hypertonic to the surrounding seawater (Hendrix *et al.*, 1981). When exposed to high salinity seawater, most marine invertebrates accumulate small organic osmolytes such as glycine-betaine (betaine) or taurine to prevent water loss and cell shrinkage (Wright and Want, 1992; Yancey *et al.*, 1982). The measurement of betaine concentrations in cephalopod blood and axoplasm of 4 mM and 75 mM, respectively (Deffner 1961), suggests that there is a betaine transporter in cephalopod neurons. A Na⁺-dependent betaine transporter has been characterized in the gills of the marine mussel *Mytilus californianus* (Wright and Want, 1992); however, betaine transport has not previously been studied in any tissue from any species of cephalopod. In the present studies, we used [³H] betaine to measure betaine uptake in giant fiber lobe (GFL) neurons from the squid *Lolliguncula brevis*.

In squid, the easily identified GFL is a triangular extension of the stellate ganglion. The

GFL contains the cell bodies of third order motor neurons that send out axons, which fuse within the stellate ganglion and form the giant axons. The squid giant axons, best known for studies that determined the ionic basis of the action potential (Hodgkin and Huxley, 1952), innervate the mantle musculature and allow the animals to make rapid jet propelled movements. Several groups have utilized the GFL as a source of neuronal cells which express many of the same proteins found in the giant axons (Gilly *et al.*, 1990, He *et al.*, 1998, Llano and Bookman, 1986, Rosenthal *et al.*, 1996). The advantage of using GFL somata for patch clamp studies is that the cell bodies are round, of suitable size for maintaining voltage control, and do not contain the Schwann cell sheath that envelops the unmyelinated giant axons. In our recent patch clamp studies of squid GFL neurons, we found that betaine activated a Cl⁻ current that showed a dependency on Na⁺ and osmolality that would be expected for a betaine transporter (Petty and Lucero, 1999). In the present study, we measured the uptake of [³H]betaine under similar ionic and pharmacological conditions as in our previous electrophysiological studies except that whole GFLs were used rather than isolated neurons. We found that a betaine transporter is

present in GFLs, and that it has ionic and pharmacological dependencies that are similar to the betaine-induced Cl^- currents in isolated GFL neurons.

MATERIALS AND METHODS

Lolliguncula brevis were flown in from the National Resource Center for Cephalopods, Galveston, Texas, and immediately killed by decapitation. The GFLs of the stellate ganglia (2 GFLs/animal) were excised under a dissecting scope, and placed in nonspecific protease (10 mg/ml, Sigma type XIV) in sterile filtered artificial seawater (ASW) for 40 min to improve betaine permeation and to mimic the protocol used in our patch clamp studies (Petty and Lucero, 1999). Following a 3–5 min rinse in ASW, the semi-intact GFLs were placed in 35 mm culture dishes containing squid culture media (Lucero and Chen, 1997). The lobes were kept in a 22 °C incubator and used for uptake studies within 12 hours. ASW in mM consisted of 340 NaCl, 10 Hepes, 17.5 glucose, 35 MgCl_2 , 4.25 NaOH, 10 KCl, 10 CaCl_2 (pH 7.4). For 50 mM betaine ASW, 50 mM NaCl was replaced with 50 mM betaine-HCl and the resulting solution was set to pH 7.4 with NaOH. For ionic dependence experiments, equimolar Li^+ or gluconate $^-$ was substituted for Na^+ and Cl^- , respectively. For all except the low osmolality experiments (540 mOsm/kg), an ASW osmolality of 780 mOsm/kg was used because it matched the average salinity of the seawater in which *L. brevis* live and our previous patch clamp experiments were performed at 780 mOsm/kg.

Uptake Studies

Tritiated betaine was synthesized from [methyl- ^3H]choline (1 mCurie/ml; New England Nuclear, Boston, MA, USA) according to the methods described in (Meister *et al.*, 1996). The specific activity of [^3H]betaine was 75 Curies/mmole; 40 picomoles of labeled betaine were loaded into 1.5 ml centrifuge tubes, lyophilized, and stored at -80 °C until used. Shortly before each experiment, the tubes were thawed, loaded with 50 μl of the incubation solution, vortexed

vigorously and centrifuged for 10 s. The concentration of [^3H]betaine in each tube was 0.6–0.8 μM . For the Na^+ or Cl^- free experiments, GFLs were washed in Na^+ or Cl^- free ASW for 10 min prior to the experiment. One GFL was placed in each tube and gently agitated for 10, 20, 30 or 45 min at room temperature (21–23 °C). Following the incubation, each tube was centrifuged briefly (15 s) and the wash supernatant (wSN) was discarded. The GFL was immediately rinsed with 1 ml ice-cold 50 mM betaine followed by two additional 1 ml rinses with ice-cold ASW. The GFL was resuspended in 200 μl of 0.25 N NaOH, sonicated, and centrifuged at 14,000 rpm for 10 min (Eppendorf Micro Centrifuge); 50 μl of the test supernatant (tSN) from each vial was counted on a 1500 TriCarb scintillation counter (Packard Instrument Co., Downers Grove, IL). The rate of betaine uptake was obtained by converting decays per min (DPM)/50 μl tSN to obtain fmoles [^3H]betaine/ μg protein/incubation time. Betaine uptake rates for each condition were averaged and are presented as a mean \pm SEM; n=number of individual GFL preparations tested under each condition. All test conditions were significantly different from control using student's T-test (two independent populations) with $p < 0.05$ or less.

Protein assays were conducted in triplicate on each tSN using a dotMETRIC 1 μl protein assay kit (Chemicon International, Inc., Temecula, CA) and following the manufacturer's protocol for the microassay.

RESULTS

The time course for betaine uptake was determined by plotting the averaged amount of [^3H]betaine/ μg protein taken up by GFLs at 10, 20, 30 or 45 min (Fig. 1, solid squares). The slope of the linear regression fit to the data yielded a total betaine uptake rate of 8.2 ± 0.3 fmoles [^3H]betaine/ μg protein/min (Fig. 1, solid line). To determine the amount of nonspecific binding of the labeled betaine to the tissue or tube, parallel experiments were run in which 50 mM unlabeled betaine was included in the [^3H] betaine mixture (Fig. 1, open circles). The slope of the linear regression fit to the non-specific binding data

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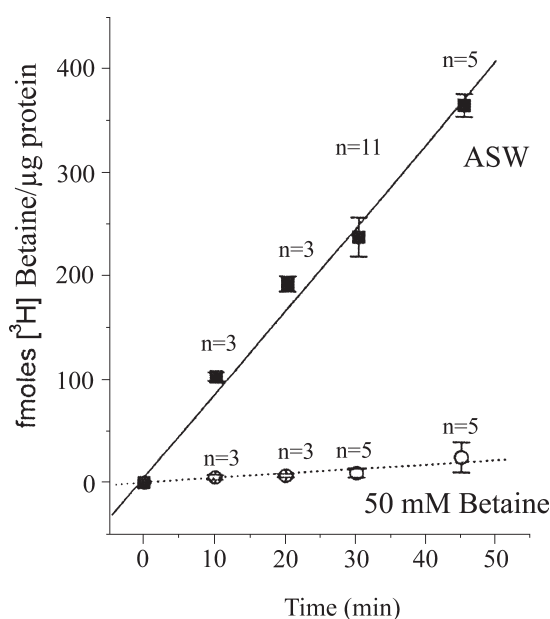


Figure 1. The rate of [^3H]betaine uptake is linear over 45 min. Squid giant fiber lobes (GFLs) were incubated in ASW containing $0.8 \mu\text{M}$ [^3H]betaine for 10, 20, 30, and 45 min (solid squares). Addition of unlabeled 50 mM betaine dramatically reduced the uptake rate of $0.8 \mu\text{M}$ [^3H]betaine (open circles). The lines are linear fits to the data. Error bars indicate SEM, n indicates number of GFL preparations.

yielded a rate of 0.6 ± 0.1 fmoles [^3H] betaine/ μg protein/min (Fig. 1, dotted line). By subtracting the nonspecific rate from the total rate, we obtained a specific uptake rate of 7.7 fmoles [^3H] betaine/ μg protein/min. As with other betaine transporters, the rate of betaine uptake was linear beyond 30 min. Therefore, the remaining experiments were performed with 30 min incubations and are reported as fmoles [^3H] betaine/ μg protein/30 min. The nonspecific betaine uptake at 30 min (11.9 ± 3.0 fmoles [^3H] betaine/ μg protein; $n=5$), determined by including 50 mM unlabeled betaine in the [^3H] betaine incubation mixture, has been subtracted from all of the following data.

Betaine uptake was dependent on the presence of external Na^+ and Cl^- . Fig. 2 shows that incubation of GFLs in 0 Na^+ ASW, in which all Na^+ was replaced with equimolar Li^+ , significantly reduced betaine uptake. Specific [^3H]betaine

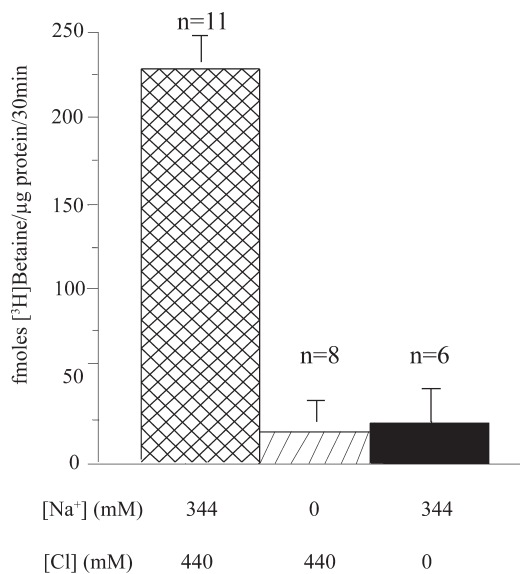


Figure 2. [^3H]betaine uptake is dependent on external Na^+ and Cl^- . GFLs were incubated for 30 min in 344 Na^+ ASW (first column), 0 Na^+ ASW (second column), or 0 Cl^- ASW (third column) containing $0.8 \mu\text{M}$ [^3H] betaine. For 0 Na^+ ASW, Na^+ was replaced by equimolar Li^+ or Tris^+ ; for 0 Cl^- ASW, Cl^- was replaced by equimolar d-gluconate. Error bars indicate SEM, n indicates the number of GFL preparations.

uptake decreased by 92%, from 228.6 ± 19.1 fmoles/ μg of tissue/30 min ($n=11$) in the control condition to 18.3 ± 19.1 ($n=8$) in the absence of external Na^+ . The Na^+ -dependence of betaine uptake was similar to the Na^+ -dependence of the betaine-induced current in GFL neurons, which was reduced by 95% in 0 Na^+ ASW (Petty and Lucero, 1999). Replacement of Cl^- in the external bath solution with equimolar d-gluconate significantly reduced the uptake of [^3H] betaine by 89% to 24.5 ± 20.1 fmoles/ μg of tissue/30 min ($n=6$). Taken together, these data indicate that like the mammalian betaine transporter (Moeckel *et al.*, 1997), the squid betaine transporter is dependent on the presence of both Na^+ and Cl^- in the external solution.

In our electrophysiological studies of the betaine transporter in squid GFL neurons, we found that the Cl^- channel blocker, niflumic acid,

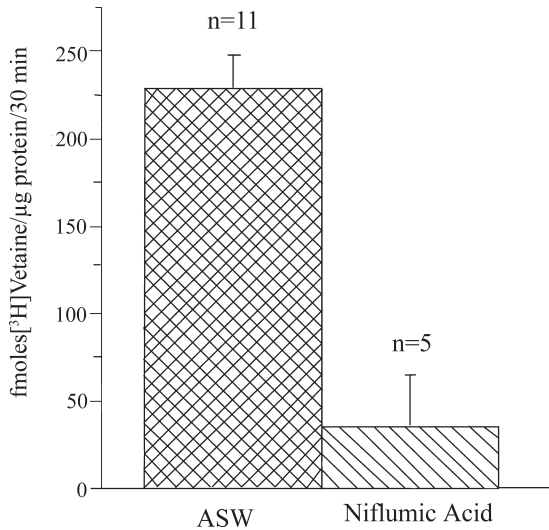


Figure 3. $[^3\text{H}]$ betaine uptake is blocked by niflumic acid. Squid GFLs were incubated for 30 min in $0.8 \mu\text{M}$ $[^3\text{H}]$ betaine in the absence (first column) and presence (second column) of $100 \mu\text{M}$ niflumic acid. Inclusion of niflumic acid significantly reduced $[^3\text{H}]$ betaine uptake ($p < 0.05$). Error bars indicate SEM, n indicates the number of GFL preparations.

reversibly blocked the betaine-induced Cl^- current (Petty and Lucero, 1999). Fig. 3 shows that $100 \mu\text{M}$ niflumic acid inhibited control $[^3\text{H}]$ betaine uptake by 85%, when incubated for 30 min with GFLs (35.0 ± 29.8 fmoles/ μg of tissue/30 min ($n=6$). This inhibition was significant at $p < 0.05$ (student's T-test, two independent populations).

Our electrical studies of the betaine transporter showed that the betaine-induced Cl^- current was inhibited by hypotonic 580 mOsm/kg ASW (Petty and Lucero, 1999). In the present studies, we found that $[^3\text{H}]$ betaine uptake was inhibited by 95% in GFLs incubated for 30 min in 580 mOsm/kg ASW (11.2 ± 13.4 fmoles/ μg of tissue/30 min; $n=6$) compared to 780 mOsm/kg ASW (Fig. 4). In order to distinguish between the effect of the 30% reduction in external Na^+ and the 30% reduction in osmolality, we added 200 mM mannitol to the 580 mOsm ASW (200 mM mannitol ASW). We observed an 8.8 fold increase in betaine uptake in the 200 mM mannitol ASW

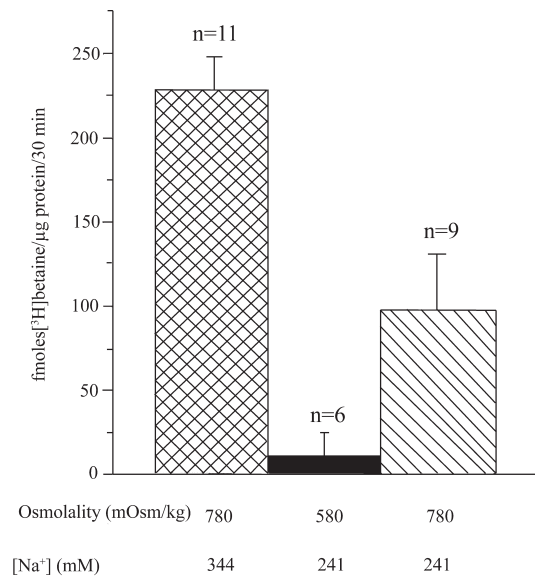


Figure 4. Hypotonic seawater suppresses betaine uptake. A 30 min incubation of GFLs in $0.8 \mu\text{M}$ $[^3\text{H}]$ betaine in ASW diluted to 580 mOsm/kg (second column) significantly reduced betaine uptake compared to 780 mOsm/kg ASW (first column; $p < 0.05$). Addition of 200 mM mannitol to the 580 mOsm ASW resulted in a partial recover of $[^3\text{H}]$ betaine uptake (third column). Error bars indicate SEM, number indicate number of GFL preparations.

compared to 580 mOsm ASW. Therefore, the 95% inhibition of betaine uptake observed in 580 mOsm ASW had two components; roughly half of the inhibition was due to reduced $[\text{Na}^+]_o$ and half was due to reduced osmolality. These data indicate that reduced osmolality is an effective inhibitor of the squid betaine transporter.

DISCUSSION

In previous electrophysiological studies, we showed that the organic osmolyte, betaine, activated a Cl^- current in GFL neurons of the squid *Lolliguncula brevis* (Petty and Lucero, 1999). Our initial hypothesis was that betaine was acting at a receptor similar to the betaine receptor that we have characterized in squid olfactory receptor neurons (ORNs; 1). To our surprise, we found that unlike the ORN betaine receptor, the electrical

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betaine responses in GFL neurons showed the characteristics of a betaine transporter; betaine-induced Cl^- currents were dependent on external Na^+ , reversibly blocked by $100\ \mu\text{M}$ niflumic acid, and completely inhibited by hypotonic seawater. As others have proposed for neurotransmitter transporters (DeFelice and Blakely, 1996; Lester *et al.*, 1994), we postulated that the betaine osmolyte transporter was capable of acting like a Cl^- channel and that the betaine-induced Cl^- currents reflected betaine transporter activity.

In the present work, we measured [^3H]betaine uptake in semi-intact GFLs and found that the uptake was specific for betaine, and could be inhibited by competition with excess amounts of unlabeled betaine. As has been shown for other betaine transporters (Moeckel *et al.*, 1997; Wright and Want, 1992), we found that betaine uptake was dependent on external Cl^- and Na^+ . Our finding that betaine uptake was inhibited by $100\ \mu\text{M}$ niflumic acid has not previously been reported for betaine transporters, however it agrees with the reversible block of the betaine-induced Cl^- current that we observed in patch-clamp experiments on GFLs (Petty and Lucero, 1999).

Betaine uptake was also inhibited by hypotonic seawater. The concentration of seawater that we chose for hypotonicity experiments ($540\ \text{mOsm/kg}$) was based on a study by Hendrix *et al.* (1981) which showed that *L. brevis* are unique among cephalopods in their ability to survive in the low salinity coastal seawater in the Gulf of Mexico. Our data indicate that a portion of the 95% reduction in betaine uptake in the hypotonic seawater was due to the 30% reduction in external Na^+ (from $344\ \text{mM Na}^+$ to $241\ \text{mM Na}^+$) and the remainder was due to osmotic effects on betaine

transport. Consistent with the present uptake studies, our previous patch clamp studies showed that hypotonic ASW eliminated the betaine-induced Cl^- current (Petty and Lucero, 1999). Under both experimental conditions (patch clamp and betaine uptake), ~50% of the 580 ASW inhibition could be reversed by increasing the osmolality without changing $[\text{Na}^+]_o$. Acute inhibition of betaine uptake by hypotonic ASW has been reported in the marine mussel *Mytilus californianus* to have similar Na^+ and osmotic components (Wright and Want, 1992). The latter proposed that the hypotonic inhibition of osmolyte transport systems is a protective mechanism to prevent the loss of metabolically expensive metabolites such as betaine, during transient exposure to reduced salinity seawater.

Although direct comparisons between charge movement in isolated GFL neurons and betaine uptake in semi-intact GFLs cannot be made, the similarities between the electrophysiological and uptake studies in terms of Na^+ dependence, inhibition by niflumic acid and effect of hypotonicity are striking. The findings in the present study support our hypothesis that the squid betaine transporter is tightly linked to the betaine-induced Cl^- currents in squid GFL neurons.

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