Monoamine transmitters and cAMP stimulation of Na transport in freshwater mussels

THOMAS H. DIETZ, JOHN I. SCHEIDE, AND DAVID G. SAINTSING
Department of Zoology and Physiology, Louisiana State University, Baton Rouge, LA, U.S.A. 70803
Received September 16, 1981

DIETZ, T. H., J. I. SCHEIDE, and D. G. SAINTSING. 1982. Monoamine transmitters and cAMP stimulation of Na transport in freshwater mussels. Can. J. Zool. **60**: 1408–1411.

The unionid mussels *Ligumia subrostrata* and *Carunculina texasensis* maintain a sodium steady state in artificial pond water. When the mussels were injected with serotonin, catecholamines, or phenylephrine ($<2 \times 10^{-5}$ *M*/L blood), the influx of Na was elevated 150–220% above controls and the animals accumulated Na from the medium. A similar response was observed with injections of dibutyryl cyclic AMP. Isolated gills of mussels accumulate Na from a pond-water bathing medium and the Na transport rate was stimulated by serotonin but not catecholamines. Serotonin was present in the gill tissue (2.26 ± 0.18 μg/g wet gill). Serotonergic neurons innervated by adrenergic fibers may be directly stimulating the Na transporting tissues in the gill of freshwater mussels.

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Les unionidés *Ligumia subrostrata* et *Carunculina texasensis* maintiennent leur concentration de sodium en eau d'étang artificiel. L'injection de sérotonine, de catécholamines ou de phenyléphrine ($<2 \times 10^{-5}$ *M*/L sang) entraîne chez les moules une augmentation de 150 à 220% de l'influx de Na et les moules accumulent du sodium extrait du milieu. Des injections de dibutyril-AMP cyclique causent les mêmes effets. Des branchies isolées de moules accumulent du Na extrait du milieu d'eau d'étang et la vitesse de transport du Na est accrue par la sérotonine, mais pas par les catécholamines. La sérotonine se retrouve dans le tissu branchial ($2,26 \pm 0,18$ μg/g de branchie (poids frais)). Il est possible que des neurones sérotonergiques innervés par des fibres adrénergiques stimulent directement les tissus transporteurs de Na dans les branchies des moules d'eau douce.
[Traduit par le journal]

Introduction

Freshwater mussels regulate blood sodium concentrations within relatively narrow limits. On a diurnal basis, the concentration of sodium in the blood oscillates between 12 and 15 m*M*/L owing to cyclic changes in the rate of sodium transport (Graves and Dietz 1980). When acclimated to a 12-h photoperiod, the freshwater unionids are most active nocturnally and the rates of oxygen consumption and sodium transport are highest at night (McCorkle et al. 1979; Graves and Dietz 1980). We have reported that exposure of mussels to deionized water causes an elevation of sodium transport when the animals are returned to dilute salt solutions (Murphy and Dietz 1976; Dietz 1978). Recently, we observed a transient stimulation of sodium transport in *Margaritifera hembeli* in response to handling (Dietz 1979). These data suggest an endogenous control mechanism capable of stimulating sodium transport in freshwater bivalves. In this report, we present evidence that several monoamine transmitter substances (catecholamines and serotonin) stimulate the unidirectional sodium influx in freshwater clams.

Materials and methods

The unionid mussels *Ligumia subrostrata* and *Carunculina texasensis* were obtained from local ponds and acclimated to artificial pond water (0.5 m*M* NaCl, 0.4 m*M* $CaCl_2$, 0.2 m*M* $NaHCO_3$, 0.05 m*M* KCl). The animals were held at room temperature (22–26°C) and laboratory photoperiod for a week before use. Drugs used in this study were obtained from Sigma Chemical Co. and all drug solutions were prepared in distilled water immediately before use. The agents used in this study were acetylcholine, adrenalin, dibutyryl-3′,5′-cyclic monophosphate (cAMP), L-3-(3,4-dihydroxyphenyl)-alanine (L-dopa), dopamine (3-hydroxytryamine), DL-isoproterenol, (−)-noradrenalin, DL-octopamine, L-phenylephrine, and serotonin (5-hydroxytryptamine).

All animals were rinsed in deionized water for 1 h before being injected with the test substance or deionized water (controls). The solutions (10 μL) were injected slowly into the anterior foot of the mussels and they were returned to deionized water for 30 min equilibration. To measure the unidirectional fluxes, each animal was placed in a separate container with 25–35 mL of pond water containing ^{22}Na (1 μCi/L; 1 Ci = 37 GBq). Bath samples were taken at time zero and 1–2 h later. Sodium was determined by flame photometry and chloride was measured by electrometric titration. ^{22}Na radioactivity was determined with a liquid scintillation counter. The animal's tissue was removed from the shell and dried (90–100°C) to determine dry tissue weight.

From the disappearance of ^{22}Na in the bathing medium and the bath sodium specific activity, the unidirectional influx (J_i) was calculated (Graves and Dietz 1982). The net flux (J_n) of sodium was determined from the changes in the sodium concentration of the pond water bath. The efflux (J_o) was estimated from the equation $J_o = J_i - J_n$. The fluxes were normalized to dry tissue, excluding the shell, and expressed as micromoles per gram dry tissue per hour.

Sodium influx was measured in isolated gills incubated in pond water. The method has been presented in detail elsewhere

0008-4301/82/061408-04$01.00/0

TABLE 1. The effect of injections of drugs on the sodium fluxes in freshwater mussels in pond water

Substance	Dose, nmol/g dry tissue	N	Na flux ($\bar{X}$ ± SE), μmol·g dry tissue^{-1}·h^{-1}		
			Net flux	Influx	Efflux
Distilled water* (10 μL)		65	0.36±0.13	1.97±0.11	1.61±0.13
Dopamine	91±6	8	3.88±0.50^c	5.26±0.58^b	1.38±0.40
Noradrenalin	71±8	9	5.19±0.67^c	6.30±0.58^c	1.11±0.13^b
Adrenalin	66±5	3	4.96±0.49^c	5.65±0.76^b	0.69±0.28^a
Serotonin	92±8	8	3.54±0.52^b	4.87±0.51^c	1.33±0.39
Dibutyryl cAMP	554±56	4	4.44±0.22^a	5.68±0.22^b	1.24±0.38
Phenylephrine	89±11	8	2.48±0.41^b	3.60±0.35^b	1.12±0.15^a
Isoproterenol	74±4	4	0.05±0.68	2.43±0.40	2.38±0.71
Dopa	109±5	5	0.71±0.26	1.94±0.30	1.23±0.12
Octopamine	111±16	8	1.34±0.51	2.09±0.51	0.75±0.08^b
Acetylcholine	95±10	7	0.75±0.36	2.80±0.63	2.05±0.62

NOTE: Superscript letters indicate values significantly different from respective controls: $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$.

*Controls for the different treatments were not different and were pooled.

and is summarized here (Dietz and Findley 1980; Dietz and Graves 1981). The isolated gills were incubated 10 min in pond water containing [^{3}H]inulin and ^{22}Na. The tissue was incubated at 25°C on a shaking water bath (100 cycles/min). After exposure to the isotopes, the gill was removed, weighed, and extracted overnight in 2.0 mL 0.1 N NHO_3 (4°C). The incubation medium and acid extract samples were counted with a liquid scintillation counter and quench corrected. The [^{3}H]inulin was used as a volume marker for pond water adsorbed onto the gill. The adsorbed ^{22}Na was subtracted from the total gill tissue ^{22}Na to obtain the ^{22}Na accumulated by the tissue. From the sodium specific activity of the incubation medium, (counts per minute per micromole) the gill tissue sodium influx was calculated and expressed as micromoles per gram dry gill per 10 min.

Serotonin content of the gills was determined using a method adapted from Snyder et al. (1965). Approximately 0.5 g of gill tissue was removed, weighed, and homogenized for 1 min (0°C) in 4.5 mL of butanol. The homogenate was centrifuged for 10 min at 1000 × g (4°C) and 2 mL of the supernatant was transferred to a centrifuge tube. Heptane (4 mL) and 50 mM phosphate buffer, pH 7, (1 mL) were added and the solution mixed for 5 min. After centrifugation for 5 min (2000 × g), 0.4 mL of the aqueous phase was removed and added to 0.8 mL of pH 7 phosphate buffer and 0.1 mL of 0.1 M ninhydrin was added. The samples were heated 30 min at 75°C, cooled for 1 h, and read on an Aminco fluorometer using an excitation wavelength of 385 nm and emission wavelength of 490 nm.

Differences between control and treated animals were determined using the Student's t-test and were considered significant if $P < 0.05$.

Results

Injection of the catecholamines (dopamine, noradrenalin, adrenalin) or serotonin stimulated the unidirectional influx and net flux of sodium in freshwater mussels (Table 1). In addition, noradrenalin and adrenalin caused a significant reduction of J_o^{Na} by increasing renal sodium reabsorption or reducing epithelial permeability. The action of the catecholamines and serotonin on sodium transport was mimicked by injection of dibutyryl cAMP. Phenylephrine, an alpha-adrenergic stimulator in the vertebrates, was an effective stimulator of sodium transport in the mussels. Phenylephrine increased the J_n^{Na} by an elevation of J_i^{Na} and reduction of J_o^{Na}. Isoproterenol, dopa, octopamine, and acetylcholine did not alter J_n^{Na} or J_i^{Na} relative to their controls. Octopamine caused a moderate decrease in J_o^{Na}.

Net chloride transport was monitored during all of the studies described above and there were no significant differences relative to controls. The animals were in a chloride steady state.

In addition to the changes in sodium transport rate, serotonin caused a marked change in activity of the mussels. The valves were gaped and the foot extended within 5–10 min postinjection and the animals moved about the container during the flux study. Phenylephrine caused a moderate valve gape with frequent valve closures and slight extension of the foot. The other drugs injected into these animals did not elicit noticeable changes in the animal activity patterns relative to controls.

Several transmitter substances were quite effective in stimulating J_i^{Na} and J_n^{Na}, however, in intact animals it is difficult to identify which substance is acting directly on

TABLE 2. Sodium influx in isolated mussel gills incubated in pond water containing transmitter substance (at 10^{-4} *M*)

Treatment	*N*	Na influx, μmol·g dry gill^{-1}·10 min^{-1}	
		Control	Treated
Serotonin	7	11.81±2.37	22.76±2.31*
Adrenalin	8	11.60±0.79	11.88±0.94
Noradrenalin	4	10.02±1.01	11.19±0.57
Dopamine	8	9.36±0.90	8.98±1.26

*Significantly different from control, $P < 0.01$.

the target tissue. Recently, we have demonstrated that the isolated gills of mussels are a major site of sodium transport (Dietz and Findley 1980; Dietz and Graves 1981). Incubating the isolated gills in pond water containing serotonin (10^{-4} *M*) caused a significant increase (93%) in J_i^{Na} (Table 2). In contrast, exposing the isolate gills to adrenalin, noradrenalin, or dopamine did not change J_i^{Na}. Chemical analysis of the gill tissue indicated a substantial amount of serotonin was present (2.26 ± 0.18 μg/g wet gill, $N = 6$). The serotonin content of blood was significantly lower (0.18 ± 0.02 μg/mL).

Both serotonin and cAMP injected into these mussels stimulate sodium influx and the response is reduced at lower doses of drugs (cf. Tables 1 and 3). When both agents are injected into the animals simultaneously, the response is not additive. Serotonin may be stimulating adenylate cyclase and generating sufficient endogenous cAMP to stimulate the sodium transport system.

Discussion

The "monoamine transmitters" (catecholamines and serotonin) stimulated sodium transport when injected into the blood of freshwater mussels. The principal action of these drugs was the significant elevation (150–220%) of J_i^{Na} relative to controls. Since 1 g of dry tissue represents about 3.9 mL of blood in these animals (Murphy and Dietz 1976), the injected dose of drugs was $<2 \times 10^{-5}$ *M* if uniformly distributed in the blood. This dosage elicits a maxmimal increase in J_i^{Na} and is 50 times higher than the blood serotonin concentration in pond water acclimated controls. However, a significant increase in J_i^{Na} was obtained with one-fifth the maximal dose of serotonin (cf. Tables 1 and 3). If serotonin is used as a transmitter substance in these animals, low endogenous blood concentrations would be expected to prevent uncontrolled interference.

Recently, we reported that the isolated gills of mussels are the principal site of sodium uptake (Dietz and Graves 1981). Sodium influx into the isolated gills was stimulated by 10^{-4} *M* serotonin but not by the catecholamines which indicates that only one transmitter substance is capable of acting directly on the target tissue.The catecholamines could stimulate J_i^{Na} in the intact mussel but not the gills, suggesting that the target tissues are controlled by serotonergic fibers which may be innervated by adrenergic pathways in the visceral ganglion of the intact animals. Phenylephrine but not isoproterenol stimulated J_i^{Na} indicating an alpha-adrenergic-like receptor is involved in the transduction mechanism.

Stimulation of sodium transport by serotonin in mussels is probably mediated through adenylate cyclase. The similarity of the sodium transport response to exogenous dibutyryl cAMP and serotonin in the intact animals and isolated gills is indirect supporting evidence. The presence of serotonin in the gill tissue indicates the availability of serotonin for stimulation of sodium transport. The nonadditive stimulation of J_i^{Na} by serotonin and cAMP suggests both agents act on the same cAMP-dependent mechanism regulating sodium transport. In addition, we have observed that theophylline, a phosphodiesterase inhibitor, injected into freshwater mussels also stimulates sodium influx (Graves and Dietz 1982). Finally, preliminary data indicate serotonin added to a crude homogenate of gill tissue stimulates adenylate cyclase 100% above controls (J. Scheide, unpublished data).

TABLE 3. The effects of dibutyryl cAMP and serotonin on Na transport in freshwater mussels

Treatment	Dose, nmol/g dry tissue	*N*	Na flux ($\bar{X}$ ± SE), μmol·g dry tissue^{-1}·hr^{-1}		
			Net flux	Influx	Outflux
Distilled water (10 μL)		7	−0.12±0.20	0.69±0.20	0.80±0.23
Serotonin	17±1	8	2.21±0.32*	3.01±0.42*	0.80±0.18
cAMP	341±28	8	1.29±0.16*	1.92±0.32*	0.63±0.23
Serotonin + cAMP	18±2 / 354±39	8	1.69±0.33*	2.43±0.39*	0.75±0.25

*Significantly different from control, $P < 0.01$.

A number of neurotransmitter substances are known to act on their target cells by increasing cyclic nucleotides (see Greengard 1978). Several monoamines have been reported to stimulate adenylate cyclase in invertebrate tissues (Gole and Downer 1979; Robertson and Osborne 1979). Cyclic AMP has been shown to stimulate chloride transport in insect rectum (Spring et al. 1978; Phillips et al. 1980). For the insect rectum, a polypeptide hormone from the corpus cardiacum is the apparent stimulator of adenylate cyclase.

Sodium transport in the freshwater bivalve is subject to rapid modulation. We have reported that sodium transport can be stimulated in response to handling (Dietz 1979). In addition, J_i^{Na} can be inhibited by prostaglandins (Graves and Dietz 1978, 1982). There is a remarkable similarity between control of sodium transport in mussels and cAMP mediaed water permeability changes in the toad bladder. Orloff and Zusman (1978) have noted that vasopressin simultaneously stimulates adenylate cyclase and phospholipase. The action of phospholipase is to release arachidonic acid which is converted into prostaglandin E_2 (PGE_2). The PGE_2 serves as a negative modulator to suppress adenylate cyclase activity. Preliminary radioimmunoassays for PGE_2 and $PGE_{2\alpha}$ in the blood of *L. subrostrata* have demonstrated these metabolites of arachidonic acid are synthesized in mussels (D. Saintsing, unpublished data).

We have reported a diurnal rhythm of sodium transport in freshwater mussels (Graves and Dietz 1980). The sodium transport rhythm correlates with a dirunal activity rhythm with the highest activities occurring during the night (McCorkle et al. 1979). It is interesting that serotonin caused a significant increase in movement in these mussels but catecholamines did not. Salanki and co-workers have reported that the pattern of filter feeding in *Anodonta* is a cyclic response associated with different monoamines (Salanki et al. 1974).

Previous reports of catecholamines altering ion transport in fish gills are complicated by the potential effects on capillary perfusion of the tissue rather than directly altering transport (Pic et al. 1975; Girard 1976). In this study we have shown that monoamine transmitters stimulate sodium transport in the mussel, an animal with an open circulatory system. In addition, serotonin is an effective stimulator of J_i^{Na} in vivo and in vitro. These data support the hypothesis that ion transporting tissues may be under direct neural control in diverse aquatic animals.

Acknowledgements

We thank Reba D. Haynes for typing the manuscript. This study was supported by National Science Foundation grants PCM 77-08818 and PCM79-21089-02.

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