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THE COORDINATE ROLES OF BRANCHIAL NERVE ACTIVITY AND POTASSIUM IN THE STIMULATION OF CILIARY ACTIVITY IN *MYTILUS EDULIS*: OBSERVATIONS WITH PHENOXYBENZAMINE, BROMOLYSERGIC ACID AND FLUORESCENCE HISTOCHEMISTRY

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SUMMARY

Potassium concentrations in excess of 30 mM increase the rate of beating of lateral cilia on the gill of *Mytilus edulis*. Cilioexcitation produced by low frequency (5 beats/s) electrical stimulation was potentiated with potassium but blocked with bromolysergic acid (a serotonergic inhibitor). Cilioinhibition produced by high frequency (50 beats/s) stimulation was decreased with potassium and phenoxybenzamine (a dopaminergic inhibitor). Phenoxybenzamine enhanced the cilioexcitation produced by potassium. Potassium doses incapable of maintaining a basal rate of beating (< 30 mM) could increase ciliary activity if phenoxybenzamine was also added. After transection of the branchial nerve, the yellow-fluorophore (serotonergic storage) and cilioexcitatory effect of potassium gradually decrease.

This study shows that the potassium effect on ciliary activity (*a*) increases with low frequency nerve stimulation, presumably through the release of serotonin and (*b*) decreases with high frequency nerve stimulation, presumably through the release of dopamine.

INTRODUCTION

Electrical stimulation of the branchial nerve in isolated ganglion/nerve/gill preparations of *Mytilus edulis* has been shown to activate quiescent lateral cilia and to increase the average rate of beating of active cilia. The mechanism probably involves the release of 5-hydroxytryptamine (5-HT, serotonin), which is cilioexcitatory. In addition to cilioexcitatory axons, the branchial nerve appears to contain axons with 3,4-dihydroxyphenyl-ethylamine (DA, dopamine) which functions as an inhibitory neurotransmitter (Aiello & Guideri, 1966). Electrical stimulation of this nerve at 2–5 pulses/s accelerated ciliary beating. However, electrical stimulation at 25–50 pulses/s inhibited ciliary activity (Paparo & Aiello, 1970). Prior treatment with bromolysergic acid (BOL) or Phenoxybenzamine (PBZ) respectively blocked the cilioexcitatory or cilioinhibitory response produced by 2–5 beats/s nerve stimulation (Paparo & Aiello, 1970).

The effects of ions on lateral cilia were studied extensively by Gray (1928). More recently, it has been shown that conditioning of gills with [K] > 30 mM enhanced the cilioexcitatory effect of exogenously applied 5-HT (Takahashi & Tsuchiya, 1971; Paparo & Murphy, 1975*a*, *b*). The purpose of this study is to investigate the effect of frequency variation of electrical stimulation on the K enhancement of lateral ciliary activity.

METHODS AND MATERIALS

All experiments were performed on mussels *Mytilus edulis* which were kept for 1–2 weeks in artificial sea water (Rila Marine Mix) in an Instant Ocean Aquarium (temperature 17 °C, pH 7·5, density 1·025). Before each experiment, mussels of 4·6–5·9 cm in length were placed in finger bowls of the same sea water, the posterior adductors were cut, and each gill with its branchial nerve, visceral ganglion, and a piece of adductor muscle for support was isolated. Gills measured 3·2–3·7 cm in length along the gill axis. This ganglion/nerve/gill preparation was pinned to rubber mats glued in the bottom of a Petri dish containing sea water, and the dish was placed in a holder fastened to the adjustable stage of a microscope. The gill was seen to consist of numerous parallel gill filaments. Three major types of ciliated cells were clearly distinguished: frontal, laterofrontal, and lateral. This study was concerned with the rate of beating of the lateral cilia which beat in such a way that metachronal waves appear to travel in opposite directions along the two sides of each gill filament.

A field was selected for observation by measuring 2·0 cm in an anterior direction from the visceral ganglion. A field of view contained about 50 filaments which were grouped for ease of observation into 3 vertical columns. By moving the stage each filament could be followed from its dorsal attachment at the axis to its free ventral end. Each column was subdivided into 4 horizontal rows, demarcated by their fixed number of interfilamentary junctions, from dorsal to ventral end. The rate of ciliary beating in beats per second was estimated by synchronizing the rate of flashing of a calibrated, stroboscopic light used in place of the substage lamp, with the rate of beating of the cilia. Synchronization was achieved when the metachronal wave appeared to stand still. Measurements were made from dorsal to ventral border, and from left to right across the field, giving 12 sets of measurements.

The Petri dish was perfused with sea water via a four channel variable speed pump with a flow rate of *ca.* 0·5 ml/min. The planetary gear mechanism of this pump ensures minimum pulsing and stable drift-free flow permitting accurate measurement of ciliary movement. A positive displacement piston metering pump with micrometric adjustment permits rapid removal of solution from the Petri dish. A continuous flow of solution can be maintained across the dish. Constant temperature is maintained by means of stainless steel tubing which cools down the movable platform (*ca.* 1·0 °C/0·5 min) by means of circulating water from a cooling system.

Nerve stimulation and perfusing of drugs follow the procedure of Paparo & Aiello (1970). A Grass stimulator supplied electrical pulses of the following characteristics: 0·1 V, 2 ms at a frequency of 5 or 50/s, for a duration of 1 min. The following drugs were used: bromolysergic acid diethylamine (BOL); phenoxybenzamine (PBZ); 5-hydroxytryptamine (5-HT, serotonin) as creatinine sulphate; and potassium (K) as a chloride.

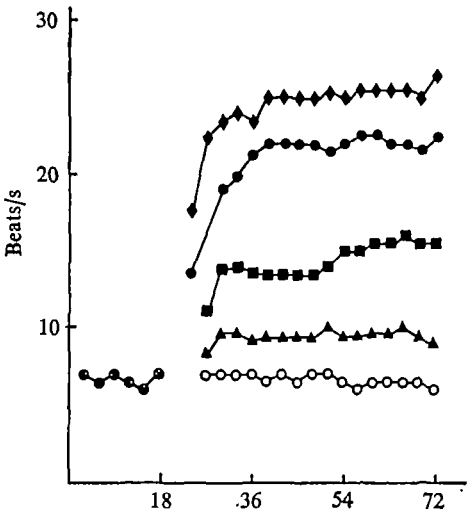

Fig. 1

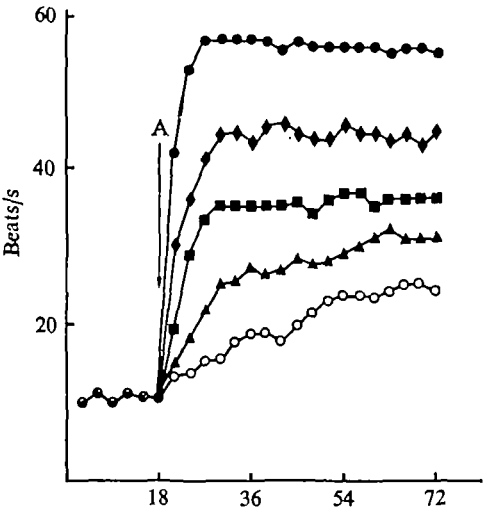

Fig. 2

Fig. 1. The effect of potassium concentration on the average rate of beating of lateral cilia. Before (⊙—⊙) and after the addition of 30 mM (○—○), 60 mM (▲—▲), 80 mM (■—■), 100 mM (●—●) and 200 mM (◆—◆) K to the perfusate.

Fig. 2. The effect of electrical stimulation (5 pulses/s) of the branchial nerve and K [60 mM (▲—▲), 80 mM (■—■), 100 mM (◆—◆), 200 mM (●—●)] on the average rate of beating of lateral cilia. The 5 pulses per sec. and perfusion with 200 mM-K was repeated with 10^{-4} M BOL (○—○). A indicates beginning of each experimental run.

Biogenic monoamines in the gill tissue from *Mytilus* were localized by means of the fluorescence-histochemical method of Falck & Owman (1965). Excised gills were immediately frozen in Freon 22, cooled by liquid nitrogen and then lyophilized for 3 days in a Virtis tissue dryer at 42 °C and 10^{-5} torr. The gills were then exposed to paraformaldehyde vapours (relative humidity 60% at 80 °C) for 2 h and embedded under vacuum in paraffin wax. Sections were examined on a Lietz MPV II fluorescence microscope equipped with a photomultiplier and digital output calibrated to read in μ-amperes. The yellow fluorophore was localized in the branchial nerve and the intensity measured in μ-amperes.

RESULTS

The addition of K to the perfusate of gill preparation increases the average rate of beating of the lateral cilia after a 3 s delay. Dosage of 30 mM-K is only sufficient to maintain a basal rate of beating. Dosages of K > 30 mM are cilioexcitatory ($P < 0.05$ in all after 72 s exposure to K, compared to control) (Fig. 1).

Low frequency (5 pulses/s) electrical stimulation of the branchial nerve enhanced the cilioexcitatory effect of K. In addition, no delay effect was initially observed with K. There was approximately a two-fold increase in the K effect with low frequency electrical stimulation ($P < 0.001$ in all cases after 72 s exposure to K, when compared with control). Low frequency electrical stimulation with 10^{-6} M BOL significantly reduced the cilioexcitatory effect of 200 mM-K. The rate of beating with 200 mM-K

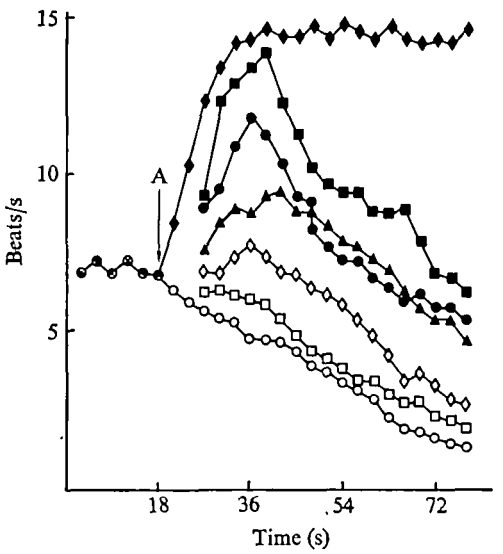

Fig. 3

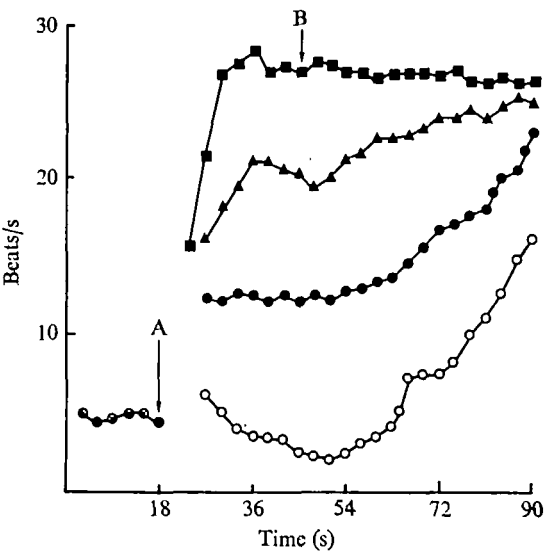

Fig. 4

Fig. 3. The effect of electrical stimulation (50 pulses/s) of the branchial nerve and K [60 mM (□—□), 80 mM (◇—◇), 100 mM (▲—▲), 200 mM (●—●), 400 mM (■—■)] on the average rate of beating of lateral cilia. The 50 pulses/s and perfusion with 400 mM-K was repeated with 10^{-6} M PBZ (◆—◆) or 10^{-6} M BOL (○—○). A indicates the beginning of each experimental run.

Fig. 4. The effect of K and PBZ on the average rate of beating of lateral cilia. A indicates experimental runs with 15 mM-K (○—○), 60 mM-K (●—●), 80 mM-K (▲—▲) and 80 mM-K + 10^{-6} M PBZ (■—■). B indicates the continuation of all previous experiments with the addition of 10^{-6} M PBZ to perfusates.

alone was $58{\cdot}3 \pm 0{\cdot}9$ beats/s (mean ± S.E.) compared with $25{\cdot}7 \pm 0{\cdot}8$ beats/s with 200 mM-K plus BOL ($P < 0{\cdot}001$; after 72 s exposure period) (Fig. 2).

High frequency (50 pulses/s) electrical stimulation of the branchial nerve decreased the cilioexcitatory effect of K. Dosages of 60 and 80 mM-K could not maintain a basal rate of beating. Dosages of 100, 200 and 400 mM-K were initially cilioexcitatory ($P < 0{\cdot}05$, comparing rates of beating to control 18 s after electrical stimulation) but soon decreased to levels that were not significantly different from control values ($P > 0{\cdot}85$; after 72 s exposure period). High frequency electrical stimulation with 400 mM-K and PBZ produced a significant sustained increase in the rate of beating ($P < 0{\cdot}05$; compare control ($6{\cdot}9 \pm 0{\cdot}3$ beats/s) to experimental ($14{\cdot}8 \pm 0{\cdot}6$ beats/s) after 72 s exposure period). However, high frequency electrical stimulation with 400 mM-K and BOL was significantly cilioinhibitory ($P < 0{\cdot}01$, compare control ($6{\cdot}9 \pm 0{\cdot}3$ beats/s) to experimental ($1{\cdot}6 \pm 0{\cdot}7$ beats/s) after 72 s exposure period) (Fig. 3).

15 mM-K is not capable of maintaining a basal rate of ciliary beating. The addition of 10^{-6} M PBZ changes this sub-basal dosage into a cilioexcitatory dosage within 54 s. Furthermore, addition of PBZ to the perfusate of 60 and 80 mM-K gradually increases the average rate of ciliary beating ($P < 0{\cdot}05$; $12{\cdot}7 \pm 0{\cdot}3$ beats/s to $22{\cdot}8 \pm 0{\cdot}7$ beats/s, and $20{\cdot}1 \pm 0{\cdot}2$ beats/s to $26{\cdot}1 \pm 0{\cdot}2$ beats/s for 60 and 80 mM-K respectively) (Fig. 4).

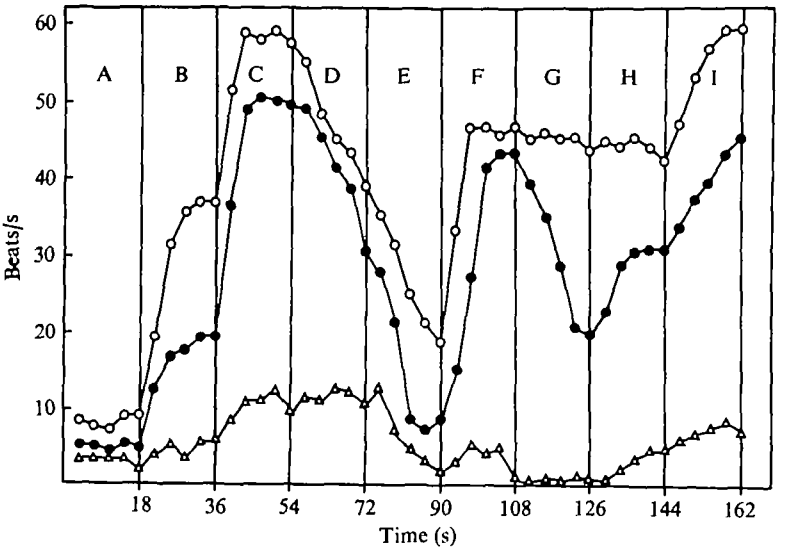

Fig. 5. The effect of electrical stimulation (5 and 50 pulses/s), K and PBZ on the average rate of beating of lateral cilia. Experiment (●—●): sea water (A), 5 pulses per sec and 100 mM-K (B), 5 pulses per sec and 200 mM-K (C), sea water (D and E), 5 pulses/s and 100 mM-K (F, G and H), and 5 pulses/s and 10^{-6} M PBZ and 100 mM-K (I). Experiment (●—●): sea water (A), 50 pulses/s and 200 mM-K (B), 50 pulses/s, 10^{-6} M PBZ and 200 mM-K (C), sea water (D and E), 50 pulses/s, 10^{-6} M PBZ and 100 mM-K (F), 50 pulses/s and 100 mM-K (G), and 50 pulses/s, and 10^{-6} M PBZ and 100 mM-K (H and I). Experiment (△—△): sea water (A), 50 pulses/s and 100 mM-K (B), 50 pulses/s and 200 mM-K (C), sea water (D and E), and 50 pulses/s and 100 mM-K (F–I).

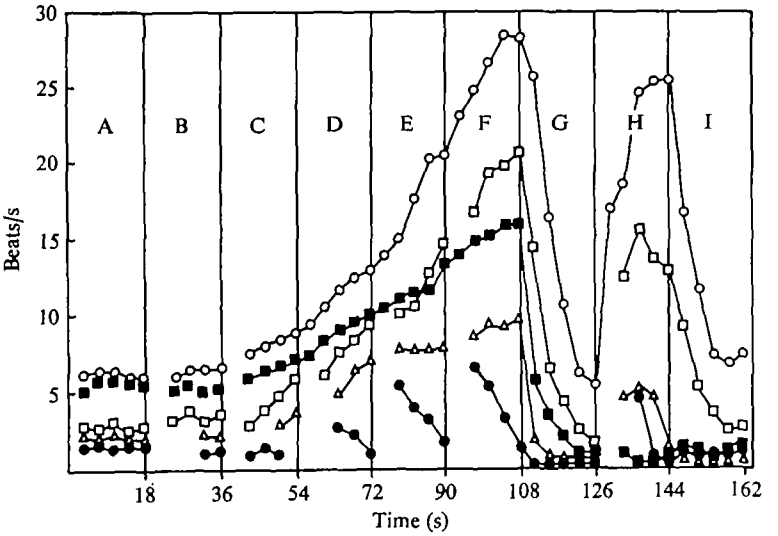

Fig. 6. The effect of transection of the branchial nerve, K and exogenous 5-HT on the average rate of beating of lateral cilia. Time of experiment after transection: zero time (○—○), 1 day (□—□), 2 days (△—△), and 5 days (●—●). Procedure in each experiment: sea water (A), 30 mM-K (B), 80 mM-K (C), 120 mM-K (D), 160 mM-K (E), 400 mM-K (F), sea water (G), 400 mM-K (H) and sea water (I). Exogenous 5-HT (10^{-6} M) added to perfusates containing gill preparations five days after transection of branchial nerve (■—■).

Table 1. *Measurement of K* (400 mM) *stimulation of ciliary activity and the yellow fluorophore in preparations with an intact branchial nerve (control) and with a transected branchial nerve (experimental) (mean* ± S.E.)

Days after perfusion of gill (control) or after transection of BN (experimental)		Yellow fluorophore (μ-amperes)	Lateral ciliary activity (beats/s)	
			Before K stimulation	After K stimulation
0	Control	15·8 ± 0·8	5·6 ± 0·3	38·5 ± 0·6
	Experimental	13·8 ± 1·3	8·3 ± 0·9	41·5 ± 0·2
1	Control	14·6 ± 1·2	6·3 ± 0·9	40·1 ± 1·8
	Experimental	10·8 ± 1·3	8·1 ± 0·5	29·9 ± 1·5
2	Control	14·8 ± 0·9	6·1 ± 0·3	36·3 ± 0·9
	Experimental	7·8 ± 0·9	6·2 ± 0·4	26·5 ± 1·9
3	Control	13·9 ± 0·6	6·2 ± 0·9	35·8 ± 0·3
	Experimental	6·2 ± 0·5	5·1 ± 0·5	15·2 ± 0·2
4	Control	15·8 ± 0·9	5·6 ± 0·5	36·9 ± 1·5
	Experimental	5·8 ± 0·3	3·9 ± 0·9	9·6 ± 0·3
5	Control	16·1 ± 0·3	7·3 ± 0·8	33·3 ± 0·5
	Experimental	3·5 ± 0·4	3·1 ± 0·7	7·6 ± 0·5
6	Control	17·5 ± 0·3	7·2 ± 0·1	39·5 ± 0·5
	Experimental	2·8 ± 0·2	3·1 ± 0·2	8·3 ± 0·2
7	Control	14·8 ± 1·2	6·8 ± 0·3	42·5 ± 0·2
	Experimental	2·1 ± 0·5	2·8 ± 0·3	5·2 ± 0·5
8	Control	13·8 ± 1·5	5·2 ± 0·8	33·5 ± 0·3
	Experimental	1·8 ± 0·5	1·9 ± 0·1	3·8 ± 0·1
9	Control	15·2 ± 0·9	6·3 ± 0·7	35·8 ± 0·5
	Experimental	1·2 ± 0·2	1·3 ± 0·1	2·9 ± 0·2
10	Control	14·3 ± 1·2	7·3 ± 0·5	39·9 ± 0·9
	Experimental	0·9 ± 0·1	1·4 ± 0·2	1·1 ± 0·1

In the presence of 100 mM-K followed by 200 mM-K, low frequency (5 pulses/s) electrical stimulation significantly increases the rate from 9·8 ± 0·2 beats/s to 58·7 ± 0·7 beats/s (control compared with last experimental reading; $P < 0{\cdot}001$). In the presence of 100 mM-K, the delivery of 5 pulses/s with 10^{-6} M PBZ significantly increased the rate of ciliary beating from 46·1 ± 0·6 beats/s to 59·1 ± 0·1 beats/s ($P < 0{\cdot}001$). High frequency (50 pulses/s) decreased the cilioexcitatory effect of K. The enhancement produced by K occurred when 10^{-6} M PBZ was added to the perfusate (Fig. 5).

Transection of the branchial nerve significantly altered the cilioexcitatory effect of serial K perfusion in gill preparations from 0 to 5 days after transection (Fig. 6). Specimens removed for fluorescence histochemical examination for the yellow-fluorophore (5-HT indicator) showed a significant decrease of this amine (compare control and experimental values in Table 1). However, when exogenous 5-HT was added to gill preparations 5 days after branchial nerve transection, there was a significant enhancement of ciliary activity (Fig. 6).

DISCUSSION

The study shows that the cilioexcitatory effect of low frequency stimulation is enhanced by K. Furthermore, inhibition of the serotonergic releasing mechanism by BOL decreases the cilioexcitatory effect of added K to perfusates containing gill preparations. 5-HT appears to be the most probable mediator of the K stimulation of the lateral cilia. This investigation also shows that high frequency (50 pulses/s) stimulation is cilioinhibitory. The cilioexcitatory effect of increasing amounts of K can be significantly reduced with high frequency stimulation. Cilioexcitation can be restored if high frequency stimulation is administered in the presence of PBZ. The selective inhibition of PBZ for high frequency and exogenously applied dopamine (DA) has been shown (Paparo & Aiello, 1970). The latter investigators believe that DA is the inhibitory transmitter released by the branchial nerve. Interestingly, this study has shown that the cilioinhibition produced by high frequency stimulation can be significantly enhanced by BOL in the presence of 400 mM-K. This can be explained on the basis of selective inhibition of 5-HT receptors. Selective inhibition of DNA by exogenously applied PBZ will increase the cilioexcitation of a particular dose of K. Furthermore, PBZ changes a sub-basal dosage of K to one that is cilio-excitatory. Transection of the branchial nerve will decrease the cilioexcitatory effect of added K. This study showed that transection of the branchial nerve results in a gradual decrease in the yellow fluorophore (a 5-HT indicator). Addition of exogenous 5-HT to gill preparations 5 days after transection of the branchial nerve restores the cilioexcitation of added K.

In conclusion, this investigation has shown that the potassium cilioexcitatory effect is brought about by low frequency (5 pulses/s) stimulation and is blocked by a selective inhibitor of 5-HT, namely BOL. In some manner, the cilioinhibition of high frequency (50 pulses/s) prevents the cilioexcitatory effect of K. Again, this latter effect can be blocked by PBZ, a DA inhibitor. Both 5-HT and/or DA are postulated to be neuroregulators in the gill of *Mytilus* (Aiello, 1960, 1962, 1965; Aiello & Guideri, 1965, 1966; Fellon & Aiello, 1972; Paparo & Aiello, 1970; Malanga, 1973; Malanga, Wenger & Aiello, 1972; Paparo, unpublished observation; Paparo & Finch, 1973; Paparo & Tate, 1973) and other molluscs (Kerkut & Walker, 1961; Walker *et al.* 1970; Woodruff, 1971). In turn a relationship between effectiveness of electrical frequency stimulation (with a specific amine released) and the K level has been established.

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