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Ionophore-Mediated Calcium Entry Induces Mussel Gill Ciliary Arrest

Abstract. *Lateral cilia of freshwater mussel gills, which normally beat with metachronal rhythm, are arrested pointing frontally by perfusion with 6.25 to 12.5 millimolar calcium and 10^{-5} molar A23187, a calcium ionophore. Arrest does not occur in either calcium or ionophore and monovalent cations alone. Activity returns with continued perfusion in potassium chloride or calcium chloride, and more slowly in sodium chloride, after removal of ionophore. These results support the hypothesis that a local rise in internal calcium causes ciliary arrest.*

Although once beating the ciliary axoneme is an autonomous motor organelle that operates via a sliding microtubule mechanism (*1*), cells can often turn their ciliary beat on and off. The cellular control of ciliary activity is perhaps best analyzed in the protozoan *Paramecium* where Ca^{2+} entry into the cell causes beat stoppage and reversal (*2*). In metazoan epithelia, such as the gill tissue of lamellibranch mollusks, mechanical or nervous stimuli can sometimes be shown to cause ciliary arrest (*3*).

Satir *et al.* (*4*) have found that local lesions 30 μm in diameter produced by laser irradiation of lateral (L) cells of freshwater mussel gill (*Elliptio* and related genera) placed in 5 to 15 m*M* $CaCl_2$ or NaCl, but not in KCl or NH_4Cl, initiate ciliary arrest, which spreads for distances greater than 1 mm to either side of the lesion. Motokawa and Satir (*5*) have shown that for *Mytilus* gill similar arrest responses occur in normal, but not in Ca^{2+}-free, seawater. We postulate that in such cases, just as in *Paramecium*, the cause of arrest is a local rise in Ca^{2+} concentration in the cytoplasm which inhibits ciliary beat at sensitive points in the stroke cycle. The L-cells are only a few micrometers wide and intracellular microelectrode recording is difficult with this system (*6*), so that my test of this hypothesis relies on an alternate method. The calcium ionophore A23187 (*7*) offers an experimental tool for controlling Ca^{2+} entry into the cell cytoplasm. For example, in concentrations of 10^{-6} or 10^{-5} *M*, this ionophore induces secretion of ATP in platelets, histamine release from mast cells, contraction of frog eggs and muscle, and so forth (*8*)—phenomena that depend on Ca^{2+} entry from either external or internal stores into the cytoplasm proper. In the experiments described here we were able to show that in the presence of Ca^{2+} and ionophore, beat of mussel gill L-cilia is arrested, but that arrest does not occur in either Ca^{2+} or ionophore alone.

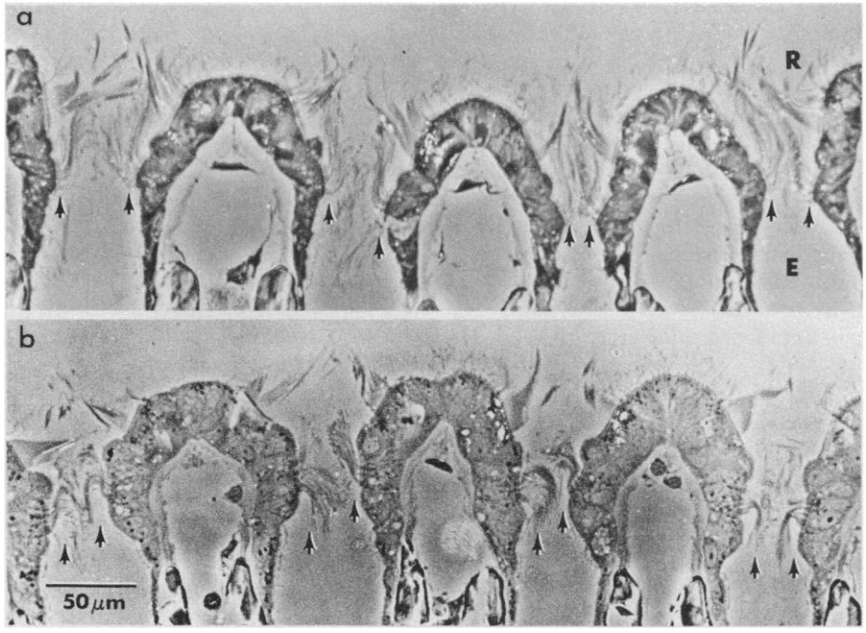

Fig. 1. Phase contrast micrographs of gill filaments embedded in Epon, showing positions of L-cilia (arrowheads) after quick fixation (*9*). (a) Tissue in 12.5 m*M* Ca^{2+} and 10^{-5} *M* A23187; L-cilia are arrested. Nearly all L-cilia have a basal bend. The distal parts of the axonemes lie straight, parallel to the epithelium, with the tips pointing in a frontal (*R*) direction. Abfrontal direction is indicated by *E*. (b) Portion of the same gill. The arrested cilia were placed in 20 m*M* KCl so that metachronism was resumed before fixation. The L-cilia are fixed in various metachronal wave positions, pointing in both abfrontal and frontal directions.

Pieces of gill tissue, excised from healthy mussels usually into 12.5 m*M* $CaCl_2$ or 20 m*M* NaCl, are stripped to give an undamaged piece of gill lamella and placed in a small perfusion chamber, as described previously (*9*). Flow through the chamber is continuous except for brief intervals (< 30 seconds) when solutions are changed. Low-power fields containing about 15 filaments whose L-cilia are beating with well-defined metachronism are selected for subsequent experimentation. Each filament bears two rows of L-cells that are measured independently with respect to metachronal activity; the number of active rows that pass metachronal waves across the entire field is used to determine percentage of metachronism in the tissue at appropriate intervals. In rows that are not active, L-cilia are usually stopped in a characteristic position shown in Fig. 1. The laterofrontal cirri and terminal and frontal cilia generally continue to beat, even when the L-cilia are stopped.

The gill is initially perfused with $CaCl_2$, NaCl, or a mixture of the two salts adjusted to approximately equal osmotic strengths. Greater than 60 percent metachronism (Fig. 2) is normally obtained in these solutions; serotonin (5-hydroxytryptamine) can initially be used to increase metachronal activity if necessary, but is always removed by perfusion for > 5 minutes before experimentation is begun. Figure 2, a and b, compares the effect of adding 10^{-5} *M* A23187 to initial perfusates of Ca^{2+} against Na^+. In the former instance (Fig. 2a) metachronism is affected almost immediately and completely abolished within less than 10 minutes of addition; in the latter case (Fig. 2b) addition

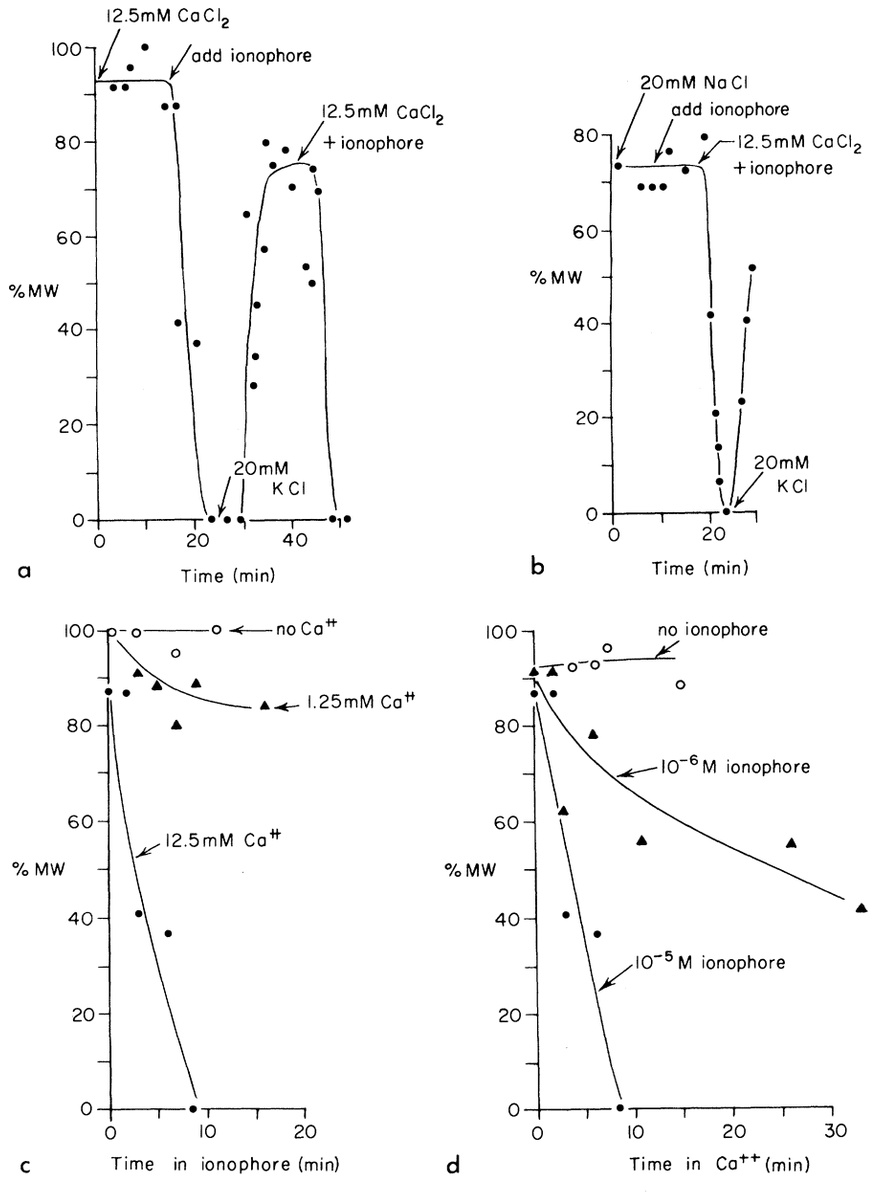

Fig. 2. (a and b) Perfusion chamber experiments. The graphs represent single continuous perfusions. The same field of cilia is counted throughout one experiment. The curves show the percentage of gill filaments beating metachronally (*%MW*) plotted against time when counts are complete. Arrows indicate times at which perfusate was changed. The ionophore (A23187) concentration is 10^{-5} *M*. (c and d) Response of L-cilia to changes in Ca^{2+} and ionophore concentrations. (c) In 10^{-5} *M* ionophore, changes in Ca^{2+} concentration affect arrest kinetics. The solution with no Ca^{2+} contains either 20 m*M* NaCl or 20 m*M* KCl; the solution with 1.25 m*M* Ca^{2+} contains 20 m*M* NaCl. (d) In 12.5 m*M* Ca^{2+}, changes in ionophore concentration affect arrest kinetics. Arrest is not obtained by perfusion with Ca^{2+} without ionophore.

does not alter activity. In a continuation of this experiment (Fig. 2b), perfusion with Ca^{2+} and ionophore abolishes metachronism, so that the negative result can definitely be described to the absence of Ca^{2+} in the medium. Perfusion with A23187 and 20 m*M* KCl, or 10^{-5} *M* gramicidin D and 20 m*M* NaCl, also has no effect on metachronism.

Dose-response curves are shown in Fig. 2, c and d. Complete inhibition is obtained in the presence of 10^{-5} *M* ionophore and 6.25 to 12.5 m*M* Ca^{2+}; partial inhibition is obtained with tenfold dilution of either Ca^{2+} or ionophore. The presence of Na^{+} in the initial perfusate does not interfere with ionophore-induced arrest, as long as Ca^{2+} is present in appropriate concentration. Arrest occurs more or less simultaneously along one row of L-cells, but occasionally metachronism will continue briefly in some short segments as others stop. Within the limit of counting time (seconds), arrest occurs cell by cell, randomly rather than in spreading or sequential fashion.

After arrest, activity is readily recovered by perfusion with KCl with or without added ionophore, or $CaCl_2$ without ionophore (Figs. 1 and 2); the tissue may be cycled several times through these solutions with identical results. Metachronal activity begins to return almost immediately after removal of ionophore, so that 50 percent of original activity is recovered in less than 5 minutes and complete recovery usually occurs within 10 minutes. If NaCl is substituted for KCl or $CaCl_2$, recovery is much slower (> 1 hour for full recovery).

These results are interpreted as follows. The ionophore intercalates into the L-cell membrane, producing temporary Ca^{2+} channels. In the presence of a high external Ca^{2+} concentration, Ca^{2+} moves down its chemical gradient, entering the cell cytoplasm faster than it can be pumped out, internal Ca^{2+} rises, and the cilia stop. A Ca^{2+} concentration adequate for arrest is not available from internal stores. The ionophore must be inactivated or removed from the cell membrane into other cellular compartments rapidly, since arrest persists only in the presence of continued ionophore, as well as Ca^{2+}, perfusion. In the absence of ionophore, Ca^{2+} is effectively pumped out of the cytoplasm and beat resumes. External K^{+} does not interfere with the recovery. External Na^{+}, which does not block Ca^{2+} entry, slows down the recovery. These experiments do not speak to the state of membrane depolarization in the various ionic solutions, but without Ca^{2+} entry membrane depolarization probably does not elicit arrest. In the animal nervous stimulation may cause the Ca^{2+} entry and ciliary arrest that is mimicked here with the ionophore. The level of Ca^{2+} in the mussel body fluids (*10*) is sufficient for the stoppage response. The effect of high Ca^{2+} on the sliding of ciliary microtubules which leads to arrest is still unknown, but it is interesting that after ionophore and Ca^{2+} treatment, as shown in Fig. 1, the cilia are arrested pointing frontally in a position in which there is a basal bend and most of the axoneme is straight, which has previously been described as a rest position in the gill (*9*).

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Dyskinesias Elicited by Methamphetamine: Susceptibility of Former Methadone-Consuming Monkeys

Abstract. *Rhesus monkeys with a history of drinking methadone but currently drug-free and control monkeys with no drug history were injected with methamphetamine hydrochloride (2 to 5 milligrams per kilogram of body weight). In six of seven monkeys which had consumed methadone the lowest dose immediately elicited pronounced oral dyskinesias virtually identical to those of human tardive dyskinesia. The control monkeys did not exhibit oral dyskinesias even after prolonged treatment with the highest dose. The clinical implications may be related to the functioning of brain dopaminergic systems.*

The use of methadone hydrochloride in maintenance regimens of former heroin abusers is now firmly established (*1*). Daily oral administration of methadone prevents craving for other narcotic drugs but leaves untouched the potential for abuse of nonnarcotic drugs. Urinalysis of patients taking methadone often reveals significant use of barbiturates and amphetamines (*2*). Chronic use of methadone combined with abuse of nonnarcotic drugs represents a potentially serious but well-recognized clinical problem. Less attention has been directed toward altered sensitivity to a drug after chronic administration of another drug has ceased. We have found that monkeys with a history of oral methadone self-administration are highly sensitive to methamphetamine administered long after methadone treatment was terminated.

Amphetamines elicit repetitive stereotyped movements in many species, including man (*3*). Ellinwood (*4*) has described, for the monkey, development of various stereotyped behaviors during long-term intoxication with methamphetamine hydrochloride (MA). Low and moderate doses (2 to 10 mg/kg) initially produce biting and chewing, searching motions, grooming, picking at the skin, and hand examining. After several months of intoxication, higher doses (10 to 20 mg/kg) elicit a more intense form of stereotyped behavior, buccolingual dyskinesias. These are virtually identical to the oral behaviors seen in tardive dyskinesia in humans and consist of repetitive mouth movements such as tongue protrusion and rolling, wide opening of the mouth, and lateral jaw displacement.

In the present study, seven male rhesus monkeys had previously drunk sub-dependence-producing doses of methadone hydrochloride once daily, for varying periods of time determined by their participation in discrimination learning experiments (*5*). Their methadone histories and duration of subsequent drug-free period are summarized in Table 1. A control group of seven males, similar in age, had never received any narcotic or amphetamine.

We administered daily intramuscular injections of MA (50 mg/ml) on a cyclic schedule of four injection days followed by three rest days (mimicking human "spree" abuse), for up to 12 weeks. Each monkey's dosage was gradually increased within the range specified in Table 2. All monkeys were observed periodically for a minimum of 6 hours after injection in their home cages on all injection days. In addition, on at least one injection day every monkey was observed continuously for 6 hours after injection in another room, where video tape recordings of representative behaviors were made.

Both experimenters independently assessed the behavioral response to MA. Although we knew each monkey's drug history, the presence or absence of criterion oral behaviors was so obvious as to minimize observational bias. These behaviors were (i) tongue protrusion, (ii) widely open mouth with lateral jaw displacement, (iii) widely open mouth with tongue rotation inside cheek, (iv) sucking the inside of cheek, (v) copious salivation probably resulting from the above movements, and (vi) expulsion of air from lungs with a loud frog-like sound. In addition, we required a criterion behavior to persist for a minimum of 4 hours after injection to be considered a dyskinesia. Lip smacking and rapid chewing or teeth-chattering movements, which are characteristic of nondrugged monkeys and are frequently elicited by low doses of MA, were not considered evidence of buccolingual dyskinesia.

Table 1. Drug history of monkeys that had been treated with methadone. Subjects are arranged in order of decreasing sensitivity to methamphetamine.

Subject	Methadone		Drug-free period (months)
	Dose (mg/kg)	Duration (months)	
M1	2.5	10	2
M2	2.0	10	2
M3	1.5	22	6
M4	3.0	12	5
M5	3.0	10	2
M6	1.0	12	17
M7	2.5	10	2

Pronounced buccolingual dyskinesias were elicited almost immediately in six of the former methadone-consuming monkeys by an MA dose of only 2.0 mg/kg. Four monkeys were affected on the very first day on which that dose was administered, and only four injection days were required for the others to develop dyskinesias. The oral dyskinesias occurred at a rate of 25 to 60 per minute. Subsequent injections of MA continued to elicit dyskinesias in these monkeys; several were tested 30 to 40 times, and the same oral behaviors were elicited on each occasion. Increasing the MA dosage prolonged the effect; at 2.0 mg/kg, dyskinesias were obvious 36 hours after injection, whereas at 4.0 mg/kg they were observed for as long as 72 hours. The monkeys did not seem to be distressed by their oral behaviors and gave no appearance of greater hyperactivity or arousal than control monkeys.

Conversely, doses of 2.0 mg/kg did not produce dyskinesias in any of the control animals, nor did these behaviors develop after lengthy treatment with doses up to 5.0 mg/kg. Some of the control monkeys would probably have exhibited oral dyskinesias eventually had we given them higher (10 to 20 mg/kg) doses (*4*). Some oral behaviors were elicited in three control monkeys late in the intoxication schedule (*6*); however, they were mild chewing movements that persisted for only 15 to 30 minutes, at which time they were replaced by other stereotyped behavior peculiar to each monkey.

Tardive dyskinesia in humans is manifested not only in the oral behaviors discussed but throughout the musculature, for example, in peculiar limb movements and abnormal postures (*7*). Three of the monkeys showing buccolingual dyskinesias also exhibited choreiform limb movements after 1 to 2 days at 2.0 mg/kg. This behavior consisted of rhythmic flailing of the up-