Cyclic AMP and calcium in the differential control of Mytilus gill cilia

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Summary. Lateral (L) cilia of Mytilus gill are activated by serotonin which, in molluscan systems, is known to activate adenylate cyclase. Triton-extracted models of L-cells, arrested at \( >10^{-6} M \) Ca\(^{++}\), are stimulated to beat by the addition of \( 10^{-5} M \) cAMP while still under Ca\(^{++}\) arrest conditions, suggesting that cAMP-activation is not mediated by alterations of Ca\(^{++}\) levels. Using isolated, permeabilized cilia, we find, independent of [Ca\(^{++}\)], that cAMP-dependent protein phosphorylation in L-cilia occurs uniquely and reversibly on three low molecular weight polypeptides of 23,000, 18,000, and 14,000 daltons. Phosphorylation is maximal at cAMP concentrations above 0.5 \( \mu M \). The phosphorylated chains partially co-extract at high salt with a 14S dynein fraction and have approximately the same molecular weights as reported for dynein light chains. Such conditions mainly extract the outer dynein arm, about 40% of the Mg\(^{++}\)-ATPase activity, and a corresponding amount of the cAMP phosphorylated chains. However, the three polypeptides sediment together at 10-11S, clearly separable from the 14S dynein ATPase. Using a gel-overlay technique, we find that calmodulin binds to axonemal polypeptides of L-cilia with molecular weights of 18,000 and 13,000, independent of Ca\(^{++}\), while in mixed-population cilia, only a 12,000 dalton chain binds calmodulin, in a Ca\(^{++}\) dependent manner. In neither case are calmodulin binding proteins found in the high salt fraction containing the cAMP-dependent phosphorylated chains, indicating that, in spite of some similarity in molecular weight, the cAMP-phosphorylated and calmodulin binding polypeptides are different. Also, double-labeling indicates that only the 18,000 dalton chains co-migrate. These data suggest that serotonin may activate lateral cilia through a cAMP-dependent phosphorylation of a dynein-associated regulatory protein complex, while Ca\(^{++}\) may inhibit ciliary movement, independently, by binding to calmodulin associated with a different class of regulatory protein.

Introduction

Lateral (L) cilia of Mytilus gill are under neuronal control and are normally beating unless struck with a foreign particle. This mechanosensitivity is a consequence of Ca\(^{++}\) influx, resulting in temporary arrest (cf. Murakami and Machemer 1982). In contrast, the abfrontal cilia of Mytilus gill activate when mechanically stimulated, also the consequence of a Ca\(^{++}\) influx (Stommel 1984a). Both the Ca\(^{++}\)-dependent arrest of lateral cilia and the activation of abfrontal cilia may be reversed by inhibitors of calmodulin (Stommel 1984b), suggesting that calmodulin may mediate these diametrically opposite effects of Ca\(^{++}\).

The lateral cilia of isolated gill filaments are usually quiescent in normal seawater, remaining at a point about two-thirds of the way into the Ca\(^{++}\) arrest position. It has long been known that serotonin (5-hydroxytryptamine, 5-HT) activates beating of quiescent lateral cilia (Aiello 1962; Paparo 1972) and that dopamine arrests the beating cilia (Paparo and Aiello 1970). In fact, it has been demonstrated that serotonin (5-hydroxytryptamine, 5-HT) activates beating of quiescent lateral cilia (Aiello 1962; Paparo 1972) and that dopamine arrests the beating cilia (Paparo and Aiello 1970). In fact, it has been demonstrated that serotonin will even activate lateral cilia that have been arrested by Ca\(^{++}\) and ionophore (Murakami and Takahashi 1975). Both serotonin and dopamine stimulate adenylate cyclase activity (Higgins 1974; Malanga and Poll 1979), increasing intracellular levels of cAMP, which normally leads to the activation of a cAMP-dependent protein kinase. Recent evidence shows...
that cAMP or theophylline (an inhibitor of phosphodiesterase), added to the extracellular medium, can activate lateral cilia to beat (Murakami 1983).

It is of interest to determine whether the implicated mechanism of serotonin action, namely to raise cAMP levels, might ultimately result in the activation of lateral cilia through some specific protein phosphorylation and also whether this phosphorylation is Ca$^{2+}$- or calmodulin-dependent. Since Mytilus lateral cilia can be isolated as a pure population by serotonin stimulation of hypercontracted shocked cilia (Stommel 1984b), we are able to explore this question. We find that cAMP is effective in activating ciliary beat in Ca$^{2+}$-arrested lateral cell models, overriding Ca$^{2+}$-arrest through the coincident specific phosphorylation of three associated axonemal polypeptides, independent of Ca$^{2+}$. In addition, we find that calcium-inhibited lateral cilia and calcium-insensitive or activated mixed population cilia bind calmodulin differentially to three low molecular weight polypeptides that are not the same as the three cAMP phosphorylated chains.

Materials and methods

**ATP-reactivated Mytilus gill cell models.** Mytilus edulis (mussels) were kept in running sea water, the temperature of which was maintained below 15 °C. Individual gill filaments were dissected from excised gills immediately before use. The extraction solution employed was modified from that of Tsuchiya (1977) by using 0.01% Nonidet P-40 instead of 0.012% and adding 9% glycerol. These solutions contained 150 mM KC1, 10 mM MgCl$_2$ (beat-reactivation and arrest-reactivation), 1 mM Tris-HCl, and either 20 mM MgCl$_2$ (extraction), or 10 mM Tris-$\cdot$HCl (beat- and arrest-reactivation), pH 7.0 (extraction) or pH 8.0 (wash and both reaction solutions). In addition, the arrest-reaction solution was 50 μM in Ca$^{2+}$. Single gill filaments were extracted for 15 min on ice with 0.25% NP-40 in 0.6 M NaCl, 5 mM MgSO$_4$, and 10 mM Tris-$\cdot$HCl (modified from Tang et al. 1981). Isolation of cilia. Lateral cilia were selectively isolated from excised, washed gills by brief hypertonic treatment, followed by serotonin stimulation (Stommel 1984b), while the remaining, mixed cilia types were removed by subsequent treatment with calcium-free sea water at normal toxicity (Sanderson and Sleigh 1981; Stommel 1984b). Isolation solutions were buffered with 10 mM Tris-HCl (pH 8.0) to maximize mucus dispersal. The isolated cilia were subjected to successive cycles of differential centrifugation at 1,000 g for 5 min to pellet cell debris and 10,000 g for 10 min to recover the cilia. Utilizing 10 mM Tris (pH 8) buffered sea water containing 0.1 mM EDTA and including 0.5 mM phenylmethylsulfonyl fluoride (PMSF) to minimize proteolytic digestion. All steps were carried out at 4 °C. Immediately before use, the pellets of purified cilia were resuspended and thoroughly washed with an artificial seawater medium consisting of 400 mM NaCl, 150 mM KCl, 10 mM MgCl$_2$, and 10 mM Tris-$\cdot$HCl, pH 8.0.

**Incubation and phosphorylation.** Before labeling, the pellets of cilia were resuspended to a final concentration of 1-3 mg/ml protein in a permeabilizing solution (Tsuchiya 1977) consisting of 150 mM KCl, 10 mM MgCl$_2$, 10 mM Tris, pH 8, and 0.012% NP-40, and then incubated on ice for 10-15 min. After permeabilization, aliquots of 0.1 ml were pipetted into a 1.0 ml incubation mixture consisting of the above solution minus the detergent, containing either 1 mM EGTA or 50 μM CaCl$_2$. Phosphorylation was initiated by addition of ATP to a final concentration of 2.5 mM, with each sample containing 25 μCi of gamma-32P-labeled ATP (New England Nuclear, NEG-0021, sp. act. 3,000 Ci/mmol; diluted 1:5 with 0.1 M neutralized carrier ATP). For membrane and axoneme analysis, the samples were incubated for 10 min at 20 °C, chilled rapidly on ice, and the cilia were recovered by centrifugation at 25,000 g for 5 min. They were then fractionated into axoneme (9+2) and membrane plus soluble matrix fractions by extraction with 0.25% NP-40 for SDS-PAGE analysis (Stommel et al. 1982).

**SDS-polyacrylamide gel electrophoresis.** The ionic system of Laemmli (1971) was used as originally formulated but modified to contain a 5-15% linear polyacrylamide gradient as the resolving gel (Stommel et al. 1982). Samples were rapidly suspended in sample buffer containing 0.5 mM PMSF, sonicated when necessary, and then quickly applied to the sample wells and run immediately. The gels were stained and destained by the method of Fairbanks and co-workers (1971), photographed, dried, and autoradiographed at −80 °C using pre-flashed Kodak XAR-2 X-ray film and DuPont Lightning Plus rare earth enhancement screens (Laskey and Mills 1977). Relative protein phosphorylation was determined from the autoradiograms by densitometry.

**Calmodulin gel overlay.** The gel overlay technique of Carlin et al. (1981) was used exactly as published, except that gel incubations were carried out at the physiological pH of 8.0 instead of 7.6. Sample sets of both lateral and mixed population cilia, or fractions thereof, were run in duplicate on the same slab gel, the gel was bisected vertically and the two identical halves were processed in parallel in either 1 mM EGTA or 1 mM Ca$^{2+}$. Iodinated (125I) calmodulin was obtained from New England Nuclear.

**Sucrose gradient analysis.** Labeled, demembranated cilia were extracted for 15 min on ice with 0.25% NP-40 in 0.6 M NaCl, 3 mM MgCl$_2$, 30 mM Tris, pH 8, 1 mM DTT, and 0.1 mM EDTA. Such a procedure rapidly removes all of the outer arms and approximately 40% of the dynein ATPase, but in a non-latent form (unpublished). After centrifugation at 45,000 g for 10 min, the pellet and extract were either analyzed by SDS-PAGE and autoradiography or else the extract was applied to a sucrose gradient for dynein separation. The 4.2 ml gradient consisted of 5-30% sucrose containing 0.6 M NaCl, 5 mM MgSO$_4$, and 10 mM Tris, pH 8.0 (modified from Tang et al. 1981). The gradient was spun at 50,000 rpm for 7 h at 4 °C in a Beckman SW 60 Ti rotor and then, by pumping from the bottom via a long syringe needle, the gradient was divided into 17-18 fractions of 0.25 ml each (including the 0.3 ml sample volume) for SDS-gel and ATPase analysis.

**Assay procedures.** Protein was determined by the method of Lowry et al. (1951) or from densitometry of stained SDS-PAGE gels. Total Mg$^{2+}$-ATPase was determined by incubating in 30 mM Tris (pH 8), 3 mM MgSO$_4$, 0.1 mM EDTA, 0.1 mM Mg$^{2+}$-ATPase was determined by incubating in 30 mM Tris (pH 8), 3 mM MgSO$_4$, 0.1 mM EDTA, 0.1 mM ATP.