Ca\(^{2+}\)-regulated guanylate cyclase in ciliary membranes from \textit{Paramecium} contained tightly bound calmodulin. Antibera against calmodulin from \textit{Tetrahymena} and soybean inhibited enzyme activity. EGTA did not easily release calmodulin; however, La\(^{3+}\) inhibited guanylate cyclase by dissociation of calmodulin. While La could not replace Ca in the activation of guanylate cyclase, it substituted for Ca\(^{2+}\) in the activation of calmodulin-dependent phosphodiesterase from pig brain independently of whether homologous or \textit{Paramecium} calmodulin was used. After removal of endogenous calmodulin from guanylate cyclase, reconstituted was achieved with calmodulin from \textit{Paramecium}, \textit{Tetrahymena}, pig brain, and soybean. Ca\(^{2+}\)-binding proteins lacking trimethyllysine like calmodulin from \textit{Dictyostelium}, parvalbumin, and troponin C failed to restore enzyme activity. The properties of the native and reconstituted guanylate cyclase/calmodulin complex were compared. Reassociation of calmodulin with its target enzyme was weak since all calmodulin remained in the supernatant after a single centrifugation. While most enzyme characteristics remained unchanged in the reconstituted complex, the inhibition by Ca > 100 \(\mu\)M was of a mixed-type compared to noncompetitive inhibition in the native enzyme. The regulation of the enzyme by cations was also altered. Whereas Ca was the most potent and specific activator of the native enzyme, in the reconstituted system Sr was far more effective.

In the cilia of the protozoan \textit{Paramecium tetraurelia} the concentration of free Ca ions is intimately related to the capability of the mechanical machinery to alter speed and direction of the ciliary beat (1). Thus, with Triton X-100-extracted models a reversal of the ciliary beat is observed, corresponding to the avoiding reaction of \textit{Paramecium}, as soon as the free Ca concentration exceeds 1 \(\mu\)M (2). The Ca sensitivity of the mechanical apparatus of the cilia, the axoneme, is complemented by specific Ca conductances of the ciliary membrane (3). There, voltage-sensitive Ca channels are localized which are responsible for the depolarization of the cell during a Ca/K action potential (4). Possibly, the Ca fluxes across the membrane are directly coupled to a particulate Ca-sensitive guanylate cyclase which has been found in the excitable ciliary membrane (5). The Ca concentration of 8 \(\mu\)M necessary for half-maximal activation of this enzyme is well within the range known to affect ciliary beating (6, 7). Since CGMP-dependent protein kinase and calcineurin\(^{1}\) have also been identified as ciliary components (8), several meaningful suggestions can be made about how a physiological stimulus translates via a Ca/K action potential into a defined mechanical and behavioral response in \textit{Paramecium}. It is conceivable that the Ca-regulated guanylate cyclase plays a mediating and amplifying role in such a reaction sequence. The Ca sensitivity of the guanylate cyclase is due to CaM\(^{2}\) (9). Next to the most interesting question of the detailed role of the Ca/CaM-regulated guanylate cyclase in ciliary function, the enzyme deserves interest in its own right since so far this particular type of guanylate cyclase has only been found in the related protozoans \textit{Tetrahymena} and \textit{Paramecium} (9, 10). In order to dissociate CaM from the guanylate cyclase, washing ciliary membranes with low concentrations of La is an efficient and gentle method (9). This enabled us to investigate the conditions necessary for reconstitution of the enzyme and the specificity and nature of the interactions between CaM and the catalytic part of the guanylate cyclase. To establish these characteristics may prove a prerequisite for successful searches for Ca/CaM-regulated guanylate cyclases in metazoan systems.

**MATERIALS AND METHODS**

**Materials**—\(\alpha\)-\[^{32}P\]GTP (specific activity 400-600 Ci/mmol), \[^{3}H\] cGMP (16 Ci/mmol), and \[^{3}H\] CaM (36 Ci/mmol) were purchased from Amersham-Buchler. Phosphoenolpyruvate potassium salt, pyruvate kinase (rabbit muscle), and all nucleotides (sodium salts) were obtained from Boehringer Mannheim. LaCl\(_3\) was from Fluka. \textit{Tetrahymena} calmodulin, antibodies against \textit{Tetrahymena} CaM (serum and purified immunoglobulin, IgG), and preimmune IgG were kindly provided by Dr. J. F. Harper, Medical Center, Houston. \textit{Dictyostelium} CaM was a gift from Dr. Y. Nozawa, Gifu University, Gifu, Japan. Serum with antibodies against soybean CaM was from Dr. J. F. Harper, Medical Center, Houston. \textit{Dictyostelium} CaM was a gift from Dr. G. A. Jamieson, Washington University, St. Louis. Carp parvalbumin and rabbit skeletal muscle troponin C were kindly provided by Dr. J. D. Potter, University of Cincinnati and Dr. F. Hofmann, University of Heidelberg, respectively.

**Cell Culture and Preparation of Cilia and Ciliary Membranes—** \textit{Paramecium tetraurelia} wild type strain 51s (from Dr. E. Kaneshiro, University of Cincinnati) and the mutants pawa/pawB and para-noic d4-147 (gifts from Dr. C. Kung, University of Madison) were grown axenically in 2-liter bioreactors as described (13). Routinely.


\(^{2}\) The abbreviations used are: CaM, calmodulin; EGTA, ethylene glycol bis (\(\gamma\)-amino-ethyl ether)-N,N,N\(^{\prime}\),N\(^{\prime}\)-tetraacetic acid.
early stationary cultures with a cell density of about 25,000 cells/ml were used.

Cells were deciliated by a calcium shock (12), pelleted at 160 × g for 10 min, and used for isolation of CaM. Cilia were further purified from the supernatant according to Thiele et al. (13). Ciliary membrane vesicles were prepared as described (7). Briefly, cilia were disintegrated by sonication on a Vortex Whirimir and membrane vesicles were separated from incompletely demembranated cilia and axonemal fragments by a sucrose step gradient (14). After a washing step at 45,000 × g for 30 min, the vesicle suspension was adjusted to 1 mg protein/ml in 2.5 mM Tris-HCl buffer, pH 7.8, frozen in liquid nitrogen, and stored at −80 °C.

Ciliary guanylate cyclase depleted of endogenous CaM was prepared by La3+ treatment prior to the assay. If not otherwise indicated, the vesicles were incubated for 15 min at 0 °C with 13 μM La3+. After centrifugation at 48,000 × g for 60 min, the pellet was resuspended in 2.5 mM Tris-HCl buffer, pH 7.8, to about 1 mg of protein/ml. Sometimes traces of contaminating La ions were removed by either two additional washes with Tris buffer alone or with Tris buffer containing 100 μM EGTA. However, no differences in results were recorded.

CaM-dependent phosphodiesterase from pig brain was partially purified according to Teshima and Kakiuchi (15) by a Sephadex G-200 column.

CaM from pig brain and from Paramecium cell bodies was prepared according to Dedman et al. (16) and Charbonneau and Cormer (17). The purification steps included ammonium sulfate fractionation, ion exchange chromatography on DEAE-cellulose, and gel filtration chromatography. Identity and purity were verified by properties generally accepted to be characteristic for CaM such as heat stability, electrophoretic mobility, and the ability to activate a CaM-dependent phosphodiesterase from pig brain in the presence of Ca2+ (18).

Protein was determined by the Lowry method using bovine serum albumin as a standard.

Enzyme Assays—Guanylate cyclase activity was determined for 8 min at 37 °C (7). The standard reaction mixture contained in 90 μl: 10–40 μg of membrane vesicle protein, 1.8 μmol of Tris-HCl, pH 7.8; 90 nmol of [1H]GMP (5 nCi) to monitor recovery; 270 nmol of MgSO4; 2.7 mM of CaCl2; 2.0 μmol of phosphoenolpyruvate; 1.25 μmol of pyruvate kinase; and 40 nmol of GTP including 0.5-1 μCi of [α-32P]GTP.

The activity of CaM-hydrolyzing phosphodiesterase from pig brain was assayed according to Teshima and Kakiuchi (15) by a Sephadex G-200 column.

The antigenic portion of this membrane-bound calmodulin indicated that in spite of several amino acid substitutions in the protozoan CaMs (20, 21) the basic conservative features of Paramecium CaM were retained. Usually, CaM is easily released from its respective enzyme when Ca ions are removed by chelating agents like EDTA or EGTA. After washing ciliary membranes with 100 μM EGTA, guanylate cyclase activity was reduced by more than 50%; however, addition of Ca2+ alone completely restored enzyme activity. Even after rinsing with buffers containing 1 mM EDTA, the inhibited guanylate cyclase (<20%) could be reactivated to about 60% of the original activity by addition of Ca ions. This suggested that CaM was not easily removed by these procedures but remained associated with the membrane by an unusual strong binding between CaM and guanylate cyclase.

Effect of Cations on CaM-mediated Enzyme Activities—La3+ was tested as replacement of Ca2+ in the CaM-mediated activation of guanylate cyclase. Even at nanomolar concentrations, La ions like the lanthanides Ce and Tb could not substitute for Ca but were inhibitory (Fig. 1). The enzyme inhibition could not be overcome by addition of a surplus of Ca2+ (range tested from 1 μM–10 mM) or by simple removal of La3+ by centrifugation and several washes of the membranes. Complete restoration of guanylate cyclase activity in the membrane pellet was only possible by simultaneous addition of Ca and CaM. In the supernatant of the La-treated membranes, the presence of CaM was demonstrated by its ability to activate a Ca/CaM-dependent phosphodiesterase from pig brain (data not shown). It seems likely that La3+ interacted with CaM resulting finally in its dissociation from guanylate cyclase. Obviously, the inactive La3+-CaM complex could subsequently be replaced by the active Ca2+-CaM complex. Next we examined whether the effect of La was causally related to the slight structural alteration of the protozoan CaM (20, 21) or whether La somehow affected a particularly intricate CaM binding site at the guanylate cyclase entity. CaM isolated from Paramecium fully activated a CaM-dependent phosphodiesterase partially purified from pig brain (Fig. 2). Activation was optimal with Ca as divalent cation.

**RESULTS AND DISCUSSION**

Calmodulin As a Component of Ciliary Guanylate Cyclase—A most prominent feature of the ciliary guanylate cyclase from Paramecium is its dependence on physiological concentrations of Ca ions for activity (7). Yet, originally a mediatory role of CaM was not evident since even 100 μM trifluoperazine, a potent inhibitory drug for almost all CaM-dependent processes, inhibited guanylate cyclase activity by not more than 30% (7). Also, addition of exogenous CaM from Tetrahymena, Paramecium, and pig brain (up to 10 μg/assay) to isolated ciliary membranes did enhance enzyme activity by at most 30%. Nevertheless, a possible involvement of endogenous CaM in guanylate cyclase regulation was further probed by the use of antibodies against CaM from the related protozoan Tetrahymena and from soybean. Both antisera (30 μl/assay) decreased guanylate cyclase activity by about 70% while preimmune IgG was completely without effect. This strong inhibition tentatively designated the ciliary guanylate cyclase as a target enzyme of a regulation by endogenous calmodulin. The antigenic portion of this membrane-bound calmodulin apparently is exposed to the surface to such an extent as to react at least partially with antibodies against nonhomologous CaMs. Furthermore, the inhibitory potency of the antibodies raised against the plant CaM indicated that in spite of several apparent amino acid substitutions in the protozoan CaMs (20, 21) the basic conservative features of Paramecium CaM were retained. Usually, CaM is easily released from its respective enzyme when Ca ions are removed by chelating agents like EDTA or EGTA. After washing ciliary membranes with 100 μM EGTA, guanylate cyclase activity was reduced by more than 50%; however, addition of Ca2+ alone completely restored enzyme activity. Even after rinsing with buffers containing 1 mM EDTA, the inhibited guanylate cyclase (<20%) could be reactivated to about 60% of the original activity by addition of Ca ions. This suggested that CaM was not easily removed by these procedures but remained associated with the membrane by an unusual strong binding between CaM and guanylate cyclase.
(ED$_{50}$ = 8 µM). Sr could substitute for Ca, although somewhat higher concentrations were necessary (ED$_{50}$ = 60 µM, Fig. 2A). Using CaM from Paramecium, La ions could at least partially replace Ca in activating pig brain phosphodiesterase (ED$_{50}$ = 5 µM), only at concentrations >15 µM, La inhibited the already enhanced enzyme activity (Fig. 2A). Using the homologous pig brain CaM instead of the protozoan protein for activation of phosphodiesterase, almost identical results were obtained (Fig. 2B). The ED$_{50}$ values for Ca and Sr were 6 and 30 µM, respectively; for La, half-maximal activation was seen at 6 µM. As with CaM from Paramecium, inhibition of pig brain CaM-activated phosphodiesterase was apparent at higher La concentrations (>20 µM). Possibly, this inhibition is unrelated to La/CaM interactions, but may involve effects of La on the phosphodiesterase itself, e.g. on a specific CaM-binding site. The functional similarities between CaMs from metazoan and protozoan sources as far as phosphodiesterase activation is concerned indicate then that the lack of any stimulatory effect of La on CaM-dependent guanylate cyclase from Paramecium is not related to a particular structural feature of the CaM from Paramecium but to a prevalent and specific site for divalent alkaline earth metal ions and La at the target enzyme. That ion binding site is thought to be directly involved in the ionic environment surrounding the hydrophobic region, which is suggested to play a critical role in the interaction between CaM and its target enzymes (22). This conclusion is further supported by the earlier finding that the inhibitory effect of high Ca concentrations on guanylate cyclase from Paramecium is most likely exerted via the same binding site as that for La ions (7). On the other hand, the stimulation of phosphodiesterase by La$^{3+}$-CaM complexes from Paramecium suggests that CaM interacts with guanylate cyclase and brain phosphodiesterase differently, the interaction with the cyclase being most sensitive to La$^{3+}$.

**Reactivation of Guanylate Cyclase by CaM—Ciliary guanylate cyclase depleted of endogenous CaM by La treatment was effectively reactivated by CaMs from various sources (Fig. 3).** CaM from the slime mold Dictyostelium, that lacks the characteristic trimethyllysine residue, and the related Ca-binding proteins parvalbumin from carp muscle and troponin C from skeletal muscle were inactive. This is in line with the well established conservative structure of CaM throughout all eucaryotes. As shown in Fig. 3A, CaM from the protozoan Tetrahymena was clearly the most potent activator with an ED$_{50}$ of 70 ng/assay corresponding to 46 nM. Using soybean and pig brain CaMs, significantly higher concentrations were necessary to activate guanylate cyclase; ED$_{50}$ doses were 1.2 and 2 µg (0.78 and 1.3 µM), respectively. Nevertheless, these CaMs could more or less completely restore guanylate cyclase activity to levels prior to removal of CaM. This observation implies that the protozoan CaM is better suited to bind to and activate the guanylate cyclase, possibly because of its slightly altered structure compared to metazoan CaM. The high potency of Tetrahymena CaM seemed to be even better than that of the CaM from Paramecium tetraurelia wild type 51s (■), from the double mutant pawnA/pawnB (△), and from the mutant paranoiac, d4-147 (○). 100% represents the guanylate cyclase activity prior to removal of calmodulin (1485 pmol of cGMP/µg × min$^{-1}$).
material on analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The mean specific activity of guanylate cyclase in the cilia from pawnA/pawnB was 200 pmol/mg x min⁻¹ and that from the paranaio was in the range of 150–200 pmol/mg x min⁻¹. These activities are comparable to values found in cilia of wild type and with a mean of 230 pmol of cGMP formed/mg x min⁻¹ (13 preparations). Therefore, it is concluded that the mutations did not affect the biological activity of CaM itself nor the CaM-regulated ciliary guanylate cyclase. However, these experiments do not finally exclude a participation of CaM in other processes involved in the altered ion conductances of these mutants, although this seems somewhat unlikely.

Properties of the Reconstituted CaM/Guanylate Cyclase Complex—As demonstrated, CaM is tightly bound to the ciliary guanylate cyclase from *Paramecium*. Even during the strenuous osmotic and ionic conditions of deciliation and isolation of cilia, which involve steps with buffers containing 150 mM sucrose, 625 mM glycerol, and 25 mM CaCl₂, or during repeated freezing and thawing, almost no CaM was split off from the guanylate cyclase. This again indicates a particularly strong and specific binding and interaction of CaM with its target enzyme. However, once CaM is removed by La treatment, reassociation of the homologous and other CaM-binding sites at the guanylate cyclase occurred once the enzyme could not quite slip back into the original hydrophobic binding pocket within the membrane fraction. By combination of the supernatant and pellet, CaM remaining in the supernatant or upon a single centrifugation (48,000 g for 60 min) while the totally inactive catalytic subunit sedimented with the membrane fraction. By combination of the supernatant and pellet, guanylate cyclase activity was fully recovered. Apparently, CaM once released from the enzyme could not quite slip back into the original hydrophobic binding pocket within the guanylate cyclase where it is otherwise protected so effectively against easy removal. Perhaps slight steric modifications of the CaM-binding site at the guanylate cyclase occur once the activator is removed.

The altered stability of the reconstituted CaM/guanylate cyclase complex necessitated a re-examination of its kinetic properties. The pH and temperature optimum (pH 8, 37 °C) were identical with the native enzyme complex (7). The reconstituted enzyme exhibited essentially the same Michaelis-Menten behavior as untreated guanylate cyclase (7). The *Kₘ* for GTP was slightly decreased (38 versus 44 μM) and *Vₕₘₐₓ* was decreased corresponding (41 pmol/min versus 49 pmol/min) so that in a Lineweaver-Burk plot parallel lines were obtained (Fig. 4A). The slopes of 1 in the Hill plot indicated that no cooperative interactions with the substrate GTP occurred neither in the native nor in the reconstituted CaM-dependent guanylate cyclase system (Fig. 4B). Ca⁺ > 100 μM was reported to reversibly inhibit ciliary guanylate cyclase from *Paramecium* in a noncompetitive way (7). An examination of the inhibitory effect of Ca on the reconstituted enzyme revealed different kinetics (Fig. 4A). Both *Vₕₘₐₓ* and apparent *Kₘ* values were altered significantly and independently compared to control resulting in a mixed-type inhibition. Since in these experiments GTP concentrations of 20–200 μM were used together with 3 mM Mg²⁺, effects of variations in free cation concentrations can almost be excluded to have affected the above results. Possibly, Ca ions can bind to an inhibitory site at the reconstituted guanylate cyclase which is not readily accessible in the native tightly coupled system. This locus may be identical with the effector site of lanthanides which have roughly the same ionic radius as Ca but carry an additional positive charge and thus often and efficiently displace Ca from its binding sites.

The alteration of the inhibitory kinetic at high Ca concentrations brought about by CaM extrication from and superficial reattachment to the ciliary guanylate cyclase raised the question whether in the reconstituted enzyme the ion specificity for activation was retained. As shown previously (7), enhancement of CaM-dependent guanylate cyclase activity was specifically achieved by Ca²⁺. Sr²⁺ was only about half as effective while Ba²⁺ had negligible potency (see also Fig. 1). With the reconstituted enzyme system several remarkable differences in the activation by divalent alkaline earth metals were found (Fig. 5). Most prominently, Ca was not anymore the best suited ion; rather Sr was most effective to enhance CaM-dependent guanylate cyclase. With 500 μM Sr²⁺ maximal activation was 1.5 times higher than that with 50 μM Ca²⁺. Ca concentrations > 60 μM were already inhibitory. Furthermore, even Ba was now capable to considerably stimulate cGMP formation, at 250 μM Ba²⁺ enzyme activity was enhanced about 5-fold (Fig. 5). The findings may indicate that in the reconstituted guanylate cyclase the stimulatory effect of Ca mediated by CaM collides early on with its rather specific inhibitory action on the enzyme moiety itself. With Sr, higher concentrations are necessary for optimal guanylate cyclase activation in both the native (7) and the reconstituted enzyme system; however, apparently Sr is much less inhibitory compared to Ca.

In view of the differences in enzyme kinetics between the native CaM-dependent guanylate cyclase and the reconstituted enzyme system some general considerations have to be made. Using the guanylate cyclase of the excitable ciliary membrane from *Paramecium* as a model for particulate Ca²⁺/CaM-regulated enzymes it becomes obvious that the CaM-binding site(s) at a target enzyme is of major importance for the enzyme regulation itself, e.g. the ion selectivity of the stimulatory process seems to critically depend on the proper fitting of CaM into its binding site. Once CaM is dissociated...
from the effector enzyme, reconstitution by soluble CaM may superficially pose no problems; however, due to minute alterations in CaM binding, the native responsiveness to divalent cations and the original regulatory properties of such reconstituted systems could be lost. It may not be unreasonable to assume that CaM as a protein of 17,000 Da is not shovelled around within the cell as are small molecules of the intermediary metabolism. Rather, it is conceivable that much of the intracellular CaM is tightly coupled to CaM-dependent enzymes, thereby conferring Ca sensitivity. In vivo removal from and reassembly of CaM to specific binding sites at target enzymes may by itself constitute a regulatory capacity of a cell by which gross levels of enzyme activity could be modulated rapidly without the need for protein degradation or synthesis. Consequently, in order to avoid experimental artifacts the integrity of CaM/target enzyme interactions must be maintained when CaM-regulated processes are investigated in vitro. This point is particularly stressed by the data presented here, where dissociation and reassociation finally resulted in altered enzyme properties. Little is known about possible modifications of CaM-binding sites during the usual steps of enzyme preparations. CaM sensitivity may decrease time dependently as with cAMP phosphodiesterase from Drosophila melanogaster (25), it may be completely lost, e.g. by proteolytic alteration of CaM-dependent phosphodiesterase from mammalian tissue (26), or the CaM-binding site may be modified to such an extent that a partly fictitious CaM specificity is displayed as seen with guanylate cyclase from Tetrahymena \(^3\) (10, 27). Considering the apparent lability of the CaM-binding site(s) at the particulate protozoan guanylate cyclases, one may conclude that special precautions should be taken when searching for similar guanylate cyclases in metazoan systems.

Obviously, our experiments do not advance the knowledge of the physiological role of the ciliary guanylate cyclase or cGMP itself in Paramecium. However, the location of this in the excitable ciliary membrane and the delicate nature of its regulation by Ca via CaM make it likely that the guanylate cyclase will serve as an immediate amplifier of an ionic signal running across the ciliary membrane. Whether it is directly coupled to the Ca conductance, which is part of the Ca/K action potential of Paramecium (2, 4), remains to be elucidated.

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