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Regulation of peptide–calmodulin complexes by protein kinase C *in vivo*

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ABSTRACT **We used the freshwater protozoan *Paramecium tetraurelia* to investigate the potential regulation by protein kinase C of calmodulin interactions with binding peptides in intact cells. In these organisms, an action potential results in membrane depolarization and a period of backward swimming; repolarization and a return to forward swimming requires the presence of normal calmodulin. We postulated that injection of high-affinity calmodulin binding peptides might interfere with repolarization and thus prolong the period of membrane depolarization. Synthetic peptides spanning the protein kinase C phosphorylation site/calmodulin-binding domains of the myristoylated alanine-rich C-kinase substrate (MARCKS) and the MARCKS-related protein (also known as F52 or MacMARCKS) were injected into cells; these caused a 2- to 3-fold increase in the duration of backward swimming. Similar changes were seen with two other calmodulin-binding peptides. This behavioral response could be prevented by coinjecting calmodulin. Activation of *Paramecium* protein kinase C with an active phorbol ester completely reversed (within 3 min) the behavioral effects of the normal MARCKS and MARCKS-related protein peptides. Injection of a nonphosphorylatable peptide, in which alanines were substituted for serines, resulted in the usual behavioral response; however, this was not reversed by phorbol ester treatment. The corresponding aspartate-substituted peptide, which has a 10-fold lower affinity for calmodulin, did not prolong backward swimming. These data suggest that these peptides can form complexes with calmodulin at the calcium concentrations that prevail in intact *Paramecium* cells and that such complexes can be disrupted by protein kinase C phosphorylation of the peptides.**

It is often difficult to prove that protein–protein interactions occur in intact cells, even though these interactions can be demonstrated readily in cell-free systems. This difficulty is magnified when complex formation and disruption are subject to rapid regulation, as by covalent modification of one of the partners. In this study, we have used a behavioral assay in intact *Paramecium* cells to demonstrate the formation of a complex involving calmodulin and a calmodulin-binding peptide; the same system was used to demonstrate disruption of the complex by protein kinase C (PKC)-mediated phosphorylation of the calmodulin-binding peptide.

The calmodulin-binding peptide used in these studies is a 25-amino acid peptide that contains the calmodulin-binding/phosphorylation site domain of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein, a prominent cellular substrate for PKC (1). In cell-free assays, the peptide binds calmodulin with high affinity (K_d < 5 nM; refs. 2 and 3); this peptide (tetra-Ser peptide) also contains the four serine residues in the MARCKS protein that are phosphorylated by PKC (4, 5). Phosphorylation by PKC of this peptide decreases its affinity for calmodulin by >200-fold and rapidly displaces calmodulin already bound to the peptide (3). Similar characteristics have recently been described for the MARCKS-related protein (MRP), a MARCKS homologue (1) [this protein has also been termed F52 (6) and MacMARCKS (7)]. These findings suggest that PKC could regulate calmodulin interactions with MARCKS and MRP *in vivo*; possible functions of such a reversible interaction include roles as a PKC-sensitive calmodulin reservoir (1–3), PKC- and calmodulin-sensitive actin binding proteins (7, 8), and others.

A fundamental difficulty with this proposal has been proving that such an interaction occurs in intact cells. We have addressed this issue by using a bioassay for "free" calmodulin in the freshwater protozoan *Paramecium tetraurelia*. In this organism, a depolarizing stimulus causes an action potential, a transient reversal of the ciliary beat direction, and a reproducible period of backward swimming; the duration of backward swimming is directly related to the strength of the membrane depolarization (9, 10). In the *cam1* mutant strain of *Paramecium*, a single amino acid mutation in the primary sequence of calmodulin leads to the inhibition of one or more calmodulin-sensitive ion channels and thus to a prolonged period of membrane depolarization and backward swimming (11); injection of normal *Paramecium* calmodulin into these mutant organisms restores the period of backward swimming to normal (12, 13).

In the present study, we have used these properties of *Paramecium* to determine whether MARCKS and MRP peptides would interact with calmodulin in a PKC-sensitive manner in intact cells.

METHODS

Cells and Culture Conditions. We used wild-type *Paramecium tetraurelia*, stock 51s, for all experiments. The cells were grown at 28°C in a wheat-grass medium enriched with stigmasterol (5 mg/liter), buffered with sodium phosphate and Tris, and containing *Enterobacter aerogenes* (14).

Behavioral Assays. The methods used to evaluate the behavioral responses of the cells were similar to those described (15). The cells were incubated in resting solution (1 mM KCl/1 mM $CaCl_2$/1 mM Hepes, pH 7.2) for 2–5 min before testing. The cells were then placed into a solution consisting of the resting solution along with the appropriate ion. The time of backward swimming was recorded while observing individual cells under a dissecting microscope. All the tests were conducted at room temperature.

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Abbreviations: MARCKS, myristoylated alanine-rich C-kinase substrate; MRP, MARCKS-related protein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MLCK, myosin light chain kinase.

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Microinjection. Cells were injected with the various peptides and proteins by using techniques as described (12, 15) with only slight modifications. Approximately 10–20 pl (which is 5–10% of the cell volume) of material was injected into logarithmic-phase cells. The injected cells were transferred to a 10% (vol/vol) wheat-grass solution (diluted with resting solution) and left at 28°C for various times before testing. Each experimental point represents the mean ± SD of determinations from 5 to 10 cells, as noted in the figure legends. In some experiments, phorbol 12-myristate 13-acetate (PMA) was added to the cells; final concentration was 800 nM in 0.1% dimethyl sulfoxide, with 0.1% dimethyl sulfoxide serving as a control.

Peptides and Proteins. The 25-amino acid MARCKS tetra-Ser peptide and its derivatives, tetra-Ala and tetra-Asp, were synthesized and purified by reversed-phase HPLC as described (ref. 5; see Table 1). The corresponding 24-amino acid MRP peptide was synthesized and purified as described (6). A peptide corresponding to the calmodulin-binding domain (residues 37–53) of bovine neuromodulin (16) (also known as GAP-43 and B-52) was synthesized and purified as described (J. Kim, P.J.B., J. D. Johnson, and S. McLaughlin, unpublished data) and was a generous gift from Stuart McLaughlin, State University of New York, Stony Brook (Table 1). A peptide corresponding to the calmodulin-binding domain (residues 342–267) of skeletal muscle myosin light chain kinase (MLCK; Table 1; see ref. 17) was synthesized and purified as described above for the MARCKS and MRP peptides. The human MARCKS protein was expressed in *Escherichia coli* and purified by sequential heat treatment, a 1% trichloroacetic acid cut, acetone precipitation, and Mono Q anion-exchange chromatography; details of this expression and purification will be described elsewhere (G. M. Verghese, J. D. Johnson, D. M. Haupt, and P.J.B., unpublished data). Chicken calmodulin was a generous gift from Genaro D'Urso, Fred Hutchinson Cancer Research Center, Seattle.

Peptide Phosphorylation by *Paramecium* PKC. Logarithmic-phase *Paramecia* were collected by centrifugation (1500 rpm in a Beckman AccuSpin FR centrifuge for 5 min at 24°C) and homogenized with 25 strokes of a Dounce homogenizer in 100 mM β-glycerophosphate, pH 7.5/0.25 M sucrose/2 mM EDTA/2 mM EGTA/2 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/aprotinin (2 mg/ml)/leupeptin (1 mg/ml). The homogenate was centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant (final protein concentration in assay ≈0.35 mg/ml) was then used to stimulate the phosphorylation of the tetra-Ser peptide (final concentration, 400 nM) in the presence or absence of phosphatidylserine (100 μM) and calcium (1.5 mM). The reaction was started by the addition of $MgCl_2$ (10 mM) and [γ-^{32}P]ATP (75 μM; specific activity, ≈9000 cpm/pmol). After 20 min at 30°C, the reaction was stopped by the addition of SDS sample buffer, and equal volumes of the reaction products were subjected to electrophoresis on a SDS/20% polyacrylamide gel. The portions of each lane corresponding to the phosphorylated peptide, or analogous portions of other lanes, were identified by autoradiography, bands were cut out, and radioactivity was measured by scintillation counting.

Table 1. Composition of peptides used for injection

Peptide	Sequence
MARCKS Peptide	
Tetra-Ser	KKKKKRFSFKKSFKLSGFSFKKNKK
Tetra-Ala	KKKKKRFAFKKAFKLAGFAFKKNKK
Tetra-Asp	KKKKKRFDFKKDFKLDGFDFKKNKK
MRP	KKKKKFSFKKPFKLSGLSFKRNRK
Neuromodulin (residues 37–51)	KIQASFRGHITRKKLKG
MLCK (residues 342–367)	KRRWKKNFIAVSAANRFKKISSSGAL

Synthetic peptides used in microinjection experiments; details of synthesis and purification of these peptides, their phosphorylation by PKC, and characteristics of their calmodulin binding are described in the text. For the MARCKS peptides, the four phosphorylated serines, or the substituted amino acids, are underlined.

RESULTS

Effects of Injected Tetra-Ser MARCKS Peptide on the Behavioral Response. Microinjection of the MARCKS tetra-Ser peptide into *Paramecium* led to an increase in the duration of backward swimming (Fig. 1). This increase was >2-fold with peptide concentrations in the injection pipette of 780 nM or higher (a 1:20 dilution is assumed upon injection); further increases in backward swimming duration were not seen with peptide concentrations up to 320 μM (data not shown). The behavioral response was seen as early as 30 min after injection of the peptide and lasted at least 4 h; by 30 h, the swimming behavior of the organisms had returned to normal. No such effect was seen when either buffer alone (Fig. 1) or bovine serum albumin (1 mg/ml; data not shown) was injected. The behavioral effect of the injected peptide was seen with two depolarizing stimuli, 30 mM KCl and 10 mM NaCl/5 mM triethylammonium chloride; 30 mM KCl was used in all subsequent experiments. A similar but more transient effect was seen upon injection of the intact human MARCKS protein (data not shown); the transience of this response may be due to active proteases present in *Paramecium* and the known proteolytic lability of the intact MARCKS protein.

Effect of PMA on Peptide-Induced Changes in the Behavior Response. The effects of activation of PKC on the behavioral responses to the peptides were assessed by exposing the cells to PMA (800 nM) for 2–3 min. As shown in Fig. 2, exposure of the cells to PMA caused a modest 10–15% increase in backward swimming duration that was seen in all experiments. As before, the tetra-Ser peptide alone caused an ≈2.5-fold increase in backward swimming duration; this effect could be completely reversed by 2–3 min of PMA treatment, suggesting that the peptide–calmodulin complex was being disrupted by PKC-dependent peptide phosphorylation. To test this possibility further, we injected a similar

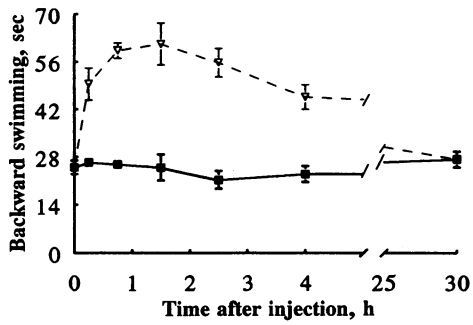

FIG. 1. Effects of the tetra-Ser peptide on backward swimming of *Paramecium*. Logarithmic-phase *Paramecia* in resting solution were injected with ≈10 pl of the tetra-Ser MARCKS peptide (780 nM; dashed line) or phosphate-buffered saline (PBS; solid line). In this and other figures, the concentration of peptide indicated represents the concentration in the injection pipette; a dilution of 1:10 to 1:20 is assumed after injection. After the injection, the cells were placed back in the resting solution. At the indicated times after peptide injection, the cells were placed in a depolarizing solution (30 mM KCl in the above resting solution), and the duration of backward swimming was recorded. Each point represents the mean ± SD of determinations from 10 cells. Not shown is another control involving injection of bovine serum albumin (1 mg/ml), which gave results essentially identical to the injection of PBS. Similar results were seen in another identical experiment.

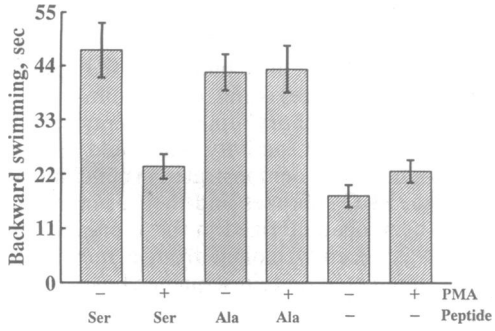

FIG. 2. Effect of PMA on backward swimming of cells injected with either the tetra-Ser or tetra-Ala peptides. Cells were injected with buffer alone (−) or 250 nM tetra-Ser (Ser) or 250 nM tetra-Ala (Ala) peptides for 1.5 h and then exposed to 800 nM PMA in 0.1% dimethyl sulfoxide (+) or dimethyl sulfoxide alone (−) for 2–3 min. The duration of backward swimming after placement of the cells in 30 mM KCl was then measured. Each bar represents the mean ± SD of determinations from five cells (see Fig. 1 for further details).

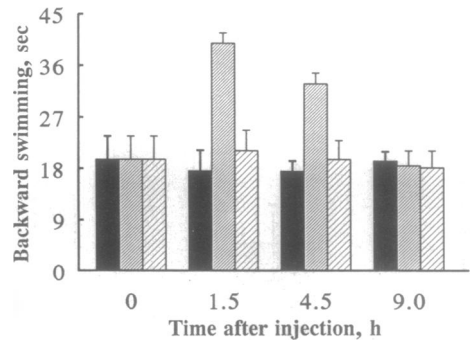

FIG. 3. Effect of tetra-Ala and tetra-Asp peptides on duration of backward swimming. Cells were injected with PBS (solid bars), 450 nM tetra-Ala (dense cross-hatching), or 450 nM tetra-Asp (sparse cross-hatching) peptides, and at the times indicated, the duration of backward swimming was monitored after the cells were placed into the 30 mM KCl depolarizing solution. Each bar represents the mean ± SD of determinations from 10 organisms (see Fig. 1 for further details).

peptide, tetra-Ala (Table 1), which binds calmodulin with identical affinity to tetra-Ser (Kim *et al.*, unpublished data) but cannot serve as a PKC substrate due to the absence of serines and threonines in the peptide. The tetra-Ala peptide had an effect similar to that of the tetra-Ser peptide in the untreated cells (Fig. 2); however, in direct contrast to the tetra-Ser-injected cells, PMA had no effect on the cells injected with tetra-Ala, consistent with the inability of this peptide to be phosphorylated by PKC.

We also tested whether the tetra-Ser peptide could serve as a substrate for a calcium/phospholipid-dependent protein kinase in extracts from *Paramecium*. Although we made no attempt to optimize the conditions of this assay, peptide phosphorylation by a *Paramecium* extract was increased ≈3.5-fold in the presence of Ca^{2+} and phosphatidylserine (data not shown). These data indicate that the peptide could be phosphorylated by a protein kinase present in cytosolic *Paramecium* extracts in a calcium- and phospholipid-dependent manner.

Effect of Tetra-Asp Peptide on the Behavioral Response. We next evaluated the ability of another related peptide to mediate the same response. This peptide, tetra-Asp (Table 1), binds calmodulin with an ≈10-fold lower affinity than the tetra-Ser peptide (Kim *et al.*, unpublished data), presumably because the acidic aspartate residues mimic, at least partially, the effects of the four phosphorylated serines on calmodulin binding. As before, the tetra-Ala peptide mimicked the tetra-Ser peptide exactly, more than doubling the duration of backward swimming that was present at both 1.5 and 4.5 h but not after 9 h (Fig. 3). In contrast, the tetra-Asp peptide had no effect on swimming direction.

Effect of Coinjected Calmodulin on the Response to the Tetra-Ser Peptide. We next examined the specificity of the peptide response by testing whether it could be overcome by coinjection of calmodulin (Fig. 4). This was indeed the case; coinjection of calmodulin, in a dose-dependent manner, prevented the increase in backward swimming duration caused by the MARCKS tetra-Ser peptide (Fig. 4). Calmodulin by itself at the highest concentration tested (600 μM) had no effect.

Effect of Other Peptides. To determine whether the behavioral response to the tetra-Ser MARCKS peptide could be mimicked by other calmodulin-binding peptides, we injected three additional peptides corresponding to the calmodulin-binding domains of the MRP protein (6), MLCK (17) and neuromodulin (ref. 16; also known as GAP-43 and B-52). As shown in Fig. 5*A*, each of these peptides at 320 μM caused a prolongation of backward swimming that was 2- to 3-fold greater than injection of buffer alone. As with the tetra-Ser peptide, addition of PMA to the cells rapidly reversed the behavioral effect of the MRP peptide (Fig. 5*B*).

DISCUSSION

The results of these experiments suggest that the MARCKS tetra-Ser and tetra-Ala peptides, as well as three other calmodulin-binding peptides, prolonged the normal period of backward swimming in *Paramecium* by interacting directly with endogenous calmodulin. Formation of peptide–calmodulin complexes could in turn prevent calmodulin from playing its presumed role in the repolarization process (10–12). This effect was not seen with the MARCKS tetra-Asp peptide, which binds calmodulin with an ≈10-fold lower affinity than the tetra-Ser and tetra-Ala peptides, presumably because the acidic aspartate residues mimic, at least partially, the effects of the strongly negative phosphate groups transferred to the peptide by PKC. Thus, at identical concentrations, the tetra-Ser and tetra-Ala peptides were effective at prolonging the backward swimming response, whereas the tetra-Asp peptide, differing only in four amino acids, was completely ineffective. These data argue strongly that the peptide effect on the behavioral response is mediated through physical interaction of these peptides with calmodulin and

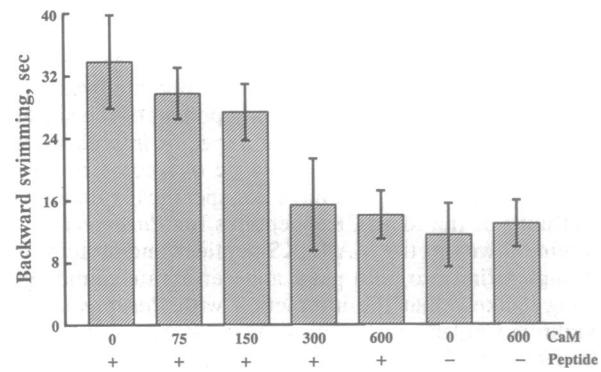

FIG. 4. Effect of injected calmodulin on the behavioral response to injected tetra-Ser MARCKS peptide. Cells were injected with buffer alone (−), chicken calmodulin alone (600 μM; CaM), or MARCKS tetra-Ser peptide (780 μM) with the indicated concentrations of calmodulin (0–600 μM). Two hours after the injections, the duration of backward swimming was measured. Each bar represents the mean ± SD of determinations from 5 to 10 individual cells (see Fig. 1 for further details).

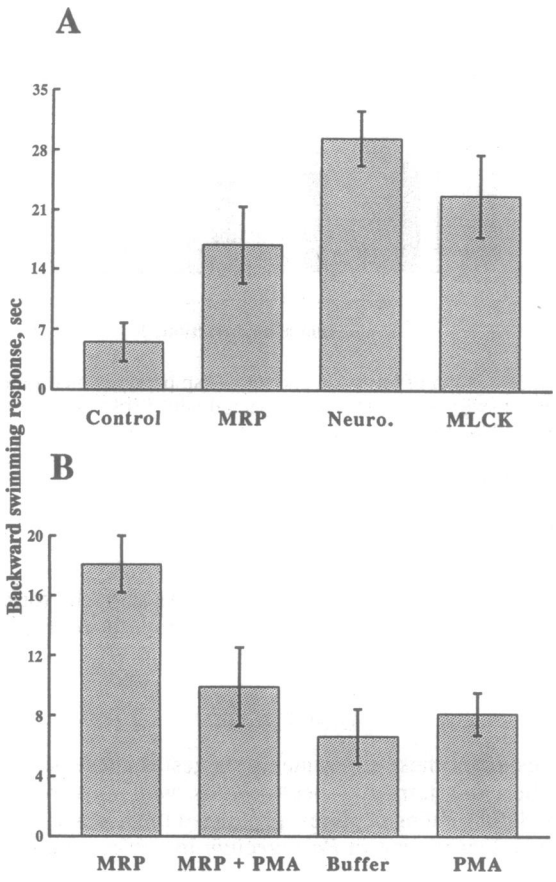

FIG. 5. Effect of other calmodulin-binding peptides. (*A*) Three other calmodulin-binding peptides, as described in the text, were injected into *Paramecia* and the behavioral responses were monitored. All peptides were at 320 μM in the injection pipette. Each bar represents the mean ± SD of results from 10 cells in each group. Neuro, neuromodulin peptide. (*B*) Buffer or the MRP peptide (320 μM) was injected into cells; some of these cells were then treated with PMA, as described in Fig. 2.

that this interaction occurs at the basal intracellular calcium concentrations in *Paramecium* (estimated to be $<10^{-7}$ M; refs. 10 and 18).

This conclusion is further supported by the experiments involving coinjection of exogenous vertebrate calmodulin. Calmodulin injection itself had little effect; however, in a dose-dependent fashion, it was able to reverse the 3-fold increase in backward swimming duration caused by the injected tetra-Ser peptide. These results suggest that the behavioral response to the tetra-Ser peptide was not a nonspecific effect on some other component of the signaling or swimming apparatus, but rather a specific interaction with endogenous calmodulin that could be overcome by excess exogenous calmodulin. We have not specifically determined the affinity of the MARCKS peptides for *Paramecium* calmodulin; however, the MARCKS peptides and proteins bind with high affinity to both plant and vertebrate calmodulins, making it likely that the interaction with *Paramecium* calmodulin is similar.

In vitro, phosphorylation of MARCKS protein and its tetra-Ser peptide by PKC rapidly disrupts complexes with calmodulin (2, 3). To evaluate whether this occurs in intact cells, the organisms were injected with the tetra-Ser or tetra-Ala MARCKS peptides and then exposed to the active tumor-promoting phorbol ester PMA. With the injected tetra-Ser peptide, only a 3-min exposure to PMA was sufficient to completely reverse the peptide-induced behavioral change. In contrast, PMA had no effect on the behavioral response of cells injected with the nonphosphorylatable tetra-Ala peptide, indicating that the complex remained intact despite PKC activation by PMA. Thus, in an intact cell, activation of PKC appeared to disrupt preexisting calmodulin–peptide complexes by rapidly phosphorylating the peptide. That these organisms contain a PKC activity that could phosphorylate the tetra-Ser peptide was demonstrated in cellular extracts; to our knowledge, PKC subtypes and their primary sequences have not yet been characterized in these organisms.

A cDNA was recently cloned from a mouse cerebellar library whose predicted protein sequence bears several striking homologies to MARCKS (19). This protein, designated MRP (1), F52 (6, 19), or MacMARCKS (7), is also a myristoyl protein that can serve as a high-affinity substrate for PKC *in vivo* and *in vitro* (6); both the equivalent phosphorylation site domain peptide (6) and the intact protein (Verghese *et al.*, unpublished data) bind calmodulin with an affinity that is essentially indistinguishable from that of MARCKS. This peptide was also effective at eliciting the behavioral response after injection into *Paramecium*, and this response was also reversed by activation of PKC with PMA. Thus, the phosphorylation site domain of MRP appears to be functionally equivalent to that of MARCKS in this cell system.

Other calmodulin-binding peptides, derived from skeletal muscle MLCK and from neuromodulin, were also effective at prolonging the backward swimming response. These data provide additional support for our contention that binding of the peptides to calmodulin is the most likely cause of the behavioral response. One possible explanation of the results with the MARCKS and MRP peptides might be that they are binding to actin and interfering in some way with ciliary movement, since both MARCKS and MRP are thought to be actin-binding proteins (7, 8). To our knowledge, neither MLCK nor neuromodulin is thought to bind actin via its calmodulin-binding domain (although, see ref. 20); since essentially the only property these peptides have in common is their ability to bind calmodulin, it seems likely that it is this property that is responsible to the behavioral response in these studies.

Thus these results provide support for our hypothesis that MARCKS and MRP could form complexes with calmodulin in vertebrate cells under conditions in which PKC is relatively inactive. We have calculated that, under conditions such as overnight serum deprivation in fibroblasts, the MARCKS protein is phosphorylated to a stoichiometry of <0.045 mol per site, thus permitting calmodulin binding (21). This proposal is further supported by the fact that resting intracellular Ca^{2+} concentrations in *Paramecium* are similar to those of vertebrate cells (9, 10). One possible result of such an interaction would be that PKC activation could displace calmodulin from proteins such as MARCKS or MRP, permitting the resulting "free" calmodulin to interact with the elevated intracellular calcium levels that almost always accompany physiological activation of PKC. A similar role has been proposed for neuromodulin (22–24). Another possibility is that phosphorylation of and calmodulin binding to the MARCKS and MRP proteins could regulate their interactions with actin or other target proteins (7).

Whatever the ultimate nature of these relationships, we believe that *Paramecium* provides a unique model system in which to study the consequences of calmodulin interacting with its binding proteins at the ambient calcium concentrations that prevail in intact cells. An interesting question is whether activation of PKC in normal *Paramecium* might have similar effects on the swimming response. It appears that the intracellular concentration of free calmodulin is not limiting in the repolarization process, since injection of high concentrations of exogenous calmodulin did not shorten the period of backward swimming. Similarly, PMA treatment of noninjected cells, which might have been expected to in-

crease free calmodulin if PKC-sensitive calmodulin-binding proteins exist in *Paramecium*, did not shorten the period of backward swimming, but lengthened it slightly. Both experiments argue against the existence of a PKC-sensitive calmodulin "reservoir" that regulates the rate of repolarization in these cells. However, it remains possible that other PKC responses in *Paramecium*, which are almost completely uncharacterized, could be mediated in part by PKC-dependent changes in local calmodulin concentrations.

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