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Hyperpolarization- and Depolarization-activated Ca^{2+} Currents in *Paramecium* Trigger Behavioral Changes and cGMP Formation Independently

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Abstract. Using 5% ethanol as a deciliating agent, 20 mM colchicine to prevent reciliation and 1 mM amiloride to affect ion fluxes in *Paramecium* we examined the compartmentation and function of Ca^{2+} fluxes employing the biosynthesis of cGMP and the stereotypic swimming behavior as indicators for Ca^{2+} entry. As a function of extracellular Ca^{2+} *Paramecia* responded to colchicine and amiloride with a short-lived ciliary augmentation (fast swimming) which indicated hyperpolarization, and formation of cGMP, i.e., the reported hyperpolarization-activated Ca^{2+} inward current in the somatic membrane is coupled to intracellular generation of cGMP. This is comparable to the coupling of the depolarization-activated, ciliary Ca^{2+} inward current and ciliary cGMP formation.

Ethanol-deciliated cells and ethanol-treated, yet ciliated control cells did not respond to a depolarization with backward swimming or formation of cGMP. Both responses recovered with similar kinetics. A persistent effect of an ethanol exposure on the axonemal apparatus or on guanylyl cyclase activity of ciliated control cells was excluded using permeabilized cells and cell-free enzyme, respectively. Further, in the presence of 20 mM colchicine ethanol-treated cells only recovered the depolarization-dependent avoiding reaction whereas the formation of cGMP remained depressed, i.e., the drug dissected both responses. Similarly, ethanol exposure of *Paramecia* did not affect the fast swimming response towards the hyperpolarizing agent amiloride whereas the cGMP formation was abrogated and recovered over a period of 7 hr, i.e., amiloride dissected the hyperpolarization-elicited behavioral response from the intracellular cGMP formation.

The data demonstrate that in *Paramecium* depolarization- and hyperpolarization-stimulated behavioral responses and cGMP formation are not coupled. The behavioral changes are triggered by smaller Ca^{2+} inward currents than the formation of intracellular cGMP.

Key words: Paramecium — cGMP — Ca^{2+} channel — Hyperpolarization

Introduction

Paramecium was the first unicell in which the regionalized localization of a distinct Ca^{2+} current has been observed [6, 18, 27]. This kind of membrane differentiation has meanwhile been shown to occur in several lower eukaryotes [1, 4, 21, 37]. In *Paramecium*, removal of the cilia leaves the denuded cell body intact and abolishes the depolarization-activated action potential. Regrowth of the cilia is associated with a recurrence of the Ca^{2+} inward current, i.e., the depolarization-activated Ca^{2+} channels of *Paramecium* are localized to the ciliary membrane [6, 18, 27]. Later it was shown that the depolarizing Ca^{2+} inward current is coupled to the generation of cGMP [34–36].

The depolarization-triggered Ca^{2+} influx across the ciliary membrane is not the only Ca^{2+} current entering *Paramecium* because (i) deciliation does not result in a total loss of Ca^{2+} influx [18, 27]; (ii) one or possibly two Ca^{2+} currents activated upon hyperpolarization have been electrophysiologically demonstrated to reside in the somatic membrane [23, 30, 31]; (iii) a Ca^{2+} resting conductance exists which is unrelated to the ciliary Ca^{2+} channel [17]; (iv) Ca^{2+}-dependent trichocyst discharge can be triggered in *pawn* mutant cells deficient in the ciliary Ca^{2+} channel [9, 14, 29]; (v) a considerable fraction of the Ca^{2+}-regulated guanylyl cyclase is present in the somatic membrane and has to be regulated by a somatic Ca^{2+} source [33], and (vi) closer inspection of the

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magnitude of the cGMP increase elicited by Ba^{2+} showed that it is inconceivable that a cGMP elevation of up to 500 pmol/mg can occur only in the tiny ciliary compartment [35, 36].

We used ethanol as a deciliating agent, colchicine to inhibit reciliation and amiloride to affect Ca^{2+} fluxes in *Paramecium* because these drugs were used formerly for these purposes [6, 11, 18, 27, 30] and examined the spatial distribution and function of Ca^{2+} fluxes using the formation of cGMP as biochemical and the swimming mode and velocity as behavioral indicators of Ca^{2+} entry. Taking the previous electrophysiological data into account which were obtained with these drugs, our data demonstrate that the Ca^{2+} requirements for the behavioral responses and for stimulation of intracellular cGMP formation differ substantially.

Materials and Methods

Reagents

[α-^{32}P]GTP (400 Ci/mmol), [^{3}H]cGMP (24.6 Ci/mmol) and carrier-free Na[^{125}]I were purchased from Amersham, UK. Phosphoenolpyruvate potassium salt, pyruvate kinase (rabbit muscle), GTP- and ATP-sodium salts were from Boehringer-Mannheim, succinyl-tyrosinyl-cGMP from Sigma (St. Louis, MO) and colchicine from Serva (Heidelberg, Germany).

Cell Culture and Deciliation

Paramecium tetraurelia strain 51s nd (nondischarge) and *pawn* A/B mutant cells (d4-500), deficient in the depolarization-activated, ciliary Ca^{2+} channel [15], were axenically grown at 25°C in Erlenmeyer flasks containing 300 ml of medium with phosphatidyl ethanolamine as the major lipid source [32]. Cells were harvested by low speed centrifugation (0.5 ml packed cells), washed twice in 100 ml of standard buffer (10 mM MOPS-Tris, pH 7.2, 1 mM KCl, 50 μM $CaCl_2$), suspended in 50 ml of the same buffer (at 120,000 cells/ml) and equilibrated at 70 rpm on a rotary shaker for 2 hr. Ethanol was added at room temperature to 5% (v/v) and cells were deciliated by vigorous shaking for 2 min. This left approximately 30% of cells broken. After dilution of ethanol to 1% by addition of buffer *Paramecia* were collected by low speed centrifugation and washed once. Pelleted cells were suspended in 120 ml of buffer and left for 10 min. After decanting the supernatant, deciliated cells were collected and suspended at 120,000 cells/ml. Stimulation was started after 30 min. Control cells were exposed to 5% ethanol for 2 min without agitation. Under those conditions, cells remained ciliated and viable. They were concentrated, washed and suspended in 120 ml of buffer as described above. After 10 min, the supernatant which contained actively swimming cells, was decanted, cells were pelleted, suspended at 120,000 cells/ml and used after 30 min as above. The viability of the cells and the deciliation procedure were monitored microscopically.

Preparation of *Paramecium* ''Ghosts''

Triton X-100 extracted *Paramecia* (ghosts) were prepared according to [22] and [25]. 0.5 ml packed cells were washed twice with 50 ml of 1 mM Tris-HCl, pH 7.2, containing 2 mM $CaCl_2$ at room temperature. All subsequent steps were carried out at 0°C. The suspended cells were added to 50 ml of 10 mM Tris-maleate, pH 7.0, 10 mM EDTA, 20 mM KCl and 0.01% (v/v) Triton X-100. After 15 min the ghosts were pelleted and washed successively in 10 mM Tris-maleate, pH 7.0, 2 mM EDTA, 50 mM KCl and in the same buffer without EDTA. Cell envelopes were finally suspended in the latter buffer. Only those preparations were used in which >95% of the cells were undamaged. Ghosts were reactivated at room temperature in a buffer containing 10 mM Tris-maleate, pH 7.0, 4 mM MgATP, 0.2 mM EDTA, and 50 mM KCl. Backward swimming was induced 3 min later by addition of 100 μM Ca^{2+}. Movement of the ghosts was videotaped and analyzed with a motion analysis system (*see below*).

cGMP Measurements

Experiments were started by addition of 150 μl of a stimulation solution to 250 μl of equilibrated cells (30,000 cells, 0.2 mg of protein) which resulted in the desired final concentration of the stimulant. Incubations were stopped by addition of 150 μl of perchloric acid (1 N final). cGMP levels were determined in triplicates by a radioimmunoassay using ^{125}I-2-O′-succinyl-cyclic GMP-L-tyrosinylmethylester as a tracer and polyclonal antibodies against cGMP [5]. Crossreactivity with cAMP was less than 3%. Protein precipitated by perchloric acid was dissolved in 0.5 N NaOH and determined by the method of Lowry using bovine serum albumin as a standard.

Assay of Guanylyl Cyclase

Guanylyl cyclase activity was determined for 10 min at 37°C [13]. The reaction contained in 50 μl: 10–40 μg of protein, 30 mM Tris-HCl, pH 7.5, 3 mM [^{3}H]cGMP (35 kBq) to monitor recovery, 3 mM $MgSO_4$, 30 μM $CaCl_2$, 1.4 mM phosphoenolpyruvate, one unit pyruvate kinase, and 0.4 mM GTP including 18.5–37 MBq [α-^{32}P]GTP.

Analysis of Swimming Behavior

The swimming velocity and direction of *Paramecia* were determined 5–10 sec after mixing with a test solution or after the indicated periods of time using the motion analysis system SM-CMA (version 4.4) from Strömberg-Mika medical equipment (Bad Feilnbach, Germany). About 50 cells were videotaped for each data point under a Leitz Labovert microscope with a CCD camera (WD-CD20), video monitor and SVHS cassette recorder (all from Panasonic). The system calculated real velocities from the video recordings by digitizing 1 or 2 sec of data at 32 frames/sec. Three types of behavior were discriminated: forward swimming, backward swimming, and turning (change of direction). Data are presented as the mean velocity or as percent of cells that display a particular swimming mode. Behavioral results were generally obtained from at least two separate experiments (>100 cells) and confirmed qualitatively many times.

Results

Effect of Ethanol Treatment on Behavior and cGMP Levels

Electrophysiological studies of *Paramecium* established the existence of hyperpolarization-activated, somatic

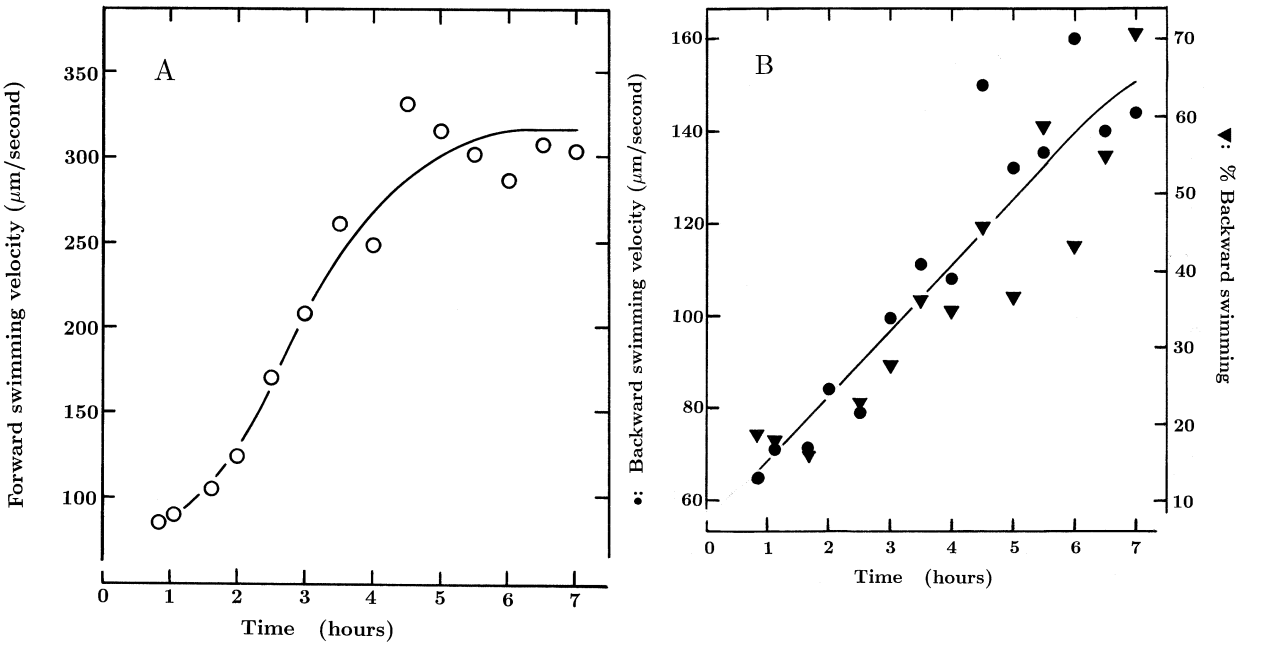

Fig. 1. Reciliation and restoration of swimming behavior of ethanol-deciliated *Paramecium.* (*A*) Increase in forward swimming speed as cilia regrow. (*B*) Recurrence of the backward swimming response upon addition of 0.6 mM Ba^{2+}. Note that the backward swimming speed (●) and the fraction of cells responding to Ba^{2+} (▼) increase concomitantly. The swimming speed and direction were determined by analysis of digitized video data. Points represent means of two independent experiments with at least 100 cells averaged for each data entry. As a control for the mechanical stress during mixing, cells were mixed with a buffer solution lacking Ba^{2+}. The percentage of backward swimming cells ranged from 0–7% (mean value 3.5%, i.e., below the right scale). This excluded the mechanical agitation as a stimulus.

Ca^{2+} channels [23, 30, 31]. Biochemical consequences of this current are as yet unknown. These should be identifiable in deciliated cells which are devoid of the depolarization-dependent Ca^{2+} channels [6, 18, 27]. For deciliation shaking with 5% (0.86 M) ethanol for 2 min is required [18, 26]. Within 7 hr cells reciliated and regained motility as determined by the increasing forward swimming velocity (Fig. 1*A*). At various points during the reciliation *Paramecia* were depolarized by Ba^{2+} and the percentage and velocity of backward swimming cells were measured. Both parameters increased in parallel (Fig. 1*B*). Since the avoiding reaction is unequivocally coupled to functional ciliary Ca^{2+} channels, these observations are in agreement with their reported ciliary localization [6, 18, 27].

As a control *Paramecia* were briefly exposed to ethanol without agitation. 10 sec after addition of 2% ethanol the forward swimming speed was increased to 123% in wild-type and *pawn* mutant cells indicating that ethanol hyperpolarized. Addition of 5% ethanol which is required for deciliation, partly caused an immediate 50% drop in forward swimming velocity (748 to 375 μm/sec) and partly immobilized the cells. We assume that this is due to Ca^{2+} leakage. Backward swimming was not observed upon ethanol addition. Cells isolated after a 2 min exposure to 5% ethanol (ethanol-treated cells) were swimming at 380 μm/sec indicating that the axonemal functions remained basically unimpaired. Surprisingly, these ethanol-treated, ciliated cells did not respond to a depolarization by Ba^{2+} with backward swimming. The avoiding response returned with a similar time course as in deciliated cells (*compare* Figs. 1*B* and 2).

One possibility might have been that ethanol affected the switch responsible for ciliary reversal. This was tested with Triton X-100 extracted *Paramecium* ghosts which were reactivated by addition of MgATP and swam forward at 40 μm/sec. Addition of 100 μM Ca^{2+} led to ciliary reversal and backward movement at 29 μm/sec. The speed of ghosts prepared from ethanol-treated cells was 37 and 28 μm/sec for forward and backward movement, respectively. Further, addition of 5% ethanol to the envelopes 2 min prior to reactivation neither impaired the forward nor backward motion. Therefore, we reason that the failure of ethanol-treated *Paramecia* to respond to a depolarization by Ba^{2+} was due to a lasting pharmacological effect of ethanol on the Ca^{2+} influx which determines ciliary switching and beating frequency [7, 8, 17, 22, 25].

A biochemical probe for a depolarizing Ca^{2+} influx into *Paramecium* is the formation of cGMP by a Ca^{2+}-dependent guanylyl cyclase [13, 34, 36]. Expectedly, deciliated cells did not generate cGMP when depolarized by Ba^{2+} (Fig. 3). cGMP formation recovered during the reciliation (Fig. 3). Cells which were isolated after a

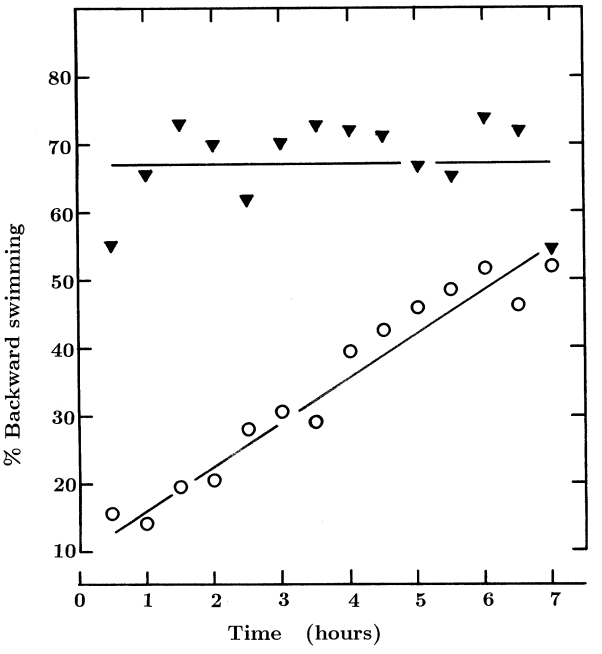

Fig. 2. Recovery of the Ba^{2+}-elicited (0.6 mM) backward swimming response of *Paramecia* which were ethanol-treated, yet remained ciliated. (▼) untreated control cells; (○) ethanol-treated cells (5%, 2 min). At least 200 cells were analyzed per point. The percentage of backward swimming control cells (mechanical agitation, no Ba^{2+}) was 0–3% (12 determinations covering all time points).

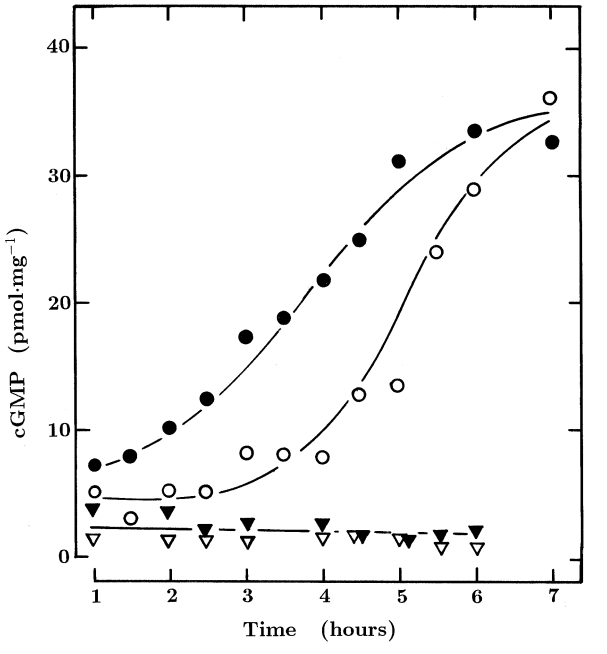

Fig. 3. Recovery of the Ba^{2+}-elicited (1 mM) cGMP formation of ethanol-treated *Paramecia.* (●) Cells treated for 2 min with 5% ethanol, no deciliation; (○) ethanol-deciliated cells; (▽) Ca^{2+}-channel double mutant *pawn A/B* cells, treated for 2 min with 5% ethanol, no deciliation; (▼) *pawn A/B* cells, deciliated (mean of two experiments). Control cGMP levels (identical mechanical handling, no Ba^{2+} addition) ranged from 1.1 to 3.1 pmol/mg. These points are omitted in the figure for clarity of presentation.

2-min exposure to 5% ethanol and which had retained their cilia, also failed to form cGMP upon depolarization (Fig. 3). The kinetics of the recovery from deciliation or merely ethanol exposure with respect to the Ba^{2+}-stimulated cGMP generation and backward swimming were rather similar (compare Figs. 1*B* and 3) and suggest a link by a common step. This was substantiated by use of Ca^{2+}-channel deficient *pawn* mutant cells which are unable to respond to depolarization by backward swimming or cGMP formation [15, 34, 35]. Deciliated *pawn* cells successfully reciliated as assessed by the increase in forward swimming speed, yet never showed a cGMP elevation (Fig. 3) nor an avoiding reaction upon Ba^{2+} stimulation (*data not shown*).

The possibility that ethanol damaged the membrane-bound guanylyl cyclase, was excluded. Although ethanol dose-dependently inhibited the enzyme in vitro this inhibition was immediately reversed upon ethanol removal. After suspension of cell membranes in ethanol-free buffer, enzyme activity was as in untreated preparations (69 *vs.* 76 pmol/min · mg^{-1}, $n = 4$). Moreover, the specific activities of guanylyl cyclase in cell membranes prepared from cells which had been exposed to 5% ethanol for 10 min (152 pmol cGMP/min · mg^{-1}) was similar to that in membranes prepared from untreated cells (122 pmol cGMP/min · mg^{-1}), i.e., ethanol treatment of *Paramecium* had no persistent effect on guanylyl cyclase activity ($n = 2$–4).

Next, we examined whether ethanol effected intracellular cGMP levels. 5% Ethanol led to a large and transient increase in cGMP (Fig. 4). The unusually high 30- to 40-fold rise was dependent on the presence of at least 50 μM extracellular Ca^{2+}, peaked within 15 sec and declined swiftly thereafter. The EC_{50} (concentration required for a half maximal effect) was 2.6% ethanol (0.44 M). Notably, the effect of ethanol was independent from the presence of functional ciliary Ca^{2+} channels because it occurred in the Ca^{2+}-channel deficient double mutant *pawn* A/B as well. The dose-response curve and the extent of cGMP formation in *pawn* mutant cells were identical to wild-type cells (*data not shown*). It should be noted that 5% ethanol certainly has effects on Ca^{2+} leakage, i.e., ethanol-stimulated cGMP formation cannot be ascribed to activation of a particular Ca^{2+} conductance or a specific membrane compartment. However, this short-lived cGMP increase may be connected to the more lasting actions of ethanol potentially mediated by cGMP-dependent protein kinases [19, 39] (*see below and discussion*).

Effect of Colchicine on Behavior and cGMP Levels

The lasting actions of ethanol on the excitability of *Paramecium* precluded biochemical studies of the somatic

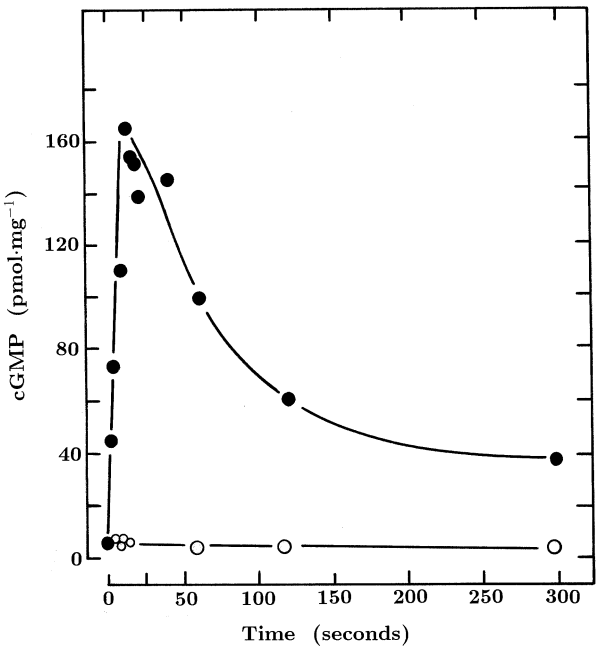

Fig. 4. Time course of the stimulation of cGMP formation in *Paramecium* by ethanol. ●: 5% Ethanol in the mixing solution; ○: control, no ethanol in mixing solution (mean of two experiments).

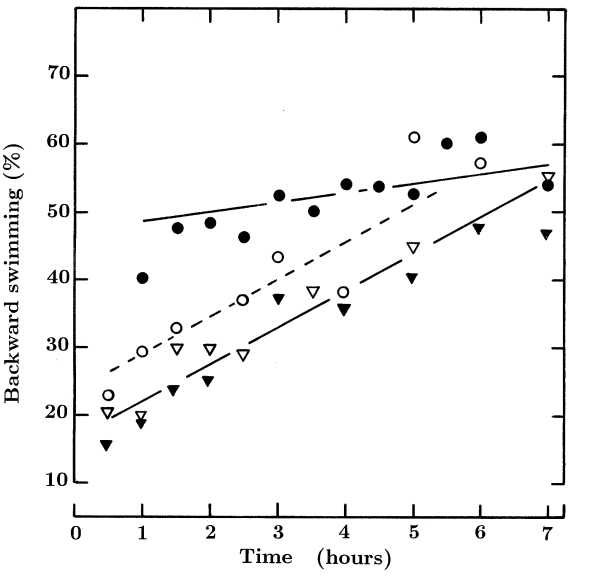

Fig. 5. Effect of colchicine on the Ba^{2+}-stimulated backward swimming of *Paramecia.* (●) control cells; (○, dashed line) cells in the presence of 20 mM colchicine; (▽) cells exposed to 5% ethanol for 2 min at the beginning of the experiment; (▼) ethanol-treated cells in the presence of 20 mM colchicine. Control and ethanol-treated cells were handled identically, colchicine was added after removal of ethanol. The final concentration of Ba^{2+} after mixing was 0.6 mM. Mechanical agitation (mixing without Ba^{2+}) resulted in backward swimming of 0–4% of all cells ($n = 17$, not shown because below of ordinate scale).

Ca^{2+} conductance after deciliation. Previously, 20 mM of the antimicrotubular agent colchicine was used to inhibit reciliation [6, 18]. Such a treatment should, therefore, result in denuded cells suitable to examine the biochemical consequences of a somatic Ca^{2+} current. Addition of colchicine to *Paramecia* dose-dependently increased the forward swimming speed, e.g., from 585 to 1219 μm/sec at 20 mM, which indicated a membrane hyperpolarization. After about 2 min the velocity returned to normal. Unexpectedly, 30 min after colchicine addition the behavioral reaction to Ba^{2+} was diminished by 60%, suggesting an action of the alkaloid on either the ciliary Ca^{2+} inward current or the switch for backward swimming. The latter possibility was excluded because *Paramecia* adapted to the presence of 20 mM colchicine and full behavioral excitability was regained 7 hr later (Fig. 5). In addition, when cells were exposed for 2 min to 5% ethanol which itself abolished the Ba^{2+} response (Fig. 2), the presence of 20 mM colchicine did not interfere with the recovery of the behavioral Ba^{2+} response (Fig. 5).

Colchicine addition to *Paramecium* dose-dependently led to a short-lived 8-fold elevation of intracellular cGMP (Fig. 6*A*). This effect was dependent on the presence of extracellular Ca^{2+}. 20 mM colchicine did not affect guanylyl cyclase activity in vitro and enzyme activity was unaltered in membranes isolated from colchicine incubated cells (*data not shown*). This excluded a direct effect of the drug on guanylyl cyclase activity. Because 20 mM colchicine strongly hyperpolarized (fast swimming) the concomitant cGMP increase is, therefore, thought to be linked to Ca^{2+} entry through the hyperpolarization-activated, somatic Ca^{2+} channels.

The continued presence of colchicine subsequently reduced the effect of a depolarization by Ba^{2+} on cGMP formation by about 60% (Fig. 6*B*). A recovery was observed only after removal of colchicine (Fig. 6*B*), i.e., colchicine affected the responses of *Paramecia* toward depolarization differentially: backward swimming was only transiently inhibited (Fig. 5) whereas cGMP generation was permanently attenuated (Fig. 6*B*). The dissection of both responses by colchicine may indicate that their quantitative Ca^{2+} requirements differ (*see Discussion*).

Dissection of the Behavioral and cGMP Responses by Amiloride

The diuretic amiloride was reported to interfere with the Ca^{2+} homeostasis in *Paramecium* and in many other cells [2, 10, 12, 30]. We investigated the short-term effects of amiloride on behavior and intracellular cGMP of *Paramecium.* Addition of amiloride dose-dependently increased the forward swimming speed (819 to 1537 μm/sec at 0.5 mM). This effect lasted about 5 min. Identical results were obtained with *pawn A/B* mutant cells defi-

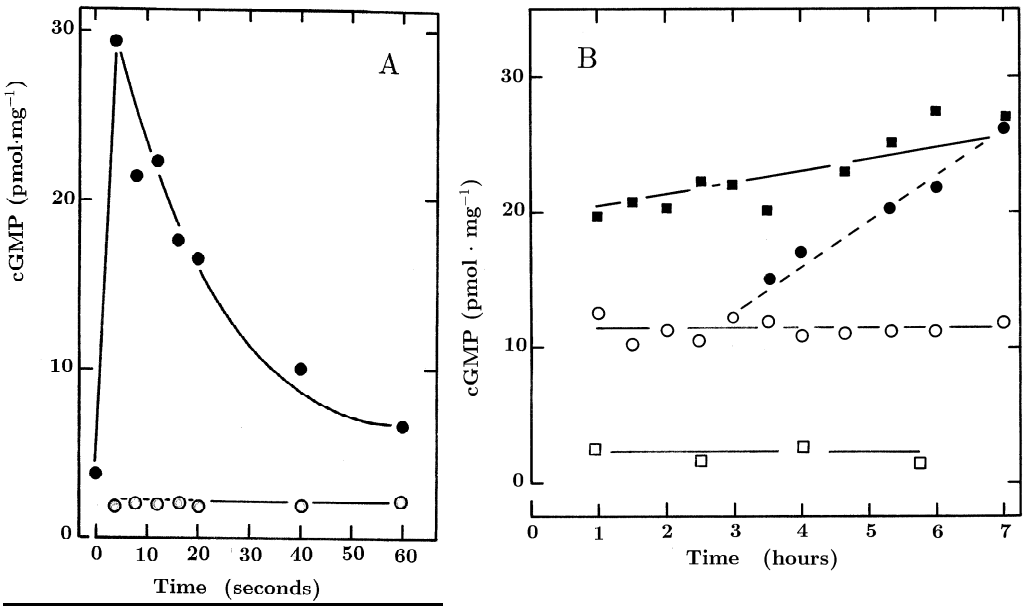

Fig. 6. Stimulation of cGMP formation in *Paramecium* by colchicine and the effect of colchicine on cGMP formation stimulated by Ba^{2+}. (*A*) ●: Time course of cGMP generation stimulated by 20 mM colchicine; ○: identical mechanical handling, no colchicine addition. (*B*) effect of colchicine on cGMP formation stimulated by 1 mM Ba^{2+}; (□): control cells in colchicine, no Ba^{2+} addition; (○): cells in 20 mM colchicine stimulated by Ba^{2+}; (●, dashed line): removal of colchicine from the cells after 3.5 hr, stimulation by Ba^{2+}; (■): control cells, no colchicine, only Ba^{2+} addition. Each point represents the mean of four experiments.

cient in the depolarization-activated, ciliary Ca^{2+} channel (804 to 1444 μm/sec at 0.3 mM amiloride). Because speeding is unequivocally associated with membrane hyperpolarization [17], we conclude that the drug hyperpolarized *Paramecium* as noted before with other cells [2]. The hyperpolarizing effect of amiloride could be titrated by concomitant addition of depolarizing Ba^{2+}-ions indicating that both agents acted on different targets (*data not shown*). Further, amiloride led to an increase in cGMP which was dependent on the presence of external Ca^{2+} (Fig. 7*A*). At 50 μM Ca^{2+} the EC_{50} was 1 mM and the effect was maximal at 3 mM amiloride (*data not shown*). Because intracellular guanylyl cyclase activity is stringently controlled by Ca^{2+}, this must mean that the crucial effect of amiloride for cGMP formation was the stimulation of a Ca^{2+} influx through a Ca^{2+} gate distinct from the depolarization-activated, ciliary Ca^{2+} channel because (i) activation of the latter invariably results in backward swimming and amiloride evoked a very large speeding response; (ii) any hyperpolarization as induced here by amiloride concomitantly inhibits existing depolarization-sensitive Ca^{2+} conductances; (iii) the drug stimulated cGMP formation in *pawn A/B* mutant cells deficient in the depolarization-activated, ciliary Ca^{2+} channel is indistinguishable from wild-type cells (*data not shown*); (iv) without the presence of extracellular Ca^{2+} amiloride could not enhance intracellular cGMP generation; (v) amiloride did not affect guanylyl cyclase activity in vitro (*data not shown*).

Next, we investigated how a brief exposure of *Paramecia* to 5% ethanol affected this site of Ca^{2+} entry. After ethanol treatment of cells amiloride did not elicit the usual cGMP accumulation (Fig. 7*B*). Full responsiveness recovered at a similar rate as the Ba^{2+} response of ethanol-treated *Paramecia* (*compare* Figs. 3 and 7*B*). This indicates that ethanol has lasting effects on the hyperpolarization-sensitive, somatic (Fig. 7*B*) as well as on the depolarization-activated, ciliary Ca^{2+} conductance (Fig. 3). In contrast, the behavioral effect of amiloride after an ethanol exposure was unaffected. The increase in swimming speed was always around 155% as determined at 11 time points up to 7 hr after the ethanol treatment. Obviously, the amount of Ca^{2+} which entered the ethanol-treated cells upon amiloride addition was always sufficient to cause ciliary augmentation, yet, it was initially insufficient for guanylyl cyclase activation.

Discussion

So far, there are two major effects of a Ca^{2+} influx into *Paramecium*, changes in behavior and increases in cGMP. One important issue is then whether both effects are functionally coupled or not. To modulate the Ca^{2+} balance in this ciliate ethanol, colchicine and amiloride have been used in electrophysiological studies because ethanol removed the depolarization-activated Ca^{2+} channels via deciliation [18, 26], 20 mM colchicine blocked

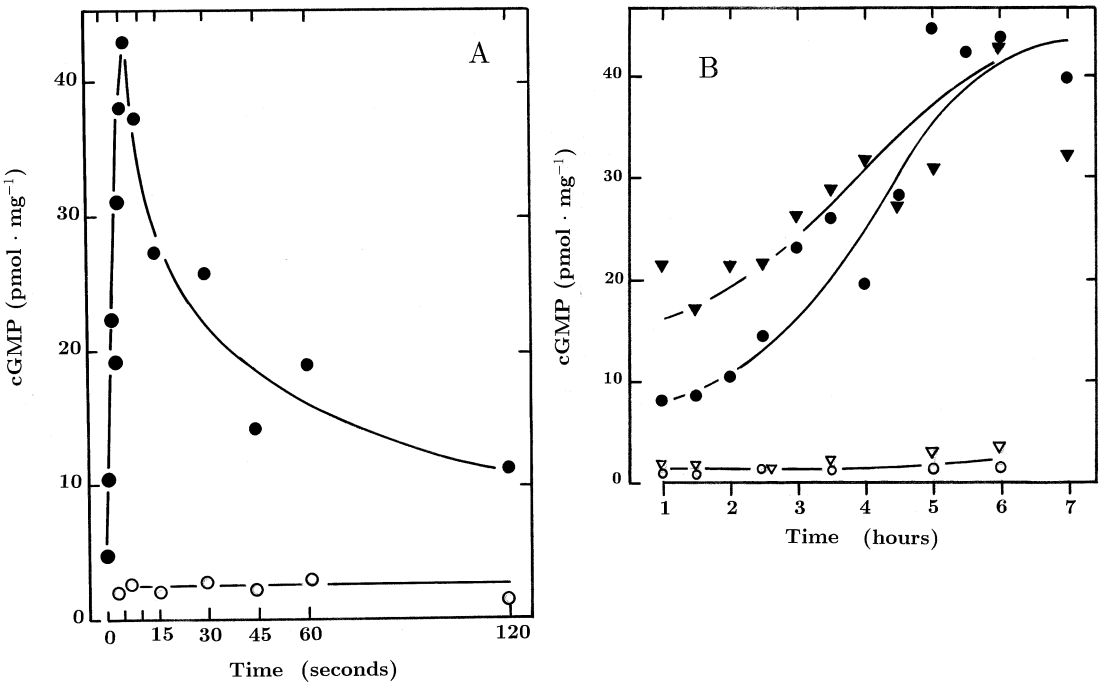

Fig. 7. Stimulation of cGMP formation in *Paramecium* by amiloride. (*A*), ●: Time course of cGMP generation stimulated by 2 mM amiloride; ○: identical mechanical handling, no amiloride addition. (*B*): Recovery of the responsiveness of the cGMP generating system toward 2 mM amiloride in ethanol-treated cells and ethanol-deciliated *Paramecia* (*see also* Fig. 3); (▼) ethanol-treated cells (5%, 2 min); (●) deciliated cells. The open symbols represent respective controls (identical mechanical agitation, no amiloride addition). Each point is the mean of two experiments.

reciliation [6, 18] and 1 mM amiloride reversibly interacted with a hyperpolarization-activated Ca^{2+} inward current [9, 30]. The biochemical, pharmacological, and behavioral effects of these agents on *Paramecium* were investigated in this study.

Paramecia are remarkably insensitive to 20 mM of the antimicrotubular drug colchicine which was employed here because this drug was often used in this system and even a long-term exposure was well tolerated [6, 11, 18, 20, 27, 28]. In contrast, 2 mM amiloride was soon noxious. After 60 min, 10% of the cells had disintegrated, 60% were sluggish, the rest was motionless. Therefore, we exposed cells to amiloride only briefly. Exposure to 5% ethanol was routinely limited to 2 min because of detrimental effects upon longer exposure [18, 26].

The common behavioral response to colchicine and amiloride was a strong, yet transient ciliary augmentation. Because the membrane potential is unequivocally coupled to the ciliary beat frequency and direction we know that the cells were hyperpolarized [7, 8, 17]. Hyperpolarization of other cells by amiloride has already been reported [2]. Further, amiloride affects different ion channels such as the L-type Ca^{2+} channel [12, 38]. Colchicine has no known effects on ion gates. However, actions on the cytoskeletal network underlying the plasma membrane certainly will influence membrane proteins such as ion channels or ATPases. The common biochemical effect to colchicine and amiloride was a rapid and short-lived increase in cGMP. A feature of this intracellular generation of cGMP was the absolute requirement for extracellular Ca^{2+}. Although it was technically unfeasible to prove that Ca^{2+} physically entered *Paramecium*, our data cannot be intelligently discussed without this conclusion. In particular, this Ca^{2+} influx cannot have occurred through the depolarization-activated channels because both, fast-swimming and cGMP formation were stimulated in Ca^{2+} channel deficient *pawn* mutant cells as well. Therefore, a Ca^{2+} influx through the somatic, hyperpolarization-activated channels [23, 31] must be causally related to the intracellular activation of the guanylyl cyclase. Although we cannot exclude the possibility that this Ca^{2+} influx may trigger a release of Ca^{2+} from internal stores which would secondarily affect guanylyl cyclase activation, so far there is no firm experimental evidence of a Ca^{2+}-induced Ca^{2+} release mechanism in *Paramecium.*

After deciliation the recovery of the depolarization-activated Ca^{2+} current coincides with reciliation [6, 18, 27]. We determined the recovery of the depolarization-triggered avoiding reaction and of cGMP generation and thereby extended the electrophysiological findings. Yet, under our experimental conditions already a brief exposure of *Paramecia* to 5% ethanol without deciliation at-

tenuated the response to Ba^{2+} for several hr (Fig. 2). For technical reasons we used only 50 μM external Ca^{2+} *vs.* 1 mM in the electrophysiological studies [6, 18, 27]. Notably, below 1 mM Ca^{2+} plateau spikes cannot be elicited in *Paramecia* due to the low rate of Ca^{2+} entry [3]. Furthermore, ethanol treatment raises the input resistance by 35% and reduces the Ca^{2+} spikes by about 20% for many hours [18]. Therefore, the unresponsiveness to Ba^{2+} after ethanol exposure in our experiments (Fig. 2 and 3) was certainly due to a decrease in the Ca^{2+} driving force. On the other hand, the reduction in external Ca^{2+} to subsaturating concentrations enabled us to ask whether the Ca^{2+} requirements for both responses are identical.

The addition of colchicine to ethanol-treated cells dissected both responses: the Ba^{2+}-induced ciliary reversal recovered (Fig. 5) whereas the cGMP response remained depressed (Fig. 6*B*). This suggests that the activation of the guanylyl cyclase requires a larger increase in internal Ca^{2+} than does ciliary reversal. Indeed, ciliary reversal was reported to occur at 1 μM Ca^{2+} [17, 22] whereas guanylyl cyclase activation only starts around 10 μM [13]. Our data provide the first in vivo evidence for these in vitro observations. Ethanol exposure also dissected the responses to hyperpolarization because ethanol-treated, yet ciliated cells did not respond normally to amiloride by cGMP formation (Fig. 7*B*) whereas the ciliary augmentation was completely unaffected. This suggests that also ciliary augmentation requires less Ca^{2+} than guanylyl cyclase activation. Again, this is in accordance with earlier in vitro studies [13, 17, 22].

In conclusion, irrespective of potentially additional pleiotropic effects of the compounds used in this study and their detailed mechanism of action our results show that (i) a brief 5% ethanol treatment of *Paramecium* results in a lasting, yet similar impairment of both, the depolarization- and the hyperpolarization-activated Ca^{2+} channels; (ii) the Ca^{2+} inward current that is activated upon hyperpolarization and localized to the somatic membrane [23, 30, 31] stimulates intracellular cGMP formation as does the depolarization-activated, ciliary Ca^{2+} current [34], (iii) those Ca^{2+} ions which enter upon hyperpolarization do not activate the switch responsible for ciliary reversal; the latter is only triggered by Ca^{2+} entering through the ciliary Ca^{2+} channels; (iv) the Ca^{2+} requirement for the behavioral responses and for cGMP stimulation differ, i.e., behavioral changes and measureable changes in cGMP formation are not coupled. This was already suggested earlier based on electrophysiological studies [24].

We are left with the question of what are the potential functions of cGMP in *Paramecium*? Obviously, stimulation of cGMP biosynthesis is a consequence of a Ca^{2+} influx in excess of what is required for the behavioral responses. Possibilities to consider are that cGMP effects electrical or mechanical properties of the plasma membrane via regulation of cyclic nucleotide-gated ion channels or structural proteins beneath the cell membrane by phosphorylation involving cGMP-dependent protein kinases [16, 19, 39] and thus stabilizes the cell under changing environmental conditions.

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