Cyclic AMP formation in *Tetrahymena pyriformis* is controlled by a K$^+$-conductance

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Abstract   Responses of the cAMP generating system of *Tetrahymena* to changes in the concentrations of external [K$^+$] or [Ca^{2+}] ions were examined. When *Tetrahymena* are equilibrated in high [K$^+$] buffers, intracellular levels of cAMP decreased to 40% within 2 h. Hyperpolarization of the cells by dilution of external [K$^+$] to one-eighth of its original concentration instantly stimulated intracellular cAMP formation. Manipulations of the K$^+$ resting conductance of *Tetrahymena* by equilibration in buffers of different K$^+$ content greatly affected the responsivity of the adenylyl cyclase. Hyperpolarization of the cell by addition of Ca^{2+} also resulted in a rapid generation of cAMP. Blockade of K$^+$ conductances by the K$^+$ channel blockers tetraethylammonium, quinine, and Cs$^+$, dose-dependently inhibited hyperpolarization-stimulated cAMP formation. The data indicate that a hyperpolarization-activated K$^+$ current is directly coupled to adenylyl cyclase regulation.

Key words: *Tetrahymena*; K$^+$ channel; cAMP; Hyperpolarization

1. Introduction

Tetrahymena is a popular model for the study of many basic biological processes. Surprisingly, the regulation of cyclic nucleotide metabolism in *Tetrahymena* has received only cursory attention. Most studies dealt with fluctuations of cyclic nucleotide concentrations during the cell cycle, i.e. on a time scale of hours [1–4]. These studies yielded conflicting data. Further, addition of glucose was reported to stimulate cAMP formation [6] and a β-adrenergic mechanism was discussed. Initial reports on the regulation of the adenylyl cyclase from *Tetrahymena* claimed an activation by the neurotransmitters adrenaline and serotonin, and by NaF, which implicated what are now known as G proteins in adenylyl cyclase regulation [4,7]. Later studies did not confirm these findings [8] and hitherto the presence of proteins in *Tetrahymena* homologous to the 40 kDa G proteins is unknown.

Because of its small size, investigations of the electrophysiology and behavior of *Tetrahymena* are limited, and behavioral and ion channel mutants, which are abundant in the larger ciliated protozoan *Paramecium* (80–200 vs. 20 μm in length, respectively), are scarce. In general the basic bioelectrical properties of *Tetrahymena* are identical to those found in *Paramecium* [9–12]. We investigated the regulation of cAMP formation in *Tetrahymena* in vivo as stimulated by sudden hyperpolarizing changes in the concentration of external K$^+$ and Ca^{2+} ions. The data define a system in which a cellular K$^+$ conductance, possibly the resting conductance, is the major controlling factor of cAMP production.

2. Experimental

2.1. Cell culture and equilibration

Tetrahymena pyriformis HSM, provided by Dr. G. Cleffmann, University of Gießen, Germany, were axenically grown with shaking (70 rpm) at 25°C in 1 l Erlenmeyer flasks containing 300 ml medium with 1% proteose peptone (Difco), 0.1% yeast extract (Difco), 0.2% glucose and 5 μg/ml Fe^{2+}. Early stationary cells (250,000 cells/ml) were harvested at room temperature by centrifugation (500 × g, 1 min) and

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transferred into 200 ml of equilibration buffer (10 mM MOPS-Tris, pH 7.2, containing between 1 and 30 mM KCl and 50 or 500 μM CaCl$_2$). Cells were equilibrated for 3–4 h on a rotary shaker (50 rpm) and washed once with equilibration buffer. The cell density in the experiments was about 200,000 cells/ml. Details of the respective stimulation protocols are given in the figure legends. Viability of the cells under the different ionic and osmotic conditions was routinely monitored by microscopic inspection: full viability was consistently maintained throughout the experiments. Behavioral observations were made with a Leitz Diavert microscope and were videotaped. Evaluation was attempted with a motion analysis system. Due to the small size of *Tetrahymena* the system could not reliably discriminate between backward and forward swimming and the reported results are from visual inspection of the videotapes.

2.2. cAMP Measurements

Stimulations were initiated by addition of 350 μl of buffer to 50 μl of equilibrated cells, which resulted in the desired final ion concentrations at room temperature. They were stopped by the addition of 150 μl of perchloric acid (1 M final). cAMP levels were determined in triplicate by a radioimmunoassay using ^{125}I-labeled 2-O'-succinylcyclic AMP-L-tyrosinylmethylester as a tracer and polyclonal antibodies against cAMP [13]. Cross-reactivity with cGMP was less than 5%. cAMP assayed was fully degradable by digestion with phosphodiesterase. Protein precipitated by perchloric acid and pelleted by centrifugation was dissolved in 0.5 M NaOH and determined by the method of Lowry using bovine serum albumin as a standard.

3. Results

3.1. Behavioral observations

Increasing the external K$^+$ concentration ([K$^+$]) during the equilibration of *Tetrahymena* resulted in a reduction in forward swimming speed estimated to be around 25%. When cells were adapted for 4 h in 16 mM [K$^+$] (and 0.5 mM Ca^{2+}) and the K$^+$ concentration was suddenly reduced to one-eighth by addition of K$^+$-free buffer, the forward swimming speed immediately increased approximately 2-fold. A similar reaction was observed when external Ca^{2+} was raised from 50 μM to 1 mM in the presence of [K$^+$] between 1 and 20 mM. These ionic stimuli correspond to two modes of hyperpolarization which elicit identical behavioral responses in *Paramecium* and *Tetrahymena* [14,15]. The forward speeding response, which is brought about by a frequency increase of the ciliary power stroke, is a reliable
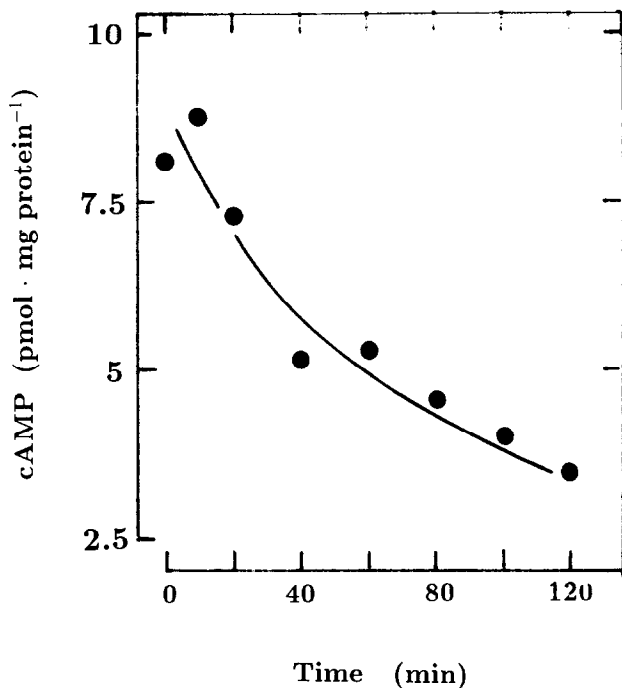
Fig. 1. Adaptive changes in cAMP concentrations in *Tetrahymena*. Freshly harvested cells in 1 mM [K⁺] were transferred at 0 min to a buffer containing 8 mM K⁺. cAMP levels were determined in aliquots at the time points indicated on the abscissa. The mean of two experiments is shown.

indicator of membrane hyperpolarization. Depolarization, i.e. a sudden increase in [K⁺] from 1 to 16 mM, resulted in repeated backward jerks, backward swimming and circling behavior of the cells.

3.2. Stimulation of cAMP formation by [K⁺] dilution

Tetrahymena in 1 mM [K⁺] were transferred into an equilibration buffer containing 8 mM [K⁺]. Over a period of 2 h cAMP levels dropped to 40% (Fig. 1). This probably reflected changes in membrane resistance which occurred under these conditions [16]. The extent of the decrease in cAMP resting levels was dependent on [K⁺] in the equilibration buffer (see controls in Fig. 3A). A sudden hyperpolarisation of cells adapted to high [K⁺] by dilution of [K⁺] to one-eighth of its original concentration resulted in an elevation of intracellular cAMP levels. The start of the rise was already measureable at 2 s after dilution (Fig. 2). At a dilution of [K⁺] from 16 to 2 mM, the rise was maximal within 10 s and declined thereafter to a new, albeit higher, resting level of cAMP which reflected the diminished external [K⁺]. The size of the cAMP response was correlated to the extracellular K⁺ concentration during equilibration (Fig. 3A). The character of the virtual dose-response relationship was particularly striking when the differences between the resting and the dilution-stimulated cAMP concentrations were plotted (Fig. 3B). For example, in cells adapted to 20 mM [K⁺], cAMP levels at rest were reduced by 41%, while dilution of [K⁺] to 2.5 mM resulted in a 2.6-fold increase in cellular cAMP within 5 s (Fig. 3B). The concentration of [K⁺] which was required during the equilibration to give a half-maximal cAMP response upon dilution of [K⁺] to one-eighth was 4 mM. No stimulatory effect on the cAMP-generating

system was apparent when *Tetrahymena* was adapted to low [K⁺] and resting cAMP levels already were quite high. Unlike in *Paramecium*, no data are available which describe the electrophysiological changes in *Tetrahymena* under these experimental conditions. Probably, a high [K⁺] concentration corresponds to a high K⁺ resting conductance, whereas at low [K⁺] cells must drastically curb their resting conductance, i.e. increase the resting resistance of the membrane, to avoid intolerable loss of [K⁺]. It is conceivable that in *Tetrahymena* the regulation of the K⁺ resting conductance and of cAMP formation are coupled, a situation comparable to *Paramecium* [17].

3.3. Stimulation of cAMP formation by a Ca²⁺ increase

Hyperpolarization of *Tetrahymena* can also be accomplished by an increase in [Ca²⁺] as observed by the fast swimming response. This anomaly is due to the fact that Ca²⁺ ions neutralize membrane surface charges [14]. Expectedly, this effect is abolished upon addition of high Ca²⁺ concentrations when the cations depolarize the membrane potential. Cells were equilibrated in buffers containing 50 μM Ca²⁺ and [K⁺] between 1 and 30 mM. [Ca²⁺] was then increased 20-fold to 1 mM (Fig. 4A). The effect of this change in [Ca²⁺] concentration on cAMP levels was consistent, but not striking. However, when the basal concentrations of cAMP were subtracted from corresponding stimulated levels, the effect of Ca²⁺ became obvious. The maximal Ca²⁺ response was obtained in the presence of 5 mM [K⁺]. Under these conditions we also observed the most prominent behavioral changes, i.e. forward speeding.

3.4. Blockade of cAMP formation by K⁺ channel blockers

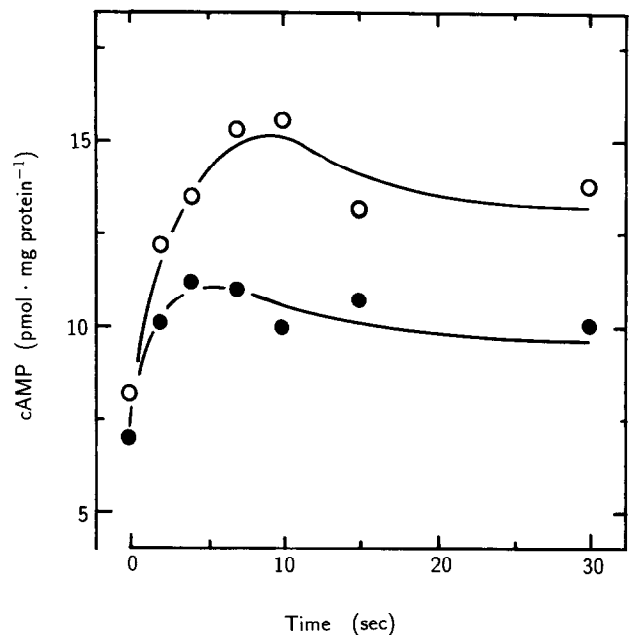Because we suspect that the K⁺ resting conductance determines the responsivity of the cAMP-generating system in

Fig. 2. Time-course of cAMP formation in *Tetrahymena* cells stimulated by hyperpolarization. (○) *Tetrahymena* were adapted to 16 mM [K⁺]/0.5 mM [Ca²⁺] (4 h). At time 0, cells were hyperpolarized by dilution of [K⁺] with K⁺-free buffer to 2 mM. (●) Tetraethylammonium was added with the dilution buffer to 30 mM final concentration. Values are means from 2–3 separate experiments.
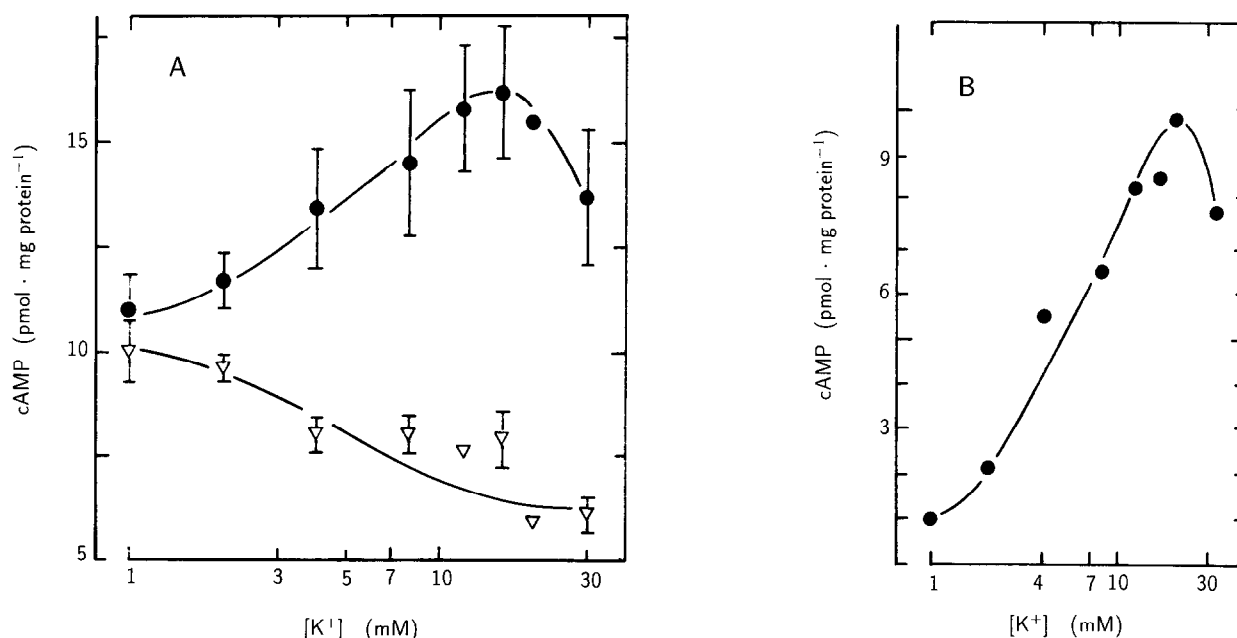
Fig. 3. Formation of cAMP in *Tetrahymena* stimulated by hyperpolarization. (A) Cells were equilibrated for 4 h at the [K$^+$] concentrations indicated at the abscissa (+0.5 mM Ca^{2+}) and cAMP levels were determined at the end of the equilibration period ($\triangledown$). Stimulation by dilution of the respective K$^+$ concentration to one-eighth of its original concentration was for 5 s ($\bullet$). Values are means ± S.E.M. of 3–4 separate experiments. (B) The differences in control and stimulated cAMP levels in A are re-plotted.

Tetrahymena, we used tetraethylammonium (TEA), Cs and quinine, which are known K$^+$ channel blockers in many cells. Neither of these compounds affected cell viability nor cAMP levels at rest. All three compounds inhibited cAMP production upon dilution of [K$^+$] in a dose-dependent fashion (Fig. 5). The inhibition was observed at all time points (see Fig. 2 for TEA). In contrast to Cs or quinine, TEA, even at 30 mM, was unable to inhibit the K$^+$ dilution response beyond 75%. This was irre-

spective of whether or not *Tetrahymena* were preincubated with TEA for 10 min. To observe a maximal effect for Cs, a preincubation of approximately 10 min was required. The IC$_{50}$ value (dose that inhibited by 50%) was 8 mM for TEA, which is rather high compared to the efficacy of TEA in inhibiting other K$^+$-channels. IC$_{50}$ values for Cs and quinine were 4 mM and 75 μM, respectively, and were in the range observed for other K$^+$ channels.
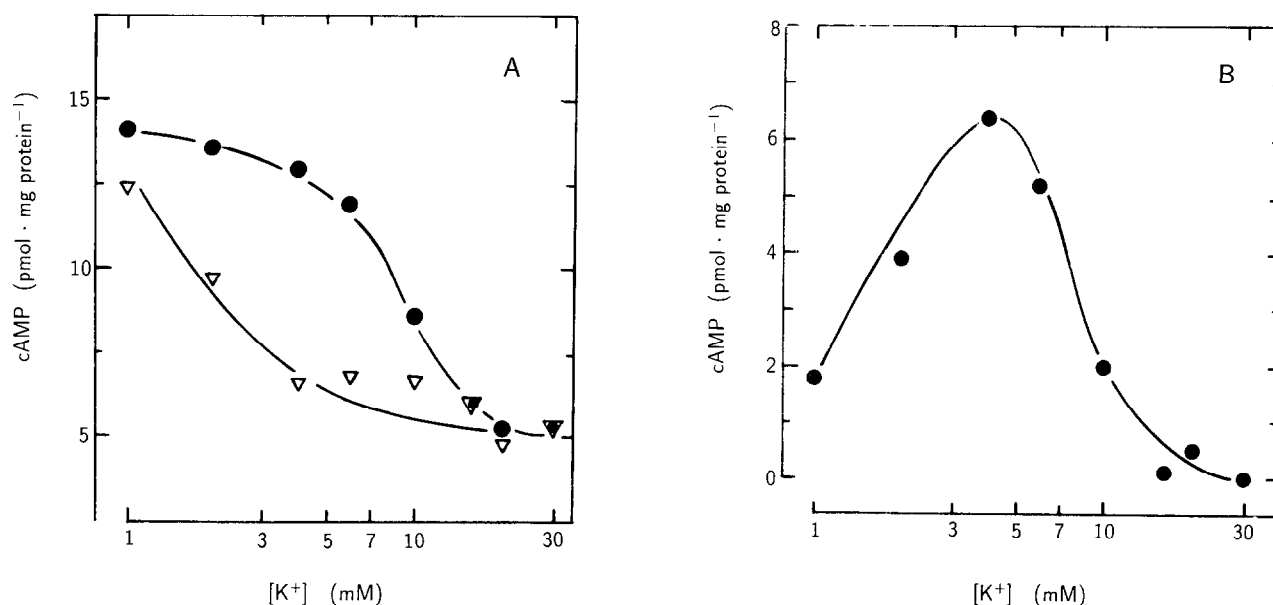

Fig. 4. Formation of cAMP in *Tetrahymena* stimulated by an increase of Ca^{2+}. (A) Cells were equilibrated for 4 h in a buffer containing 50 μM Ca^{2+} and [K$^+$] concentrations indicated at the abscissa, and cAMP levels were determined at the end of the equilibration period ($\triangledown$). cAMP formation was stimulated by an increase in external Ca^{2+} to 1 mM for 5 s ($\bullet$). Values are means of 2 separate experiments. (B) The differences in control and stimulated cAMP levels in A are re-plotted.
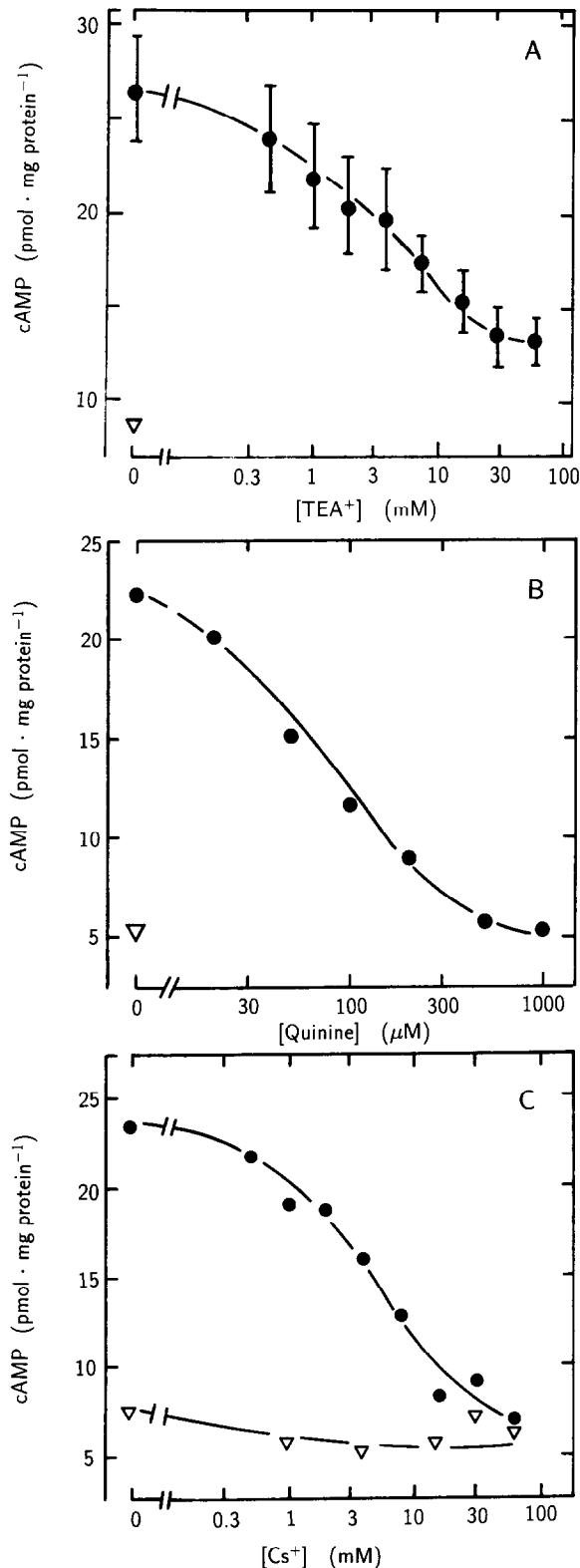
Fig. 5. Inhibition of cAMP formation by K+ channel blockers TEA, Cs and quinine. Cells were adapted for 4 h in buffer containing 16 mM K+ and 0.5 mM Ca²⁺. For stimulation, external K+ was diluted to one-eighth of its original concentration. TEA+ (A) and quinine (B) at the indicated concentrations were added with the dilution stimuli. Cs+ (C) at the indicated concentrations was added 15 min prior to stimulation. In A, mean values ± S.E.M. of 4 experiments are shown, in B and C representative experiments are depicted. (∇) Amount of cAMP in un-stimulated cells. In all instances, stimulations were for 5 s.

4. Discussion

Hitherto, the metabolism of cAMP in *Tetrahymena* was investigated on a time scale of minutes-to-hours. In synchronized cultures cAMP levels were reported to either increase or decrease at cell division [1,4]. The reasons for the discrepancies are unknown. Nandini-Kishore and Thompson reported that 1% glucose stimulates cAMP formation over 90 min via a Ca^{2+}-and adrenaline-mediated process [6]. In our experiments, all growth-related processes and nutritional responses (glucose) were excluded.

For the purpose of the discussion we extrapolate the electro-physiological properties of the ciliate *Paramecium* which are known in detail (for review see [9,10]), to *Tetrahymena*. This is permissible considering that all known electrophysiological properties of *Tetrahymena* [11,12] are similar to those of *Paramecium*. Regulation of cAMP in *Tetrahymena* displayed two components, a slow, adaptive response at a time scale of hours, and an instantaneous one within seconds. Both were triggered solely by changes in the external ion concentrations. Adaptation of *Tetrahymena* to high [K+] lead to a striking decline of cAMP levels, much more so than in *Paramecium* [18]. Because of the smaller size of *Tetrahymena* (≈ 20 pl/cell) compared to *Paramecium* (≈ 100 pl/cell) it probably has even less capacity to intracellularly buffer changes in the external ion composition. As a freshwater ciliate living in changeable environments *Tetrahymena* must actively regulate its resting conductances, mainly carried by K+, to maintain its membrane potential and intracellular ion composition within narrow limits and compensate the initial shifts in membrane potential. An increase in external [K+] leads to a reduction in membrane resistance (e.g. by more than 50% in *Paramecium*; [16]). Yet, because of the smaller K+ gradient between the intra- and extracellular space the resting current is smaller in high [K+] than in low [K+] [18]. The time–course of the drop in cAMP levels in *Tetrahymena* in high [K+] was indistinguishable from that of the membrane potential and resistance reported for *Paramecium* under identical conditions [16]. The mere correlation does not necessarily establish a cause–effect relationship. However, the stimulation of cAMP formation in *Tetrahymena* solely by a hyperpolarizing dilution of [K+] indicates a close correlation between a K+ conductance and adenylyl cyclase regulation. The extent of the cAMP response upon dilution of different K+ concentrations looks like a classical pharmacological dose–response curve while in fact it is not, because the strength of each stimulus was identical (dilution of [K+] to one-eighth of its original concentration). Obviously, the sensitivity of the adenylyl cyclase to hyperpolarization is determined in a dose–reponse-like manner by the external K+ concentration, indicating that the K+ resting conductance and the capacity for cAMP generation may be co-regulated. This is supported by the fact that three K+ channel blockers, TEA, Cs, and quinine, dose-dependently inhibited cAMP biosynthesis, and that an elevation of [Ca²⁺] which causes a hyperpolarization-induced fast-swimming response also elicited cAMP formation. The hyperpolarizing effect of Ca²⁺ that is also observed in *Paramecium* [17] is more difficult to interpret because one would expect that every increase in the concentration of external cations invariably results in depolarization. The unequivocal behavioral response, forward speeding, is testimony to the contrary. It is rationalized in terms of a screening of membrane surface charges which prevails at

modest Ca^{2+} concentrations over the depolarizing effect [14,15]. The dependance of the Ca^{2+} effect on the external K^+ concentration lends credence to this explanation, which is termed Ca^{2+} paradox [14]].

Tetrahymena is the second system in which the regulation of adenylyl cyclase activity is coupled to an ion conductance rather than a hormone receptor. The question remains as to how universal this system is. In *Paramecium* the adenylyl cyclase itself seems to have an intrinsic ion conductance [17], a property which did not come as a surprise considering the domain structure of cloned metazoan adenylyl cyclases [19]. Purification of the membrane-anchored adenylyl cyclase from *Tetrahymena* would further prove the analogy. The emerging techniques of transformation of *Tetrahymena* by electroporation [20] may, however, make this ciliate a more suitable model to resolve the nature of the relationship between the K^+ resting conductance, K^+ currents, and regulation of the adenylyl cyclase by molecular biological methods.

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