Ionic regulation of cyclic AMP levels in *Paramecium tetraurelia* in vivo

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cAMP levels in *Paramecium* increased dose dependently after a step increase of [Ca] or [Sr] in the incubation, provided K was present. Two mM Ca or Sr tripled cAMP concentrations within 3 s and induced an increase in forward swimming speed. The increase in cAMP formation was strictly dependent on the Donnan ratio $[K]:\sqrt{[Ca]}$. Na, Li, or tetraethylammonium could not replace K. The data provide evidence for regulation of cAMP in *Paramecium* by the membrane surface charge as determined specifically by the K:Ca ratio.

Adenylate cyclase *Excitable membrane* *Calcium* *Cyclic AMP* Paramecium

1. INTRODUCTION

Paramecium has been nicknamed 'swimming neuron' or 'swimming receptor' [1] since it reacts to a host of external stimuli with sophisticated behavioral responses. For example, if stimulated at the anterior end, *Paramecium* swims backward (ciliary reversal) in association with a Ca/K action potential, while posterior stimulation increases the speed of forward locomotion (ciliary augmentation) related to membrane hyperpolarisation [2]. In *Paramecium* the presence of several enzymes prominent in nervous tissues has been demonstrated. The cilia contain an adenylate cyclase [3], a guanylate cyclase [4], a cyclic GMP- and two cyclic AMP-dependent protein kinases and endogenous substrate proteins [5–7], phosphoprotein phosphatase 2B (calcineurin) [8], phosphodiesterase and calmodulin [4]. The guanylate cyclase of the ciliary membrane is regulated by Ca/calmodulin [4]. In spite of much effort, the physiological regulation of the membrane-bound adenylate cyclase remains mysterious. Neither did we observe an effect of NaF or GTP [3] nor did we find in cilia protein substrates for choleratoxin or pertussis toxin catalyzed adenoribosylation indicative of regulatory components of adenylate cyclases in other tissues [9]. Therefore, we studied the in vivo regulation of cyclic AMP (cAMP) levels in *Paramecium* to possibly obtain a hint for the regulation of adenylate cyclase. Here we report that ionic factors play a paramount role in the regulation of cAMP concentrations.

2. EXPERIMENTAL

Paramecium tetraurelia wild-type strain 51S were grown axenically as in [10]. Stationary cells were equilibrated at 40000 cells/ml for at least 2 h in buffer containing 50 μM $CaCl_2$, 5 mM K^+, 10 mM 4-morpholinepropanesulfonic acid (Mops), pH 7.2. Viability and behavior were microscopically checked prior to, and during the experiments. Cations were added as chlorides, the final concentrations in the incubations were as indicated. Cells were killed with 11 N perchloric acid and cyclic nucleotides were determined by radioimmunoassay with rabbit antibodies raised against cAMP and cyclic GMP (cGMP) and iodinated 2′-*O*-succinyl-cyclic nucleotide-L-tyrosylmethylesters [11].

Cross-reactivity of the antibodies was $<5\%$. To test the identity of the assayed immunoreactivity,

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samples were occasionally digested with phosphodiesterase prior to the assay. No cyclic nucleotides were found under these conditions. Also, in no instance did we find cyclic nucleotides in the incubation media. All values are expressed as pmol cAMP/mg protein as determined by the Lowry method; about 90000 cells correspond to 1 mg protein.

3. RESULTS AND DISCUSSION

3.1. *Time dependence of cyclic AMP changes in* Paramecium

Paramecia left undisturbed for 5 min had cAMP and cGMP levels of 5.2 ± 0.2 and 2.3 ± 0.1 pmol/mg, respectively ($\bar{x}$ ± SE, n = 12). Random mechanical stimulation, e.g. shaking on a vortex (setting 2) for 3 s or pipetting with an Eppendorf pipette resulted in statistically highly significant increases in cAMP and cGMP concentrations to 7.0 ± 0.5 and 7.0 ± 0.7 pmol/mg (n = 11). Excitation of *Paramecium* by a sudden increase of [Na] or [K] in the incubation buffer to 20 mM initiated a series of wild backward jerks for up to 1 min indicating repeated firing of Ca/K action potentials. Yet, cAMP levels were not affected and cGMP concentrations had only a slight, statistically not significant tendency to increase within 1 min and to return to basal values thereafter. When [Ca] was suddenly elevated from 50 μM to 2 mM a dramatic increase in cAMP levels occurred (fig.1). A rise was seen as early as 40 ms after Ca^{2+} addition, at 160 ms cAMP concentrations were already more than doubled (fig.1, inset), and they peaked around 3–10 s before declining to a new steady state level of about 175% of the original basal value (fig.1). The behavioral response to the increase in [Ca] was an immediate acceleration of forward swimming speed lasting for almost 20 min. Sudden addition of 2 mM Mg^{2+} also caused a vigorous behavioral response of *Paramecium* like that of Ca^{2+} and doubled cAMP levels from 4.8 to 9.7 pmol/mg within 3–10 s. Sr^{2+} at 2 mM was as effective as Ca in inducing cAMP accumulation (fig.1) and a long lasting increase in swimming speed. However, 2 mM Ba^{2+} did not raise intracellular cAMP levels (fig.1) and induced tumbling of the animals and weak avoiding reactions. Under the experimental conditions cGMP

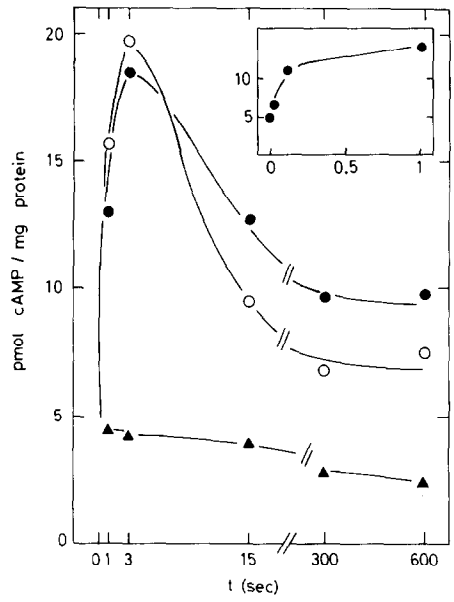

Fig.1. Time-dependent increase of cyclic AMP levels in whole *Paramecium* after a sudden increase of Ca^{2+} (●), Sr^{2+} (○), and Ba^{2+} (▲). Cells were equilibrated in 50 μM Ca, 5 mM K, 10 mM Mops buffer (pH 7.2), and at 0 min M^{2+} was increased to 2 mM by addition of corresponding buffers such that otherwise the buffer composition remained unchanged. The inset depicts the increase in response to a change in [Ca] in the ms range as measured with a continuous-flow setup. n = 6, SE was <0.7 pmol for all points.

concentrations were least increased by Ca^{2+} and most by Ba^{2+} (not shown).

Acceleration of forward swimming of *Paramecium* is traditionally thought to be associated with membrane hyperpolarization [1,2]. In view of the ciliary augmentation in response to Mg^{2+}, Ca^{2+}, and Sr^{2+} it would, therefore, be tempting to associate hyperpolarization with cAMP accumulation. However, all externally added cations depolarize the membrane at mM concentrations [1,2,12]. We now established dose–response curves to further probe the effect of sudden changes in divalent cation concentrations on behavior and cAMP formation in *Paramecium*.

3.2. *Cyclic AMP changes as a function of* $[M^{2+}]$

Sudden increases of [Ca] and [Sr] up to 1 mM in the incubation barely increased cAMP levels in *Paramecium* in correlation with only a modest acceleration in swimming speed. On additions of

higher concentrations (>2 mM) high contents of cAMP were found (fig.2). Even with large depolarizing concentrations of Ca^{2+} and Sr^{2+} we found exclusively a pronounced increase in forward locomotion in all cells during the entire incubation period with no apparent cell damage due to osmotic stress. With Mg^{2+} a biphasic effect was found, maximal stimulation of cAMP formation occurred at 2 mM, the effectiveness decreasing at lower and higher concentrations (not shown). Changes in [Ba] raised cAMP levels only at high, toxic concentrations of >10 mM and induced frequent avoiding reactions during the 10 s incubation.

The data virtually exclude a direct correlation between cAMP increases and membrane potential in *Paramecium*, since additions of >10 mM Ca^{2+}, Sr^{2+} and Mg^{2+} depolarize the membrane by 20–30 mV concomitant with a drop in membrane resistance [1,12,13] and induce ciliary augmentation while addition of 10 mM Ba^{2+} depolarizes the membrane and induces firing of Ca/K action potentials and ciliary reversal [2,12]. A correlation between ciliary augmentation and increased cAMP content seems possible, however. It has been suggested that certain electrical properties of *Paramecium* might be more directly related to the Donnan ratio than to absolute concentrations of monovalent and divalent cations [12,14]. Therefore, it may be asked whether the increase in [Ca] alone is responsible for changes in behavior and cAMP levels or rather the alteration of the [K]:[Ca] ratio.

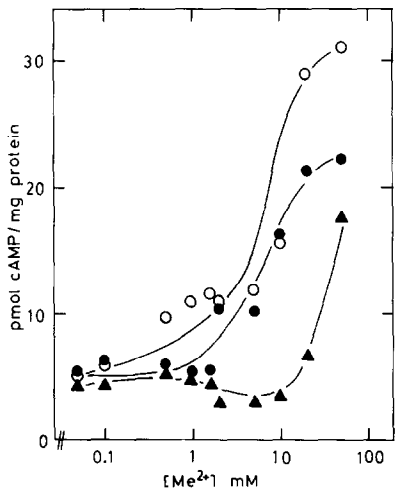

Fig.2. Dose–response curves for Ca^{2+} (●), Sr^{2+} (○), and Ba^{2+} (▲) stimulated accumulation of cyclic AMP in *Paramecium*. Cells were equilibrated in buffer containing 50 μM Ca, 5 mM K, 10 mM Mops (pH 7.2). Additions were made to yield the final concentrations of M^{2+} without effecting the concentration of other buffer constituents. Measurements were made after 10 s.

3.3. *Role of Donnan ratio in cyclic AMP regulation*

The Donnan ratio is defined as the concentrations of monovalent cations divided by the square root of the concentrations of divalent cations [14]. This ratio is changed from 22.3 to 3.5 when *Paramecia* are transferred from the equilibration buffer (50 μM Ca^{2+}, 5 mM K^+) to a buffer with 2 mM Ca^{2+} and 5 mM K^+. We now altered the ionic composition of the incubation medium by additions of monovalent cation solutions of different concentrations which simultaneously step increased [Ca] to 3 mM. The Donnan ratio $[K]:\sqrt{[Ca]}$ was varied from 22.3 to 2.9. As seen in fig.3, the cAMP increase was not a result of the stepped-up [Ca] but was due to changes in the Donnan ratio. The more the ratio of $[K]:\sqrt{[Ca]}$ in the final buffer approached that of the equilibration buffer, the smaller the increase in cAMP levels, in spite of the considerable osmotic stress exerted and the large decrease in membrane-resting potential as reported in [12,13]. Closely correlated with this effect was the reduction of the behavioral response as checked microscopically. The next question is then: are we dealing with a specific ion antagonism between K^+ and Ca^{2+} or can other monovalent cations substitute for K^+? Rb^+ could completely replace K^+, i.e., maintenance of the Donnan ratio with Rb led to cAMP levels and behavioral responses identical to those with K (not shown). However, Li^+, Na^+ and the K-channel blocker tetraethylammonium (TEA) [13] had quite different effects. Step changes of buffer cation composition from 50 μM Ca^{2+}, 5 mM K^+ to 3 mM Ca^{2+}, 5 mM K^+ and 5 mM Li^+, Na^+ or TEA did not increase cAMP levels markedly (fig.3). In addition, instead of a ciliary augmentation as seen with K^+ as sole cation, all cells showed ciliary reversal with frequent backward jerks alternating with periods of backward swimming which is characteristic for entry of Ca^{2+} through voltage-

operated Ca-channels during Ca/K action potentials [2]. In addition, cells equilibrated in K-free buffers containing 50 μM Ca^{2+}, 10 mM Mops, and either 5 mM Li^+, Na^+ or TEA for 2 h did not increase cAMP levels or change swimming behavior in response to a step increase of [Ca] to 3 mM.

Apparently, a rather specific antagonism exists between Ca^{2+} (or its congener Sr^{2+}) and K^+ (or Rb^+) on the *Paramecium* membrane which seems essential in regulation of cAMP levels. Addition of phosphodiesterase inhibitors such as isobutylmethylxanthine or rolipram has little effect on cAMP levels in vivo and on *Paramecium* phosphodiesterase in vitro. Thus, the accumulation of cAMP is most likely due to adenylate cyclase activation. The adenylate cyclase itself or a regulatory component should be able to detect changes in surface charge patterns specifically established by the Ca:K ratio. This was particularly evident in animals equilibrated in K-free buffer where an increase in [Ca] did not elicit cAMP accumulation. Differential alterations of surface charge by various ions have been implicated in sensory transduction and can selectively effect voltage sensitivity of ion channels [1,2,15]. A mutant of *Paramecium* with a reduced surface charge is available [15] to further study details of the ionic regulation of cAMP in vivo and in vitro.

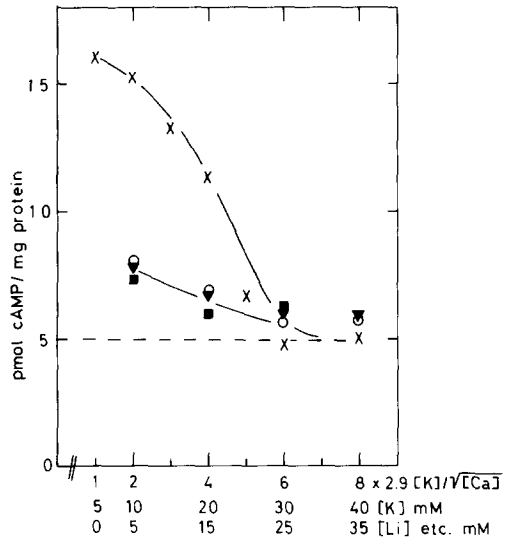

Fig.3. Effect of Donnan ratio $[K]:\sqrt{[Ca]}$ on cAMP accumulation in *Paramecium* stimulated by a step increase of [Ca] to 3 mM. The dashed line represents the cAMP level of cells in the equilibration buffer (50 μM Ca, 5 mM K, 10 mM Mops, pH 7.2, $[K]:\sqrt{[Ca]} = 22.3$) before [Ca] was stepped up with concomitant increases of [K] from 5 up to 40 mM (×). In experiments with Li^+ (■), Na^+ (○), and tetraethylammonium (▼) [K] was kept at 5 mM as in the equilibration buffer while the other monovalent cations were stepped up as indicated to yield final cation concentrations corresponding to respective K steps. Measurements were made after 10 s. $n = 3$.

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