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Calcium Channel Activation and Inactivation in *Paramecium* Biochemically Measured by Cyclic GMP Production

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Summary. The Ca-inward current of *Paramecium* is related to cGMP production by a Ca-dependent guanylate cyclase. Excitation with Ba^{2+} increases cGMP levels about ninefold to 45 pmol/mg within 15 sec. Inhibition of cGMP hydrolysis reveals a large rate of synthesis of up to 25 pmol cGMP/mg · sec^{-1}, or about 1.2 · 10^8 molecules/cell · sec^{-1}. Because no other factors than the Ca-inward current were found to affect cGMP formation in *Paramecium*, we used it as a quantitative measure of Ca^{2+} channel activity. After a transient stimulation of cGMP formation by 1 mM Ba^{2+}, an additional increase of Ba^{2+} to 5 mM did not result in a renewed elevation of cGMP levels. The extent of desensitization towards a second stimulus was graded with the strength of the first stimulus. Termination of the first stimulus after various time intervals and restimulation after 3 min with 1 mM Ba^{2+} revealed a time-dependent inactivation of the Ca^{2+} channel, which could be fitted by a single exponential. The inactivated form of the channel was stable for a few minutes at room temperature. The partial desensitization of *Paramecium* reduced the maximal response, but did not shift the dose-response curve for Ba^{2+}. Veratridine, which activates the Ca^{2+} channel, was also used as a first stimulus. It effectively and transiently inactivated the channel resulting in a complete loss of both a behavioral response of *Paramecium* and cGMP elevation towards a second stimulus. The time course of reactivation of channel excitability was studied at different temperatures. Half times of recovery were 51 and 7.5 min at 12 and 25°C, respectively. Reactivation curves can be described by a single exponential, indicating a first order reaction. The activation energy was 100 kJ/mol.

The extremely high rate of cGMP turnover in *Paramecium* is reminiscent of findings in visual cells. A model for regulation of the voltage-dependent Ca channel of *Paramecium* is proposed.

Key Words *Paramecium* · cyclic GMP · turnover · calcium channel · rod outer segment

Introduction

A voltage-operated Ca^{2+} channel is central to the behavior of the protozoan *Paramecium*. Following a threshold depolarization, the Ca^{2+} action current increases the intracellular Ca^{2+} concentration of 50 nM up to 40 μM, which causes the ciliary power stroke to reverse [24]. The Ca-inward current stimulates a rapid and transient increase in cGMP by a Ca-dependent guanylate cyclase [21, 31]. Most of our knowledge about the voltage-operated Ca^{2+} channel of *Paramecium* is derived from electrophysiological voltage-clamp experiments using *Paramecium* wildtype and Ca^{2+} current deficient pawn mutant cells [8, 24, 30]. Ca-dependent Ca^{2+} channel inactivation occurs during depolarization within tens of milliseconds due to the rapid buildup of intraciliary Ca^{2+} [3, 8]. This fast inactivation is absent when depolarization is elicited by addition of Ba^{2+} ions, which enter as charge carriers together with Ca^{2+} [26]. In addition, a slow inactivation process of the Ca^{2+} channel with time constants of tens of seconds was characterized behaviorally [15] and electrophysiologically under voltage clamp with long conditioning depolarizations [13]. This slow inactivation process is not dependent on internal Ca^{2+}, but is described to depend on voltage and time [13]. A similar slow inactivation and reactivation has been reported in squid axon [2].

Biochemical studies of Ca^{2+} channel regulation in vivo are usually restricted to time periods, which exceed those routinely used during electrophysiological experimentation. In our experiments, 0.5 to 1 sec is the shortest time interval at which reproducible biochemical measurements can be carried out with intact cells. Furthermore, biochemical measurements cannot be made with single cells or using an electrical stimulus comparable to electrophysiological tests. However, uniform responses of an entire population of *Paramecium* that are suitable for biochemical studies, can be elicited after sudden changes in the ion concentration. For example, an increase in Ba^{2+} depolarizes the cell by an influx of Ba^{2+} and Ca^{2+} through the voltage-operated Ca^{2+} channel and induces avoiding reactions. Due to the concomitant Ca^{2+} influx during a Ba-induced depolarization, the Ca-dependent formation of cGMP in

Paramecium is stimulated in a way strictly proportional to the extent of excitation [26, 31]. This indicates that changes in cGMP concentration in *Paramecium* reflect changes in the Ca^{2+} permeability of the membrane, i.e., Ca^{2+} channel activation and inactivation.

In this study, we used the intracellular level of cGMP as biochemical indicator of Ca^{2+} channel activity. Under conditions where the fast Ca-dependent inactivation is abolished or greatly reduced, it should be possible to biochemically follow the slow Ca^{2+} channel inactivation and reactivation. This can be accomplished by applying Ba^{2+} ions as stimulus. Ba^{2+} ions serve as charge carriers and enter the cell together with Ca^{2+} through the voltage-operated Ca^{2+} channel. However, Ba^{2+} inhibits the fast inactivation of the ion channel as caused by Ca^{2+} [8, 24, 26]. We adapted a double stimulus protocol from electrophysiological experiments for our biochemical purposes. Evidence was obtained that slow Ca^{2+} channel inactivation and reactivation are enzymatic processes with defined time and temperature dependences. A model for Ca^{2+} channel regulation is presented.

Materials and Methods

Cell Culture and Equilibration

Wildtype *Paramecium tetraurelia* 51s were axenically grown at 25°C in 1 liter Erlenmeyer flasks containing 300 ml medium with phosphatidylcholine as major lipid source [33]. Early stationary cells were harvested by centrifugation (500 × *g*, 1 min) and transferred into 200 volumes of equilibration buffer (10 mM MOPS adjusted to pH 7.2 with Tris, 1 mM KCl, 50 μM $CaCl_2$). Cells were equilibrated at 25°C for 2 hr on a rotary shaker (50 rpm) and washed once with equilibration buffer. The cell density in the experiments was 20,000 cells/ml.

Stimulation Protocols

Unless otherwise stated, all operations were carried out at room temperature. We used two different stimulation protocols. First, *Paramecia* were stimulated by the addition of Ba^{2+} (up to 1 mM). After various periods of time, a second, higher concentration of Ba^{2+} (5 mM) was added. Care was taken that only one parameter was altered, while the concentrations of other buffer ingredients remained constant. In a different protocol, Ba^{2+} or the plant alkaloid veratridine were used as first stimulus. *Paramecia* were stimulated in centrifugation test tubes. At the indicated time points, aliquots were withdrawn for cyclic nucleotide determinations and the remainder was centrifuged to remove the first stimulus (500 × *g*, 1 min). The supernatant was quickly aspirated and the cells were flushed with 200 volumes of equilibration buffer. After different time intervals a second stimulus was added.

Incubations were stopped by the addition of perchloric acid (1 M final).

cAMP and cGMP levels were assayed by radioimmunoassay using ^{125}I-2-O'-succinylcyclic nucleotide-L-tyrosinylmethylesters as tracers and respective antibodies against cAMP and cGMP with crossreactivities of less than 5% [7]. Veratridine (50 mM, freshly dissolved in 50 mM HCl) and 1 M $BaCl_2$ stock solutions were diluted with equilibration buffer prior to use. Protein precipitated by $HClO_4$ 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. Veratridine was purchased from Sigma. All other chemicals were of analytical grade from usual sources.

Straight lines were fitted by linear regression.

Results

cGMP Turnover Rate in *Paramecium*

In order to use intracellular levels of cGMP in *Paramecium* as a correlate for the actual fraction of open Ca^{2+} channels, first, the intraciliary processing of Ca^{2+}, and, second, the turnover of cGMP must be rapid compared with Ca^{2+} channel inactivation. Only then, activation, inactivation and reactivation processes of the voltage-operated Ca^{2+} channel will be rate limiting for cGMP levels in vivo. Third, the exclusively Ca-dependent guanylate cyclase [21, 31] and the phosphodiesterase activities should be under no regulatory control during depolarization other than Ca^{2+}.

First, Ca^{2+} removal from *Paramecium* is extremely rapid. Browning and Nelson [4] biochemically measured a stimulated Ca^{2+} influx at 4°C. They attributed the lack of a biochemically detectable accumulation of Ca^{2+} at room temperature to an extremely efficient Ca^{2+} removal system of *Paramecium* [4, 26, 27].

Second, to determine the rates of cGMP turnover, we inhibited cGMP degradation by the simultaneous addition of the depolarizing stimulus Ba^{2+} and the competitive phosphodiesterase inhibitor isobutylmethylxanthine. Under these conditions, initial rates of cGMP synthesis were 25 pmol/sec · mg^{-1} and occasionally maximal levels of even more than 500 pmol cGMP/mg protein were obtained within 30 sec (Fig. 1). After a few seconds, the rate of cGMP synthesis and, thus, cGMP levels decreased. We interpret this as an indication of Ca^{2+} channel inactivation and rapid Ca^{2+} sequestration, which reduced the intracellular Ca^{2+} concentration and, hence, Ca-dependent cGMP formation (Fig. 1). The fall in cGMP levels was considerably slower compared to the increase. This was due to the competitive inhibition of cGMP phosphodiesterase by isobutylmethylxanthine, which permitted hydrolysis of cGMP to GMP to proceed only at a reduced

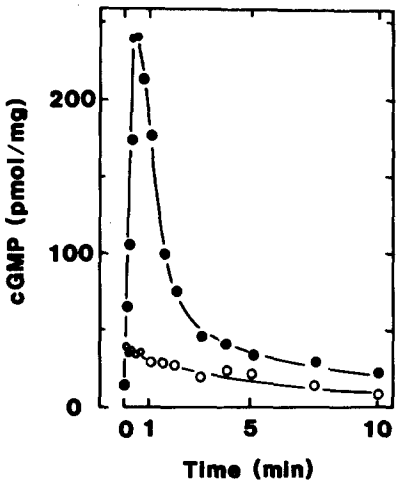

Fig. 1. Potentiation of the Ba^{2+}-elicited increase of cGMP levels in *Paramecium tetraurelia* wildtype cells by the phosphodiesterase inhibitor isobutylmethylxanthine: (○) 5 mM Ba^{2+}; (●) 5 mM Ba^{2+} + 5 mM isobutylmethylxanthine. One of three representative experiments is shown

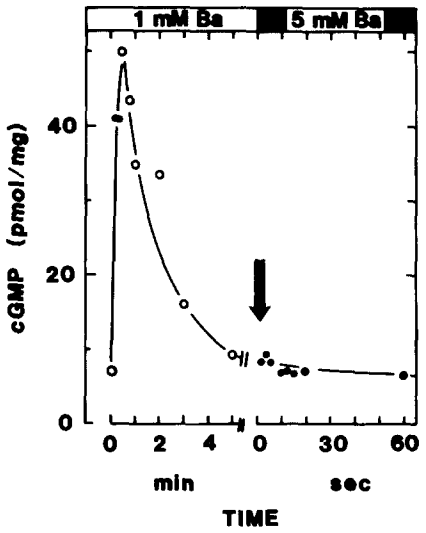

Fig. 2. Desensitization of *Paramecium* towards a second Ba^{2+} stimulus. Cells were stimulated by addition of 1 mM Ba^{2+} and cGMP levels were monitored for 5 min (○). After 5 min, the Ba^{2+} concentration was increased to 5 mM (indicated by the big arrow) and cGMP levels were monitored for 60 sec (●). Note the different time scales. One of three representative experiments is shown

rate. No cGMP was found in the medium [31]. That indeed the rate of cGMP formation was reduced in spite of the continued presence of the excitatory agent, was demonstrated by the next experiment. In the absence of the phosphodiesterase inhibitor, but otherwise identical stimulation conditions, maximal levels of only 40 to 50 pmol of cGMP/mg were measured (Figs. 1 and 2). Addition of 5 mM isobutylmethylxanthine after 5 min to such an incubation did not result in a huge increase of cGMP as observed upon concomitant addition. Under these conditions, cGMP was raised only about fourfold above basal levels within 30 sec (*data not shown*). Such an increase is also observed by addition of the phosphodiesterase inhibitor alone and indicates a basal rate of cGMP turnover of approximately 1 to 2 pmol/sec · mg^{-1}. These data demonstrate that upon stimulation by Ba^{2+}, the rate of cGMP turnover is greatly increased. Considering that in the presence of the competitive phosphodiesterase inhibitor isobutylmethylxanthine cGMP levels initially increase at a rate of 25 pmol/sec · mg^{-1}, it can be concluded that the levels of 45 pmol cGMP/mg, which were obtained under standard depolarizing conditions (1 mM Ba^{2+}, 50 μM Ca^{2+}) in the absence of a phosphodiesterase inhibitor, turned over at least once every 2 sec.

Third, we studied the kinetics and regulation of guanylate cyclase and phosphodiesterase activities in vitro. Cell-free extracts were prepared at 0°C prior to and immediately after stimulation. No differences in the properties of guanylate cyclase and phosphodiesterase activities were found between these preparations (*data not shown*). We interpret this result to indicate that, as reported earlier [21, 31], guanylate cyclase is only regulated by Ca^{2+}, not affected by Ba^{2+}, and not subject to an additional, long lasting regulatory control, e.g., by phosphorylation. cGMP phosphodiesterase activity has a K_m of 2.5 μM and is not affected by Ca^{2+}, Ba^{2+} or by any other known regulatory process [10, 17]. The rate of cGMP hydrolysis appears to follow standard enzyme kinetics.

Thus, all three criteria, as outlined above, for using cGMP as sensitive indicator for Ca^{2+} channel activity in *Paramecium* are fulfilled. In all subsequent incubations, no phosphodiesterase inhibitor was added to allow for a high metabolic turnover of cGMP such that under submaximal conditions of stimulation the prevailing cGMP levels are proportional to Ca^{2+} channel activity at any point in time.

cGMP Stimulation by Consecutive Ba^{2+} Additions

The time course of a Ba-elicited cGMP elevation in *Paramecium* is characterized by a rapid increase culminating around 15 sec and a fast decline afterwards (Fig. 2, [31]). cGMP concentrations return to prestimulation levels of about 7 pmol cGMP/mg after 3 to 5 min despite the continued presence of the Ba^{2+} stimulus (Fig. 2). After stimulation with 1 mM

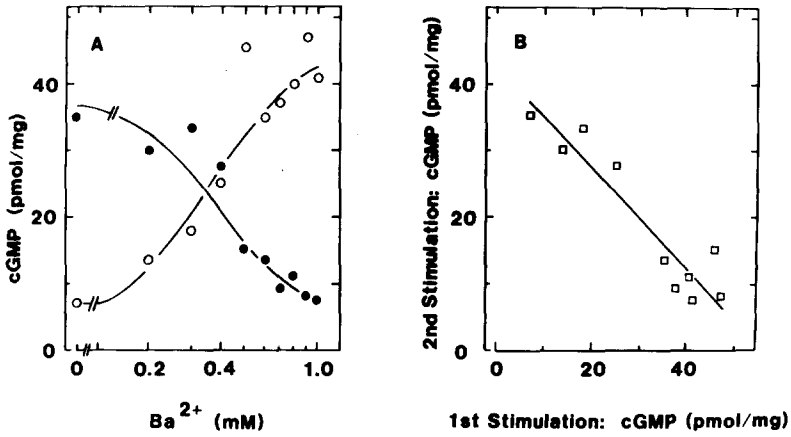

Fig. 3. Desensitization of *Paramecium* towards a second stimulus is graded to the strength of the first stimulus. (*A*) Various concentrations of Ba^{2+} were added as first stimulus and cGMP levels were determined after 15 sec (○). Five min later, when cGMP had declined to basal levels (range 4.2–9.3 pmol/mg; *not shown*), the Ba^{2+} concentration was increased in all samples to 5 mM and cGMP concentrations were measured after 15 sec (●). (*B*) cGMP levels in response to the first Ba^{2+} stimulation are plotted against the cGMP concentrations obtained during the second stimulation. The correlation coefficient of the computed line was 0.9292. One of two experiments is depicted

Ba^{2+} for 5 min ($[Ba^{2+}]:[Ca^{2+}] = 20$), an increase of the Ba^{2+} concentration to 5 mM ($[Ba^{2+}]:[Ca^{2+}] = 100$) was without further effect (Fig. 2). The lack of a cGMP response coincided with a lack of a behavioral response. We interpret this result to indicate that all voltage-operated Ca^{2+} channels are locked during the first Ba^{2+} stimulation in an inactivated state. In fact, the cells even survived for several minutes a concentration of 20 mM Ba^{2+}, which otherwise would immediately be lethal. Because 1 mM Ba^{2+} at a background concentration of 50 μM Ca^{2+} resulted in a rather strong excitation, we tested whether the extent of Ca^{2+} channel inactivation depended on the strength of the first stimulus. *Paramecia* were stimulated with various concentrations of Ba^{2+}. An aliquot was removed after 15 sec and cGMP levels were determined. A typical Ba^{2+} dose-response curve was obtained (Fig. 3*A*, open circles). The incubations were then continued for 5 min such that cGMP concentrations returned to basal levels. At this point, the Ba^{2+} concentration was suddenly raised to 5 mM and cGMP was measured after a further 15 sec incubation (Fig. 3*A*, solid circles). The magnitude of the cGMP increase in response to the second stimulus depended on the Ba^{2+} concentration of the first stimulus. A small increase during the first stimulation period was followed by a correspondingly large cGMP increase elicited by the second stimulus and vice versa. Plotting the amplitude of the first cGMP response against that of the second response, we obtained a straight line (Fig. 3*B*). This documented the strict correlation between both events. Apparently, the extent of Ca^{2+} channel inactivation during the first stimulation period was graded to the intensity of the primary excitation in a manner more or less identical to that previously observed in electrophysiological voltage-clamp experiments [13].

Similarly, cAMP concentrations were determined in all samples. Neither the first nor the second stimulus affected intracellular cAMP levels (*data not shown*).

cGMP Stimulation after Removal of the Prestimulus

Two questions arose (*i*) is the inactivation of the Ca^{2+} channel as measured by a decrease in cGMP formation, an instantaneous event due to the concomitant entry of Ca^{2+} and Ba^{2+} or does it require a longer time period? (*ii*) is the continuous presence of the first stimulus necessary for the Ca^{2+} channels to stay inactivated? These questions were addressed in the following experiments.

Paramecia were stimulated with 1 mM Ba^{2+} for 1, 3, 5 and 7 min. Aliquots were withdrawn for cGMP determinations (Fig. 4*A* left) and the remaining cells were transferred by centrifugation into Ba^{2+}-free equilibration buffer. After 3 min, cGMP levels in all samples were back to basal (Fig. 4*A* right). 1 mM Ba^{2+} was once again added to each incubation and cGMP levels were quantitated after 15 sec (Fig. 4*A* right). The results demonstrated that the desensitized state of the Ca^{2+} channel (*i*) required time to develop and (*ii*) was stable at room temperature for a brief period of time. Another notable observation was that cGMP levels obtained upon the second stimulation were almost identical

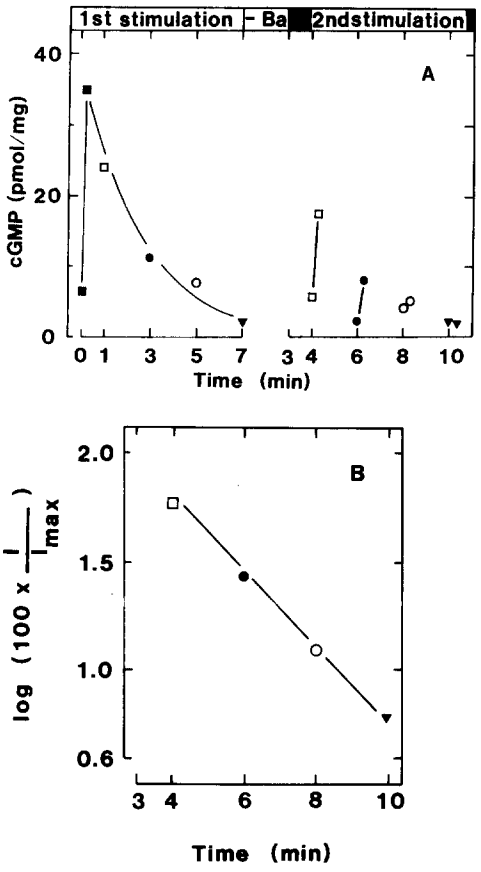

Fig. 4. Desensitization of *Paramecium* towards a second stimulus is dependent on the length of time of exposure to the first stimulus. (*A*) 1 mM Ba^{2+} was added as first stimulus. cGMP concentrations were measured in aliquots of the incubations (after 1, 3, 5 and 7 min; *A*, left). At these time points, Ba^{2+} was removed from the remaining samples by a brief centrifugation and the cells were immediately resuspended in equilibration buffer. After 3 min, cGMP levels were determined in a second aliquot after 4, 6, 8 and 10 min (*A*, right) and the rest of the samples was again challenged with 1 mM Ba^{2+} for 15 sec. Identical symbols in the left and right side of *A* indicate the same origin of the cells in various stages of the experiment. (*B*) Single exponential indicating a first order reaction. I_{max} was measured 15 sec after stimulation of naive cells with 1 mM Ba^{2+}. *I*-values are the cGMP levels obtained during the second stimulation (*A*, right side). The log of these cGMP concentrations as a percentage of I_{max} was plotted vs. the period of exposure towards the first stimulus (r = 0.9950). $t_{1/2}$ was about 70 sec. The symbols correspond to those in *A*. One of two representative experiments is shown

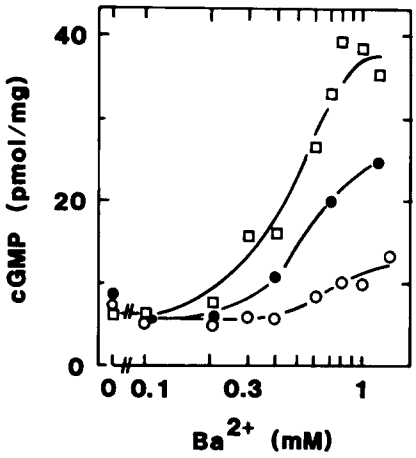

Fig. 5. Desensitization of *Paramecium* reduces the maximal response but not the sensitivity towards Ba^{2+}. *Paramecium* was stimulated for 90 (●) and 300 sec (○) with 1 mM Ba^{2+}. This primary stimulus was then removed by centrifugation, cells were resuspended in equilibration buffer and stimulated again after 5 min for 15 sec with the indicated concentrations of Ba^{2+}. Control without Ba^{2+} pretreatment (□). One out of two experiments is shown

to those measured at the break off point of the first stimulation (*compare* stimulated cGMP levels in Fig. 4*A*). This indicated that the slow inactivation process of the Ca^{2+} channel instantly stopped on removal of the stimulus. The fraction of channels not yet inactivated could be reopened immediately. The rate of inactivation could be fitted with a single exponential (Fig. 4*B*). This indicates that a single molecular event is responsible for Ca^{2+} channel inactivation.

Next, we tested whether the fraction of Ca^{2+} channels not inactivated by a first stimulus had a similar sensitivity towards Ba^{2+} stimulation as channels in unstimulated control cells. *Paramecia* were stimulated for only 90 sec with Ba^{2+}. The stimulus was then removed by centrifugation and cells placed in equilibration buffer. After 5 min, a Ba^{2+} dose-response curve was established using the partly desensitized cell population.

The concentrations of Ba^{2+} necessary for a half-maximal cGMP response were almost identical in naive and prestimulated cells (EC_{50} = 510 and 570 μM, respectively). However, the maximal response was reduced by 40% in the pretreated cell population (Fig. 5). We interpret this result to indicate that the sensitivity to activation by Ba^{2+} was unchanged for those Ca^{2+} channels not yet locked in an inactivated state. When Ca^{2+} channels of *Paramecium* were inactivated during a 5-min preincubation period with Ba^{2+} rather than 90 sec as described before, no cGMP formation could be elicited anymore, i.e., most of the Ca^{2+} channels were in a temporarily inactivated state and could not be opened even at high concentrations of Ba^{2+} (Fig. 5).

Prestimulation of cGMP with Veratridine

We have presented evidence that the plant alkaloid veratridine, which opens voltage-operated Na^+

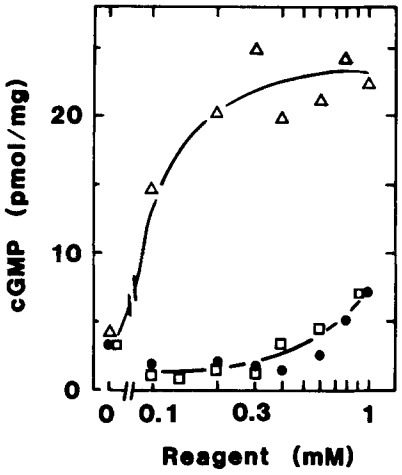

Fig. 6. Desensitization of *Paramecium* occurs with veratridine as a first stimulus. Cells were stimulated with 1 mM veratridine for 5 min. The alkaloid was then removed by centrifugation and the cells were resuspended in equilibration buffer. After 5 min, cGMP formation was stimulated by addition of various concentrations of either veratridine (□) or Ba^{2+} (●). For control, naive cells were stimulated directly with veratridine (△). One of two representative experiments is shown

channels in metazoans, can also open the voltage-operated Ca^{2+} channels of *Paramecium* [32]. It induces a similar behavioral response and an increase in cGMP virtually identical to a Ba^{2+} stimulation. We were interested to learn whether veratridine used as a first stimulus also causes a long-lasting desensitization of *Paramecium*. Cells were exposed for 5 min to 1 mM veratridine. At the end of this period, cGMP levels had returned to basal levels from a concentration of 40 pmol/mg at 15 sec [33]. Veratridine was then removed by centrifugation and 5 min later the pretreated cells were stimulated with increasing concentrations of Ba^{2+} or veratridine. The cells were almost completely unresponsive to either agent (Fig. 6). The lack of a cGMP response after prestimulation with veratridine coincided with a lack of a behavioral response. *Paramecium* could survive 20 mM Ba^{2+} after a prestimulation with veratridine. We conclude that the inactivation process of the voltage-operated Ca^{2+} channel is (*i*) not restricted to stimulation by Ba^{2+} and (*ii*) not dependent on the presence of intracellular Ba^{2+}, which remains long after a Ba^{2+} stimulus is removed [26].

Time Course of Ca^{2+} Channel Reactivation

We now asked how long it takes for the inactivated Ca^{2+} channel to be processed into a closed state. *Paramecia* were incubated at 25°C for 5 min with 1 mM Ba^{2+}. The stimulation was terminated by centrifugation and the cells were suspended and kept in equilibration buffer of 12, 16, 18 and 25°C, respectively. After various periods of time, cells were quickly warmed to 25°C and stimulated for 15 sec with 1 mM Ba^{2+} (Fig. 7*A*). The reactivation of the Ca^{2+} channel in vivo was strongly dependent on the temperature at which the cells were kept after the inactivating first stimulation. For example, the cGMP response was halfway up after 51 and 7.5 min at 12 and 25°C, respectively. The reactivation curves could be fitted with high statistical significance to single exponentials (Fig. 7*B*), which indicated first order kinetics. This is strongly suggestive of a single enzymatic reaction. Using these data we obtained a Q_{10} for the temperature range of 15 to 25°C of 2.9. The $t_{1/2}$ values obtained from Fig. 7*B* were used to construct an Arrhenius plot (Fig. 7*C*), which yielded an activation energy of 100 kJ/mol, compatible with an enzymatic reaction.

The reactivation of the inactivated channel was also investigated after a prestimulation with 1 mM veratridine for 5 min. We found a $t_{1/2}$ of 13 min at 22°C (*data not shown*). This further substantiates the earlier claim that veratridine opens the voltage-operated Ca^{2+} channels in *Paramecium* [32].

Discussion

The intracellular concentrations of cGMP were used in this study as a sensitive and quantitative indicator of Ca^{2+} channel activation in *Paramecium*. This was justified because the stimulated levels of cGMP reflected a very high rate of cGMP turnover of at least 25 pmol/sec · mg^{-1}. This high turnover became only apparent when cGMP hydrolysis was decreased by competitive inhibition of phosphodiesterase (*see* Figs. 1 and 2). It must be concluded that in the absence of a phosphodiesterase inhibitor stimulated cGMP levels turned over at least once every 2 sec. With 45,000 cells corresponding to 1 mg protein, we calculated a turnover of 1.2×10^8 molecules/sec · $cell^{-1}$. Since voltage-dependent Ca^{2+} channels and a considerable fraction of guanylate cyclase are localized in the ciliary membrane, much of this turnover is likely to occur in the cilia. This high turnover of cGMP in *Paramecium* is reminiscent of rod outer segments. To our knowledge, this is the only other system in which comparable turnover rates of cGMP have ever been reported [1, 6, 11]. Interestingly, the rod outer segment is originally derived from a ciliary structure, it even contains a remnant axoneme of unknown function. cGMP regulates an ion conductance in rod outer segments by binding to a channel, which is

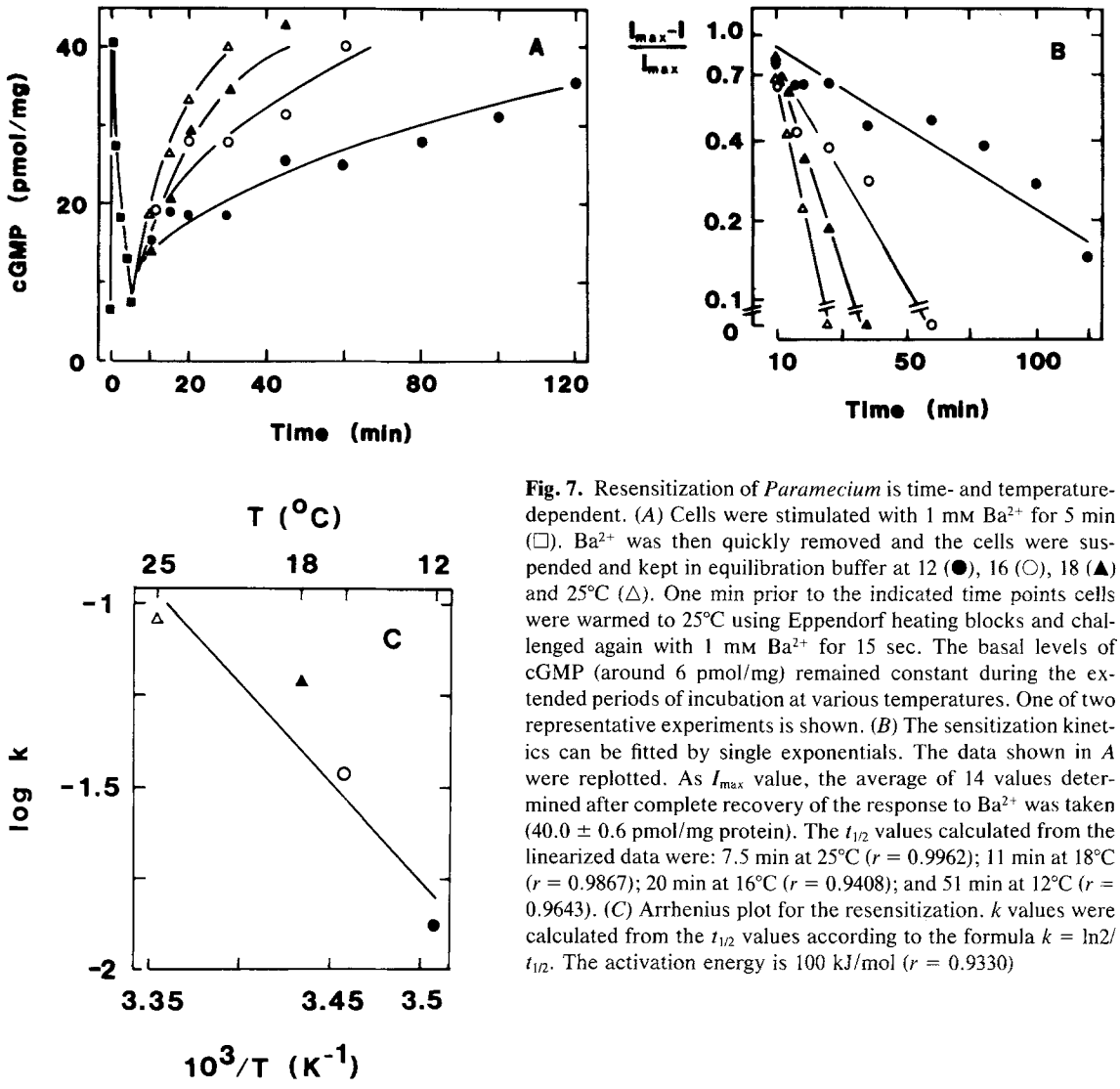

Fig. 7. Resensitization of *Paramecium* is time- and temperature-dependent. (*A*) Cells were stimulated with 1 mM Ba^{2+} for 5 min (□). Ba^{2+} was then quickly removed and the cells were suspended and kept in equilibration buffer at 12 (●), 16 (○), 18 (▲) and 25°C (△). One min prior to the indicated time points cells were warmed to 25°C using Eppendorf heating blocks and challenged again with 1 mM Ba^{2+} for 15 sec. The basal levels of cGMP (around 6 pmol/mg) remained constant during the extended periods of incubation at various temperatures. One of two representative experiments is shown. (*B*) The sensitization kinetics can be fitted by single exponentials. The data shown in *A* were replotted. As I_{max} value, the average of 14 values determined after complete recovery of the response to Ba^{2+} was taken (40.0 ± 0.6 pmol/mg protein). The $t_{1/2}$ values calculated from the linearized data were: 7.5 min at 25°C (r = 0.9962); 11 min at 18°C (r = 0.9867); 20 min at 16°C (r = 0.9408); and 51 min at 12°C (r = 0.9643). (*C*) Arrhenius plot for the resensitization. k values were calculated from the $t_{1/2}$ values according to the formula $k = \ln 2/t_{1/2}$. The activation energy is 100 kJ/mol (r = 0.9330)

permeable to monovalent and divalent cations [5, 9, 12, 22]. In vertebrate rods, a hyperpolarizing receptor potential is mediated by cGMP [9], while in invertebrates, a depolarization is produced [19]. cGMP levels are regulated by an enzyme cascade involving rhodopsin and the G-protein transducin, which controls a cGMP phosphodiesterase [20]. A guanylate cyclase, which supposedly is inhibited by Ca^{2+}, has recently been reported [23]. In spite of the striking and unique similarities in turnover rates of cGMP in rod outer segments and *Paramecium*, no further analogies can be drawn since at present no tentative function can be assigned to cGMP in *Paramecium*. We were unable to unequivocally demonstrate the presence of G-proteins (*unpublished*).

A phosphodiesterase specific for cGMP hydrolysis has been identified and partially purified from *Paramecium* [10, 17]. The enzyme has a K_m of 2.5 μM. A regulation by ions or other factors was not found [10, 17]. The membrane-bound guanylate cyclase activity of *Paramecium* is activated by Ca^{2+} [21, 31] rather than inhibited as is the guanylate cyclase from rod outer segments [23]. Despite these discrepancies between *Paramecium* and rod outer segments, we believe that the unusually high turnover rate of cGMP in *Paramecium* is of great functional significance and represents a basic feature possibly used in many sensory cells. It is certainly attractive to speculate that like in rod outer segments, cGMP in *Paramecium* is somehow involved in the intricate fine-tuning of ion conductances. Our data certainly makes studies into that question a worthwhile endeavor.

Our findings concerning the slow Ca^{2+} channel inactivation permit us to propose a model for the regulation of the voltage-dependent Ca^{2+} channel of

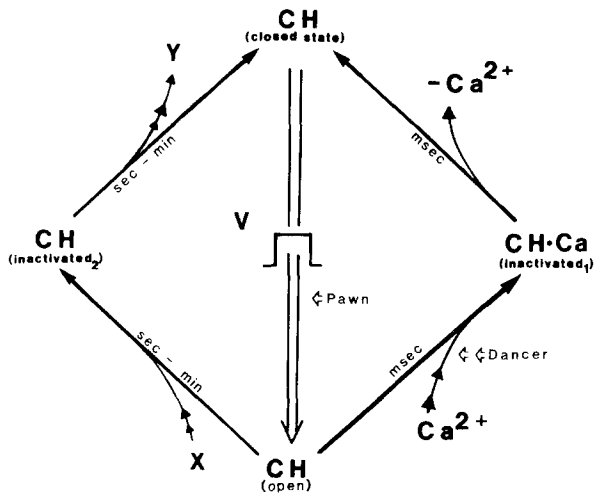

Fig. 8. Model for the regulation of the voltage-dependent Ca^{2+} channel in *Paramecium* as outlined in the discussion. *CH* is used as abbreviation for channel

Paramecium, which incorporates several behavioral, electrophysiological, genetic and biochemical findings (Fig. 8). With the membrane potential at rest, the Ca^{2+} channel is in a closed state, which is susceptible to activation by depolarization. As demonstrated in the preceding paper [32], it is this activation process, which is afflicted in the pawn mutation. There, the protein components, which constitute the Ca^{2+} channel, seem to be present. However, the channel complex cannot be activated by depolarization or by modest concentrations of Ba^{2+} [24, 26]. The plant alkaloid veratridine is able to activate this Ca^{2+} channel in wildtype cells (Fig. 6) and in all pawn mutant lines tested so far [32]. The molecular mechanism of the veratridine action remains to be elucidated.

Once the channel is open, it can be inactivated by two independent mechanisms, which have been described in several organisms [2, 14, 28]. There exists a rapid, Ca-dependent inactivation, which occurs within milliseconds (Fig. 8, right; [3, 16]) and a slow Ca-independent inactivation with time constants of tens of seconds [2, 13]. The fast Ca-dependent Ca^{2+} channel inactivation in *Paramecium* has been investigated in detail by electrophysiological techniques [8, 24, 30]. This process is responsible for Ca^{2+} channel inactivation in other organisms as well [28]. The inactivation is proposed to rapidly lead to an "inactivated-1" configuration, which might represent a complex of the channel protein and Ca^{2+} (Fig. 8). In the group of dancer mutants, this rapid inactivation process is delayed resulting in a sustained Ca-inward current [16], an enhanced formation of cGMP [31], and an exaggerated behavioral response to environmental stimuli. The transition from the inactivated-1 configuration of the Ca^{2+} channel to the closed state occurs within hundreds of milliseconds [3]. This cycle operates chiefly in what has formerly been termed type I and type II membrane excitation of normal *Paramecium* [29]. The behavior during these states of excitation is characterized by spontaneous stops and turns, which are displayed in usual hay media or fresh waters of constant composition.

In the type III excitation, the excited state is prolonged to hundreds of seconds, e.g., by a strong Ba^{2+} current, extensive removal of Ca-dependent inactivation or under long voltage-clamp depolarization [26, 29]. Under these conditions, a slow, voltage-dependent inactivation of the Ca^{2+} channel with time constants of tens of seconds has electrophysiologically been observed [13]. From a biochemical point of view, the increase of the fraction of Ca^{2+} channels, which are open per unit time, can be viewed as an increase of the substrate concentration for an enzyme, which catalyzes the transition of the activated channel (Ch_{open}, Fig. 8) to an inactivated-2 configuration. The time course of this inactivation process observed in electrophysiological as well as biochemical experiments is fully compatible with the proposed model. Since the inactivation process can be fitted by a single exponential (Fig. 4*B*, and [13]), only a single enzymatic step may be required. Preliminary evidence in our laboratory indicates that this reaction is a dephosphorylation by a protein phosphatase predominantly localized in the cilia from *Paramecium*. This is comparable to higher systems [14, 18, 25]. The "semipermanent" inactivation of the Ca^{2+} channel in *Paramecium* occurs only during prolonged depolarizations, e.g., by voltage clamp or by chemical agents such as Ba^{2+} or veratridine. The formation of the inactivated-2 configuration may, therefore, be related to adaptational processes of membrane ion conductances required under changing environmental conditions.

The transition of the inactivated-2 state to the closed-channel configuration was time- and temperature-dependent. The kinetics indicated a single enzymic reaction, possibly a phosphorylation. The activation energy of approximately 100 kJ/mol was rather high. Although our experiments can only yield a tentative value, a high energy barrier may make physiological sense in that it tends to dampen the effect of rapid oscillations of environmental conditions on cellular processes.

The proposed model not only incorporates in a plausible manner virtually all what is hitherto known of the Ca^{2+} channel regulation of *Paramecium*, it also provides the basis for further experiments designed to biochemically identify the channel protein itself.

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