Electrophysiological Studies of Activation and Inactivation of Ca channels by Ionic Stimulation in Paramecium caudatum

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CONTENTS

GENERAL INTRODUCTION

It is well known that specimens of the ciliate protozoan Paramecium show backward swimming due to reversed beating of cilia (ciliary reversal), when they are transferred into a solution with high K^+ concentration (Jennings, 1906). The backward swimming which continues for several ten seconds (depending on stimulus condition) is followed by forward swimming due to renormalization of beat direction of cilia. Mast and Nadler (1926) examined effects of various chemicals on the behavior of Paramecium. They reported that 1) an increase in concentration of monovalent cations induced ciliary reversal, though its degree depended on cation species, 2) anions were less effective than cations and 3) Ca^{2+} ions antagonized the effects of monovalent cations. On the other hand, when Paramecium is exposed to electric current, ciliary reversal occurs at cell surface facing the cathode (Verworn, 1889). Based on the analogy with Pflüger's cathodal excitation in nerve and muscle cells, Bancroft (1905) thought that the cathodal ciliary reversal might correspond to membrane excitation.

Ciliary reversal and ionic factors in the external solution

Kamada and Kinosita (1940) examined effects of external K^+ and Ca^{2+} concentrations on ciliary reversal. The duration of ciliary reversal was affected by both $[K^+]/[Ca^{2+}]$ ratios of adaptation medium and stimulation medium. Jahn (1962) introduced the Gibbs-Donnan principle to analyze relation between the amount of cations bound to the membrane and the duration of ciliary reversal. He proposed that release of bound Ca by an increase in

the $[K^+]/\sqrt{[Ca^{2+}]}$ ratio was responsible for ciliary reversal. Naitoh and Yasumasu (1967) demonstrated that external cations such as Ca^{2+} or K^+ bind to anionic sites of Paramecium membrane according to the law of mass action and the membrane can be regarded as an ion-exchanger. Naitoh (1968) found that factors which affect the duration of the ciliary reversal response are 1) the initial amount of Ca bound to the cation-exchange system of the membrane, 2) the amount of Ca^{2+} released from binding sites in the stimulation medium and 3) the concentration of Ca^{2+} in the stimulation medium.

Electrophysiology of Paramecium membrane

Electrophysiological researches of Paramecium membrane using intracellular electrode technique were started by Kamada (1934). By making use of quartz capillary microelectrodes, he measured the membrane potential of Paramecium in various ionic solutions, and found that the potential, which was usually negative with reference to the external solution, depended strongly on the external cation concentration. Ciliary reversal was elicited in association with a transient depolarization of the membrane (Yamaguchi, 1960; Kinosita et al., 1964, 1965; Naitoh, 1966).

Naitoh and Eckert (1968a) examined in detail membrane electrical properties of in Paramecium equilibrated in solutions with various concentrations of several kinds of monovalent and divalent cations. The results can be summarized as follows: 1) The membrane is depolarized more effectively by cations with higher binding affinity to the membrane (K^+, Rb^+, Ba^{2+}) than those with lower binding affinity (Na^+, Mg^{2+}). An increase in

bound Ca reduces depolarizing effects of external cations. 2) Characteristics of the electrical responses to current injection are strongly affected by external $[K^+]/\sqrt{[Ca^{2+}]}$ ratio, rather than by the absolute concentrations of these cations. 3) The current-voltage relationships also depends on the amount of membrane bound Ca. 4) The membrane resistance depends on the membrane potential and the bound Ca. These facts suggest that Ca-binding system of the membrane strongly affects not only ciliary responses but also membrane electrical properties. In response to outward current injection, Paramecium exhibits a graded action potential in $CaCl_2$-containing solutions, but it shows an all-or-none action potential in solutions containing $BaCl_2$ (Kinosita et al., 1965; Naitoh and Eckert, 1968a). Characteristics of the all-or-none action potential in Ba-containing solutions is dependent on the amounts of Ba and Ca bound by the membrane (Naitoh and Eckert, 1968b). Mechanism of action potential generation by outward current injection in solutions containing K^+, Ca^{2+} or other cations was investigated electrophysiologically in more detail by Naitoh et al. (1972). The saturated peak value of the action potential increases with increase in the external Ca^{2+} concentration by a slope of about 25 mV to 10-fold increase in the Ca^{2+} concentration. This suggests that the action potential is caused by a transient increase in Ca conductance of the membrane, which is accompanied by an inflow of external Ca^{2+} into the cell (Eckert, Naitoh and Machemer, 1976).

On the other hand, responses of Paramecium membrane to mechanical stimulation was investigated by Naitoh and Eckert (1969), Eckert et al. (1972) and Naitoh and Eckert (1973). A

mechanical stimulation of the anterior portion of the cell produces a depolarizing receptor potential, which in turn causes an action potential. The receptor potential is caused by an increase in Ca conductance. Stimulation of the posterior portion produces a hyperpolarizing receptor response due to an increase in K conductance. The hyperpolarization is accompanied by an increase in beating frequency of cilia. Simultaneous recording of regenerative Ca response and ciliary movement revealed that ciliary reversal response was closely related to the Ca action potential (Eckert and Naitoh, 1970; Machemer and Eckert, 1973).

Ciliary reversal and intracellular Ca^{2+} ions

Relationships between intracellular ionic environment and ciliary movement were investigated by making use of glycerol- (Naitoh, 1969) and Triton- (Naitoh and Kaneko, 1972) extracted models of Paramecium caudatum. Ions and other chemicals in the external solution can flow freely into the cell through the extracted membrane. Ciliary reversal was found to occur when Ca^{2+} concentration was more than 10^{-6} M in the presence of ATP. Eckert (1972) explained the membrane control of ciliary reversal response as follows: Depolarizing stimuli such as outward current injection or high K^{+} stimulation cause an increase in Ca conductance of the membrane. External Ca^{2+} ions flow into the cell down the electrochemical gradient of Ca^{2+} ions across the membrane. A resultant increase in the intracellular Ca^{2+} ions activates the mechanism for ciliary reversal.

Modification of the function of Ca channels in behavioral mutant cells or deciliated cells

Kung (1971) obtained a behavioral mutant of Paramecium tetraurelia, which was insensitive to an increase in K^+ concentration and continued to swim forward. This mutant was named "pawn" because of its behavior. The mutant was found not to produce an action potential in response to depolarizing stimulus, while other electrical properties of the mutant were almost the same as wild type (Kung and Eckert, 1972). However, the mutant showed ciliary reversal in response to an application of Ca^{2+} ions together with ATP, when its membrane was disrupted by Triton X-100 (Kung and Naitoh, 1973). Schein et al. (1976) suggested that among genes relating to the membrane excitability, pw A gene was responsible for the depolarization sensitivity (i.e. gating) of Ca channels, and pw B gene was for the characteristics of the wall of Ca channels. In Paramecium caudatum, mutants with malfunction in the voltage sensitivity of Ca channel were reported by Takahashi and Naitoh (1978).

Paramecium whose cilia were removed by its treatment with chloral hydrate or ethanol lost its ability to produce the Ca action potential. The action potential was found to resume gradually as the cilia regenerated (Ogura and Takahashi, 1976; Dunlap, 1976, 1977; Machemer and Ogura, 1979). These results suggest that depolarization-sensitive Ca channels were localized in the ciliary membrane. In contrast, mechanoreceptor potential did not disappear even after removal of cilia. This suggests the mechanoreceptor channels are present in somatic membrane (Ogura and Machemer, 1980).

Detailed characterization of ion channels

Recently, ion channels of Paramecium membrane have been investigated by means of voltage clamp technique and micro-injection of ions and other chemicals into the cell. Since EGTA-injected cells become to produce a prolonged action potential with a plateau, inactivation of Ca channels and activation of delayed K channels may be caused by an increase in the intracellular Ca^{2+} concentration (Brehm et al., 1978; Satow, 1978). Decrease in the resting membrane resistance seen in Ca^{2+} injected cells suggests that the resting K conductance is also affected by internal Ca^{2+} ions (Satow, 1978). Electrophysiological examinations of Paramecium membrane with voltage clamp technique were reported first by Naitoh and Eckert (1974). Oertel et al. (1977) analyzed membrane currents in wild type cells and mutant "pawn" under voltage clamp condition (see also Satow and Kung, 1980a,b). Oertel et al. (1978) confirmed the previous finding that activation of K conductance was produced by hyperpolarization. Satow and Kung (1979) examined concentration effects of external Ca^{2+} ions (i.e. Ca-stabilizing effects, see below) on the depolarization-sensitive Ca channels (Eckert and Brehm, 1979; Satow and Kung, 1981).

Brehm and Eckert (1978) demonstrated Ca-dependent inactivation of Ca channels which caused relaxation of transient inward Ca current during sustained depolarization (Brehm et al., 1980; Eckert and Chad, 1984). Voltage sensitive inactivation of Ca channels was also found by Hennessey and Kung (1985).

Other excitable membranes

Hodgkin and Katz (1949) suggested that the action potential in squid giant axon was caused by selective increase in Na^+ permeability of the membrane. Hodgkin and Huxley (1952) quantitatively described time courses of the changes in Na and K conductances during membrane depolarization under voltage clamp condition. The action potential can be interpreted by a transient increase in the Na conductance, which is followed by an increase in the K conductance. Voltage sensitivity of Na channels responsible for the Na conductance was confirmed by detecting a voltage dependent gating current (Armstrong and Bezanilla, 1973, 1974; Bezanilla and Armstrong, 1974; Keynes and Rojas, 1973).

Membrane excitation by rapid increase in the external K^+ concentration were observed in Nitella cell (Hill and Osterhout, 1938), Ranvier node (Tasaki, 1959) and in squid giant axon (Tasaki et al., 1966, 1968; Inoue et al., 1973). Tasaki (1968) noted that excitable membrane has characteristics as a cation-exchanger. He suggested that the ratio of divalent cations to monovalent cations in the membrane was important for the membrane excitation. The liberation of membrane bound divalent cations (Ca^{2+} ions) causes membrane excitation (see also Tobias, 1964). However, there are few studies on transient changes in the membrane electrical properties caused by changes in ionic condition of the external or internal solution of the cell.

On the other hand, relationship between membrane excitability and external Ca^{2+} concentration was examined in squid giant axon under voltage clamp by Frankenhaeuser and Hodgkin

(1957). They concluded that an increase in Ca^{2+} concentration was equivalent to hyperpolarization of the membrane. This hyperpolarization-like effect of Ca^{2+} ions on the membrane was termed "Ca-stabilizing effects", and seen in some excitable cells other than giant axon (Frankenhaeuser, 1957; Hille, 1968; Ohmori and Yoshii, 1977; Cota and Stefani, 1984; Byerly et al, 1985). The Ca-stabilization is attributable to a hyperpolarizing shift of the transmembrane diffusion potential by Ca binding to the membrane.

Problems in the membrane excitation caused by ionic stimulation in Paramecium

Paramecium membrane exhibits an increase in Ca permeability (membrane excitation) in response to an increase in the external $[K^+]/\sqrt{[Ca^{2+}]}$ ratio, therefore, to a decrease in the amount of membrane bound Ca. The permeability increase is rather independent of the membrane potential (Naitoh, 1968), although Eckert (1972) proposed that an increase in Ca conductance is caused by membrane depolarization (Naitoh and Eckert, 1974). To find out causes for this discrepancy, relationships between ciliary reversal, changes in $[K^+]/\sqrt{[Ca^{2+}]}$ ratio and membrane potential changes due to changes in ionic strength should be carefully examined. In response to an appropriate ionic stimulation, cilia reverse their beat direction for about 1 min, then they gradually resume normal beat direction. This suggests that an increase in Ca permeability (activation of Ca channels) by ionic stimulation is followed by its decrease (inactivation of Ca channels). However, there have been little electrophysiological evidences to

show the increase in Ca permeability (Kung and Saimi, 1982).

Primary objective of the present studies is to reveal electrophysiological characteristics of the membrane corresponding to activation and inactivation of Ca channels in Paramecium seen during its ionic stimulation. Part I will deal with changes in the membrane electrical properties and concomitant ciliary responses when the external ionic concentration was quickly changed. Part II will deal with relationships between ionic stimulation and electric current stimulation, time courses of activation and inactivation of Ca channels and dependence of the inactivation on the internal Ca^{2+} ions. Part III will deal with detailed descriptions of characteristics of inactivation of Ca channels during ionic stimulation. Part IV will deal with effects of ruthenium red (R.R.) on generation of Ca action potential and ciliary reversal response by ionic stimulation. R.R. is thought to bind to negative charges of sialic acid occupying the terminal region of polysaccharides on the cell surface. Negative charges of mucopolysaccharide layer of surface coat of the membrane were found to be closely correlated with the ion-exchanger property of Paramecium membrane. Part V will deal with theoretical considerations about electrogenesis in Paramecium membrane. Transmembrane diffusion potential was found to be a function of the external $[K^+]/\sqrt{[Ca^{2+}]}$ ratio, and the potential decreased with an increase in $[K^+]/\sqrt{[Ca^{2+}]}$ ratio.

Part I

Activation of Ca Channels by Ionic Stimulation in *Paramecium caudatum*

INTRODUCTION

It is well known that specimens of the ciliate protozoan Paramecium caudatum show backward swimming due to reversed beating of cilia (ciliary reversal), when they are transferred into a solution with high K^+ concentration (Jennings, 1906; Kamada and Kinosita, 1940; Naitoh, 1968). Ciliary reversal occurs when depolarization-sensitive Ca channels in the ciliary membrane are activated and the intraciliary Ca^{2+} concentration is raised thereby (Eckert, 1972; Eckert and Naitoh, 1972; Naitoh and Kaneko, 1972; Eckert, Naitoh and Machemer, 1976; Naitoh, 1979). An increase in the external K^+ concentration always depolarizes the membrane of Paramecium (Kamada, 1934; Yamaguchi, 1960; Naitoh and Eckert, 1968). It has been, therefore, believed by many authors that the K^+-induced ciliary reversal is mediated by the K^+-induced membrane depolarization. However, there is no electrophysiological evidence to show the activation of Ca channels during the long-lasting ciliary reversal (Kung and Saimi, 1982).

On the other hand, an increase in the external Ca^{2+} concentration produced a membrane depolarization in Paramecium, whereas this depolarization did not produce ciliary reversal (Yamaguchi, 1960; Kinosita, Dryl and Naitoh, 1964; Naitoh and Eckert, 1968). Moreover, Naitoh (1968) found that ciliary reversal occurred in association with a decrease in the ionic strength, if the ratio of K^+ concentration to the square root of Ca^{2+} concentration (Ja ratio; Naitoh and Yasumasu, 1967: see also Jahn, 1962) was raised concomitantly with the ionic strength decrease. A decrease in the ionic strength always produces a

membrane hyperpolarization in Paramecium (Kamada, 1934; Naitoh and Eckert, 1968). These results read a paradoxical conclusion that activation of the depolarization-sensitive Ca channels by ionic stimulation (raise in the Ja ratio) is independent of the membrane potential (Naitoh and Eckert, 1974).

In order to solve the paradox, I examined changes in the electrophysiological characteristics of the membrane during ciliary reversal caused by an increase in the Ja ratio. Present results clearly demonstrate that the Ca channels are activated by an increase in the external Ja ratio independently of membrane depolarization. Involvement of the surface potential in the measured membrane potential in Paramecium was suggested by Eckert and Brehm (1979) and Hook and Hildebrand (1980). Theoretical analysis of surface potential and transmembrane diffusion potential described in part V suggests that the membrane depolarization seen upon increasing external ionic strength does not coincide with a decrease in the diffusion potential. It is concluded that an increase in the Ja ratio, which produces an increase in the amount of membrane-bound K in exchange with membrane-bound Ca, causes a decrease in the diffusion potential (true depolarization of the membrane), thereby activates the voltage sensitive Ca channels. Preliminary communication has been published in abstract form (Onimaru and Naitoh, 1982).

MATERIALS and METHODS

Specimens of Paramecium caudatum (strain kyk201, wild type and 16A601, CNR mutant) reared in bacterized wheat straw infusion, were washed well with a standard saline solution (4 mM KCl, 1 mM $CaCl_2$ and 1 mM Tris-HCl buffer; pH 7.2), and equilibrated in the solution for more than 30 min prior experimentation.

As shown in Fig. I-1, two glass capillary microelectrodes (less than 0.5 μm in tip diameter filled with 1 M KCl; about 10^8 ohms), one for recording of the membrane potential (e1) and the other for current injection (e3), were inserted into a specimen (P). Tip of another microcapillary electrode of similar type (e2) was placed closely to the cell surface. External solution was virtually grounded through a thicker glass capillary electrode (e4; 0.5 mm inner diameter, filled with 3% agar-3 M KCl; $5x10^3$ ohms), which was placed 20 mm apart from e1 and connected in series with a current-voltage converter (Naitoh and Eckert, 1972).

The standard saline solution in the experimental vessel was first replaced with a given equilibration solution. An inserted specimen was immersed in the equilibration solution for more than 10 min. To stimulate the inserted specimen chemically, a test solution in a thicker glass capillary pipette (0.5 mm in inner diameter; T) was gently squirted (running rate; 5 mm/s) against the specimen and kept running throughout. The opening of the capillary was placed less than 0.5 mm apart from the specimen. When the test solution reached the surface of the specimen, a potential change, which was due to mainly tip potential of e2 but

included a small liquid junction potential (less than several mV) between the test solution and the equilibration solution, appeared between e2 and e4. This was served for monitoring approximate time of onset of the chemical stimulation (CH2).

To examine electrical property of the membrane, an electric current pulse (200 ms) was injected into the specimen every 4 seconds. Potential responses to the currents (potential difference between e1 and e2) were amplified, displayed on the CRT and photographed (CH1 - CH2). The electrical examination of the specimens was made through three minutes after the onset of the chemical stimulation. A potential difference between e1 and e4 was also recorded by a pen-recorder to monitor long-term effects of chemicals on the membrane potential (CH1).

Behavioral responses to ionic stimulation was examined according to methods described in Naitoh (1968) (Fig. I-2). A specimen equilibrated in the medium (equilibration medium) was pipetted into a large vessel of stimulation medium, and the duration of its reversed swimming (duration of ciliary reversal, Fig. I-2-2) was measured with a stopwatch. This measurement was repeated 10 times with 10 different specimens under each condition, then calculated a mean and its standard error.

PH of all the experimental solutions was adjusted to 7.2 by 1 mM Tris-HCl buffer. All the experiments were done under room temperature ranging from 20 to 22 ^{o}C.

RESULTS

A. Membrane potential response and ciliary reversal to an increase in the external ionic strength.

In the first series of experiments the specimens were equilibrated in a mixture of 1 mM KCl and 1 mM $CaCl_2$ (in final concentration; hereafter the solution will be described as 1 K + 1 Ca), then subjected to a mixture of 20 mM KCl and 1 mM $CaCl_2$ (20 K + 1 Ca). The Ja ratio, therefore, was increased from 1 to 20 upon solution exchange. In the second series of experiments the solution was changed from 1 K + 1 Ca to 4 K + 16 Ca, so that the Ja ratio was kept unchanged at 1. In the third series it was changed from 4 K + 1 Ca to 0 K + 20 Ca, so that the Ja ratio was decreased from 4 to 0.

As shown in Fig. I-3, the membrane was depolarized as soon as the external ionic concentration was raised, and kept depolarized in the solution with higher ionic concentration. Degree of depolarization, measured 2 minutes after solution exchange, was 32 $\pm$ 1.4 mV (mean $\pm$ S.E., n=15) in the first series, 33 $\pm$ 0.8 mV (n=13) in the second series and 26 $\pm$ 0.9 mV (n=13) in the third series of experiments. Long-lasting (scores of seconds) ciliary reversal took place only when the Ja ratio was raised upon increasing the ionic strength (the first series; Fig. I-3A). The long-lasting depolarization was always preceded by a spike-like potential response (small arrows in Fig. I-3). The spike was the most conspicuous in the first series and the least conspicuous in the third series. A transient (fraction of a second) ciliary reversal was always accompanied by the spike.

B. Effects of an increase in the external Ja ratio.

In this series of experiments the external Ja ratio was increased from 4 to 20 upon changing solution. The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca, so that an increase in the ionic strength was accompanied in the first series, from 10 K + 6.25 Ca to 20 K + 1 Ca, so that the ionic strength was kept more or less unchanged in the second series and from 12 K + 9 Ca to 10 K + 0.25 Ca, so that a decrease in the ionic strength was accompanied in the third series.

Membrane potential and ciliary responses

A typical result in each series is shown in Fig. I-4. In the first series (A) the membrane suddenly depolarized as the external ionic strength was raised. Then the degree of depolarization increased rather slowly to its maximum, then decreased slowly to reach its steady value (26 $\pm$ 0.6 mV; n=14) in about one minute. In the second series (B) the membrane depolarized slowly to its maximum value after changing the solution. Then the degree of the depolarization decreased slowly to its steady value (2.7 $\pm$ 1.0 mV; n=7) in about one minute. In the third series (C) the membrane suddenly hyperpolarized as the external ionic strength was lowered. Then the degree of the hyperpolarization decreased slowly to its minimum, then increased slowly to its steady value (-21 $\pm$ 0.9 mV; n=14) in about one minute. The resting potentials were -24 mV in 4 K + 1 Ca and -16 mV in 10 K + 0.25 Ca.

A long-lasting ciliary reversal always took place in association with an increase in the Ja ratio. Duration of the ciliary reversal was 42.9 $\pm$ 1.0 sec (n=13) in the first series, 44.5 $\pm$

1.0 sec (n=13) in the second series and 32.7 ± 2.4 sec (n=13) in the third series of experiments. Each duration (shaded bars in Fig. I-4) seems to approximate the duration of the initial slow membrane potential shift (1-5 mV) towards depolarizing direction, which was superimposed on the membrane potential change seen after changing the solution (a portion of each potential record above half dotted line in Fig. I-4). A spike response was observed when the membrane was depolarized by an increase in the ionic strength as mentioned previously. The spike, however, was not recorded in Fig. I-4A because of larger time constant of the pen-recorder employed.

Change in the membrane electric response to an outward current pulse

In each series the Ja ratio increased from 4 to 20 upon solution exchange. Membrane potential response to an outward electric current pulse (0.6-0.8 nA; 200 ms) injected every four seconds were recorded before and after changing the external solution. When an outward current pulse was injected into a specimen in 4 K + 1 Ca (Ja=4), the membrane produced a Ca action potential which was followed by a more or less passive steady IR depolarization. The steady depolarization decreased quasi-exponentially after the current was turned off (Fig. I-5A1). Upon changing the external solution to 20 K + 1 Ca (Ja=20) the amplitudes of the action potential and of the steady depolarization quickly decreased to their respective lowest levels (Fig. I-5A2), then gradually increased to reach their respective steady levels in a minute or two (Fig. I-5A3, 4). Detailed time courses

of the changes are shown in Fig. I-5A' together with the time change in the maximum rate of rise of the action potential. Time changes in the membrane potential response to an outward current in two other series were essentially similar to those in the first series (Fig. I-5B, B'; from 10 K + 6.25 Ca (Ja=4) to 20 K + 1 Ca (Ja=20): C, C'; from 12 K + 9 Ca (Ja=4) to 10 K + 0.25 Ca (Ja=20)). Duration of the long-lasting ciliary reversal (indicated by shaded bars in Fig. I-5A', B' and C') approximated the period from the beginning of the solution exchange to the time when the membrane potential response became steady in each new solution.

Change in the membrane electric response to an inward current pulse

Membrane potential response to an inward electric current pulse (0.8-1.0 nA; 200 ms) injected every four seconds were recorded before and after changing solution. As shown in Fig. I-6A1, when an inward current pulse was injected into a specimen in 4 K + 1 Ca, the membrane was hyperpolarized to a peak level, then degree of the hyperpolarization decreased to reach its steady value. The hyperpolarization decreased quasiexponentially to 0 after the inward current was turned off. The hyperpolarizing response changed immediately after changing the external solution to 20 K + 1 Ca, i.e. the degree of membrane hyperpolarization came to continue to increase during the inward current pulse, and the membrane produced a Ca action potential upon turning off the inward current pulse (Fig. I-6A2). This anode-break Ca action potential gradually became inconspicuous with time after the solution exchange, and unobserved in about one minute (Fig. I-

6A3, 4). Detailed time course of the change in the response to an inward current pulse associated with increasing the Ja ratio is shown in Fig. I-6A'. Time changes in the hyperpolarizing response essentially similar to those in the first series were observed in the second (Fig. I-6B, B') and the third series (Fig. I-6C, C') of the experiments. The period of time when the anode break action potential had been seen after raising the Ja ratio approximated the duration of the long-lasting ciliary reversal (shaded bars in Fig. I-6). The long-lasting ciliary reversal was intermittently (every 4 sec) inhibited during each inward current pulse. However, the overall duration of the ciliary reversal and time change in the membrane potential response were scarcely affected by the current pulse themselves.

Changes in the membrane electrical properties in response to an increase in the external Ja ratio in CNR mutants

CNR mutant has malfunction in its voltage-sensitive Ca channels (Takahashi and Naitoh, 1978). When a specimen of CNR mutant was subjected to an increase in the external Ja ratio together with an increase in the ionic strength (from 4 K + 1 Ca to 20 K + 1 Ca), it showed no ciliary reversal. The time course of the membrane potential change was essentially identical with that shown by a wild type specimen (Fig. I-7A, see also Fig. I-8A). However, it should be noted that the initial transient slow depolarizing shift such that seen upon raising the Ja ratio (Fig. I-4) was not observed in CNR. Amplitude of passive I-R responses followed by a delayed increase in K^+ conductance to outward current pulses (0.8 nA; 200 ms) suddenly decreased to a certain

level immediately after an increase in the external Ja (Fig. I-7B, B'). Amplitude of passive I-R responses to inward current pulses (1 nA; 200 ms) also decreased to a certain level after an increase in the external Ja (Fig. I-7C,C'). The anode break response was not observed.

C. A change in the ionic strength at constant Ja.

In the first series of experiments, the external solution was changed from 4 K + 1 Ca to 10 K + 6.25 Ca, and in the second series from 10 K + 6.25 Ca to 4 K + 1 Ca. The Ja ratio, therefore, was kept unchanged at 4 upon solution exchange.

Membrane potential and ciliary responses

A typical result obtained from each series of experiments is shown in Fig. I-8. The membrane depolarized when the ionic strength was raised (A), while hyperpolarized when the ionic strength was lowered (B) as previously described. The long-lasting ciliary reversal never took place in either cases. It should be noted that the initial transient slow depolarizing shift such that seen upon raising the Ja ratio (Fig. I-4) was not observed.

Change in the membrane electric response to an outward current pulse

As shown in Fig. I-9, the membrane electric response to an outward current pulse (0.8 nA; 200 ms) was not affected by the solution exchange, while the membrane potential level shifted much upon solution exchange. Transient drops of the amplitude and of the maximum rate of rise of the action potential seen immediately after changing the solution (Fig. I-9A' and B') seem

to be caused by mechanical agitation of the specimen due to the solution exchange.

Change in the membrane electric response to an inward current pulse

As shown in Fig. I-10, the membrane potential response to an inward current pulse (0.8 nA; 200 ms) did not change much by the solution exchange. Neither the anode break response nor the long-lasting ciliary reversal were observed.

D. A decrease in the external Ja ratio.

In this series of experiments the external Ja ratio was decreased from 20 to 4 upon solution exchange. The external solution was changed from 10 K + 0.25 Ca to 12 K + 9 Ca, so that an increase in the ionic strength was accompanied by the decrease in Ja in the first series, from 20 K + 1 Ca to 10 K + 6.25 Ca, so that the ionic strength was kept more or less unchanged in the second series and from 20 K + 1 Ca to 4 K + 1 Ca, so that a decrease in the ionic strength was accompanied by the decrease in Ja in the third series.

Membrane potential and ciliary responses

A typical result in each series is shown in Fig. I-11. In the first series (A) the membrane suddenly depolarized as the external ionic strength was raised. Then the degree of depolarization increased slowly to reach its steady value within one minute. In the second series (B) the membrane potential was almost unchanged, while fluctuation in the membrane potential level tended to increase. In the third series (C) the membrane

suddenly hyperpolarized as the external ionic strength was lowered. Then the degree of the hyperpolarization decreased slowly to its steady value within one minute.

The long-lasting ciliary reversal never took place in all cases. Contraction of the cell body was observed for several seconds after a decrease in Ja.

Change in the membrane electric response to an outward current pulse

Membrane potential response to an outward electric current pulse (0.6-0.8 nA; 200 ms) injected every four seconds were recorded before and after changing the external solution. In the first series, Ca action potential was extremely suppressed upon changing the external solution, then gradually recovered (Fig. I-12A1,2,3). Detailed time courses of the changes are shown in Fig. I-12A' together with the time change in the maximum rate of rise of action potential (M.R.R.) and in the steady depolarization. The time course of change in the M.R.R. clearly indicates that Ca action potential was almost perfectly suppressed immediately after changing the external solution. The steady depolarization gradually increased to a certain steady level. Time changes in the membrane potential responses to outward current pulses in two other series were essentially similar to those in the first series (Fig. I-12B, B'; from 20 K + 1 Ca to 10 K + 6.25 Ca: C, C'; from 20 K + 1 Ca to 4 K + 1 Ca).

Change in the membrane electric response to an inward current pulse

Membrane potential response to an inward electric current pulse (0.5-0.8 nA; 200 ms) injected every four seconds were

recorded before and after changing solution. Upon solution exchange, inward going rectification became conspicuous in the first series (Fig. I-13A4). The peak value and the steady state voltage of the electric response decreased to their lowest levels, then gradually increased to reach their respective steady levels in a minute or two. Detailed time course of the change in the response to an inward current pulse associated with decreasing the Ja is shown in Fig. I-13A'. Time changes in the hyperpolarizing response essentially similar to those in the first series were observed in the second (Fig. I-13B, B') and third series (Fig. I-13C, C') of the experiments.

DISCUSSION

Increase in the external ionic strength and activation of Ca channels

An increase in the external ionic strength always produced a sustained membrane depolarization in Paramecium. The depolarization was preceded by a Ca action potential (Fig. I-3) which is always associated with a transient ciliary reversal similarly to the case of outward current stimulation (Naitoh and Eckert, 1968; Eckert, 1972; Eckert and Naitoh, 1972). The Ca action potential is thought to be produced by a local outward current generated by a localized depolarization of the membrane due to unequal application of the test solution to the specimen. The transient ciliary reversal was followed by a long-lasting ciliary reversal only when the Ja ratio was increased in association with an increase in the ionic strength (Fig. I-3A). The long-lasting ciliary reversal also took place, even when the membrane was kept hyperpolarized by decreasing the external ionic strength, if the Ja ratio was increased in association with the decrease in the ionic strength (Fig. I-4C). These results are consistent with those obtained by earlier workers (Naitoh, 1968; Naitoh and Eckert, 1968), suggesting that the long-lasting ciliary reversal is closely correlated with an increase in the Ja ratio and not with the membrane depolarization.

Increase in Ja ratio and activation of Ca channels

It should be noted that an initial, small (1-5 mV), slow depolarization was always superimposed upon the sustained membrane potential change produced by a change in the ionic strength, when the long-lasting ciliary reversal took place (Fig.

I-4; portion of each potential trace above the half dotted line). The initial slow depolarization was not observed when the long-lasting ciliary reversal did not take place (Fig. I-7A and Fig. I-8). These facts, together with the previous findings that ciliary reversal of Paramecium is produced by an increase in the intraciliary Ca^{2+} concentration (Naitoh and Kaneko, 1972; 1973) suggest that an increase in the Ja ratio produces a long-lasting activation of the Ca channels.

Amplitude of the action potential and the following steady shift in the membrane potential level in response to an outward current pulse were smaller during the long-lasting ciliary reversal than after cessation of the reversal (Fig. I-5). On the contrary, they had been kept unchanged, when the ciliary reversal did not take place in such cases as constant Ja series (Fig. I-9) and CNR series (Fig. I-7B). These results indicate that the membrane resistance decreases in association with the long-lasting ciliary reversal. The decrease in the membrane resistance is consistent with the long-lasting activation of the Ca channels. Ca-mediated activation of K channels (Satow and Kung, 1980) might also be partially responsible for the decrease in the membrane resistance.

The Ca action potential took place upon turning off the inward current pulse during the long-lasting ciliary reversal. The peak level and the rate of rise of the anode break Ca action potential decreased with time, and the action potential became inconspicuous after the ciliary reversal ceased (Fig. I-6). The ciliary reversal was inhibited during passage of the inward

current pulse. This suggests that the activated Ca channels are quickly closed (deactivated) by the inward current (or by a resultant hyperpolarization). Ca channels open again upon turning off the inward current. This causes a regenerative activation of the Ca channels. Decrease in the peak and the rate of rise of the anode break Ca action potential with time implies gradual inactivation of the Ca channels which is not removed by the inward current pulse. Termination of ciliary reversal concomitantly with disappearance of the anode break responses (Fig. I-6A',B',C') suggests that this slow inactivation of the Ca channels is a key factor for determination of duration of ciliary reversal. The inward current pulse presently employed was not long enough to remove the inactivation. Application of an inward current for several seconds was needed for removal of the inactivation (see part III). These results, therefore, support the idea that an increase in the Ja ratio produces activation of the Ca channels, which are slowly inactivated with time (in minutes). The inactivation of Ca current during sustained membrane depolarization by current injection similar in its time course to the present inactivation was reported by Hennessey and Kung (1985) (discussed in part III). Hildebrand (1978) had reported an inactivation of ciliary reversal developing very slowly (in several 10 min) in a high-K^+ solution.

Mechanism of Ca channel activation by Ja ratio increase

As already noted, present results clearly demonstrate that the activation of the voltage-sensitive Ca channels was closely correlated with an increase in the Ja ratio independently of the membrane depolarization. Eckert and Brehm (1979) suggested

involvement of the surface potential on the membrane in the potential difference recorded through an inserted microelectrode in fresh water protozoans such as Paramecium and Tetrahymena (see also Satow and Kung, 1979; 1981). Based on the Gouy-Chapman's theory on the electric double layer, Hook and Hildebrand (1980) suggested that the transmembrane diffusion potential was kept almost constant irrespective of the external ionic concentration, if the Ja ratio was kept constant. In part V, I analyzed the relation of the Ja ratio in the external solution to both the transmembrane diffusion potential and the surface potential (Donnan potential) in Paramecium based on the theories on the membrane potential of charged membrane proposed by Teorell (1935) and Meyer and Sievers (1936). The formulation clearly showed that the diffusion potential was dependent of the Ja ratio. An increase in the Ja ratio produces a membrane depolarization, while its decrease brings about a membrane hyperpolarization. Therefore, it can be said that the activation of Ca channels by an ionic stimulation is caused by a membrane depolarization due to an increase in the Ja ratio as conventional voltage-sensitive ion channels in nerves and muscles. Apparent independency of the activation from the membrane depolarization shown in the present paper is attributable to the presence of a surface potential at the outer surface of the membrane of Paramecium.

An increase in the Ja ratio produces release of Ca^{2+} bound by the membrane in exchange with K^+ ions (Naitoh and Yasumasu, 1967). The membrane depolarization, therefore, can be said to occur in association with liberation of Ca^{2+} from the membrane

anionic sites. The idea that the liberation of Ca^{2+} directly activates the Ca channels (Naitoh, 1968; Tasaki et al, 1968; Hildebrand and Dryl, 1976) is not contradictory to the present findings.

Decrease in Ja ratio and suppression of Ca-channel activation

When the external Ja ratio was reduced, the Ca action potential induced by injection of outward current pulse was extremely suppressed, then it was gradually recovered. This response was independent of the direction of the membrane potential change in association with change in the external ionic strength (Fig. I-12). Membrane is truly hyperpolarized upon a decrease in Ja ratio, independently of change in the observed membrane potential (part V). Therefore, the suppression of Ca action potential in response to the decrease in Ja ratio is partly explained from rise of the threshold by hyperpolarization of membrane. Gradual recovery of action potential generation is attributable to removal of Ca channel inactivation by hyperpolarization (see part III).

FIGURE LEGENDS

Figure I-1. Upper portion; schematic presentation of the experimental arrangement. e1; intracellular potential electrode. e2; external potential electrode. e3; current injection electrode. e4; potential reference and current drain electrode. cp; a current pump system. P; Paramecium. T; a glass capillary for chemical stimulation of Paramecium. Other symbols have conventional meanings. Middle portion; potential recordings by a pen recorder. CH 1; membrane potential responses to an ionic stimulation recorded through e1. The external solution was changed from 4 mM KCl + 1 mM $CaCl_2$ to 20 mM KCl + 1 mM $CaCl_2$. Hyperpolarizing spikes on the potential trace are electric responses to inward current injected every 4 sec. CH 2; potential change at the tip of e2 occurred in association with solution exchange. Lower portion; three examples of membrane potential responses (Vm) to an inward current injection. Each trace was recorded at a time indicating by an arrow with corresponding number on CH1 trace in the middle portion. $\dot{V}m$; first order time derivative of Vm.

Figure I-2. Schematic presentation of examination of behavioral responses of P. caudatum to ionic stimulation. Upper portion: A specimen in an equilibration medium (K1, Ca1) was transferred into a stimulation medium (K20, Ca1), then the duration of backward swimming due to ciliary reversal was measured with a stopwatch. Lower portion: Change in beating direction of cilia in response to ionic stimulation. 1; a non-stimulated forward

swimming specimen in the equilibration medium. 2; a backward swimming specimen due to ciliary reversal immediately after its transfer into the stimulation medium. 3; a specimen rotating about its posterior end due to ciliary beat around the peristome, just before cessation of its backward swimming. 4; a forward swimming specimen after due to recovery of normal ciliary beat. Arrows indicate swimming directions. Anterior end of the body is marked "a". (modified from Naitoh, 1968).

Figure I-3. Membrane potential response of P. caudatum (Vm) to an increase in the external ionic strength. The external solution was changed from 1 K + 1 Ca to 20 K + 1 Ca in A, from 1 K + 1 Ca to 4 K + 16 Ca in B, and from 4 K + 1 Ca to 20 Ca in C. St; change in the potential at the tip of the external microelectrode (e2 in Fig. 1) upon increasing the external ionic strength, showing approximate time course of the increase around specimen. Shaded bars under Vm traces show approximate duration of ciliary reversal. Broken lines; the reference level for the Vm. Small arrows indicate Ca action potentials.

Figure I-4. Membrane potential responses of P. caudatum (Vm) to a change in the Ja ratio from 4 to 20. The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca in A, from 10 K + 6.25 Ca to 20 K + 1 Ca in B and from 12 K + 9 Ca to 10 K + 0.25 Ca in C. Broken lines; membrane potential level before increasing the Ja ratio. Shaded bars under the Vm traces; approximate duration of the long-lasting ciliary reversal produced by an increase in the

Ja ratio. Half-dotted lines under the Vm traces; steady potential level after increasing the Ja ratio.

Figure I-5. Change in the membrane potential response of P. caudatum to an outward current pulse (200 ms; 0.6 nA in A, 0.8 nA in B and 0.7 nA in C) after increasing the Ja ratio from 4 to 20. The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca in A and A', from 10 K + 6.25 Ca to 20 K + 1 Ca in B and B' and from 12 K + 9 Ca to 10 K + 0.25 Ca in C and C'. A, B, C; the membrane potential responses (Vm) and their first order time derivatives ($\dot{V}m$). A', B', C'; time changes in the amplitude (open triangles) and the maximum rate of rise (solid triangles) of the Ca action potential, and in the steady IR shift of the membrane potential level measured at the end of the pulse (open circles) (see the inset in A'). Abscissae in A', B' and C' are time after the increase in the Ja ratio. Small arrows under the abscissae indicate the moments when the membrane potential responses shown in A, B and C were recorded. Number under each arrow corresponds to the number at the upper left portion of each potential trace in A, B and C. The potential responses with number 1 were recorded just before the Ja ratio was increased. Shaded bars in A', B' and C' show approximate duration of the long-lasting ciliary reversal. Vertical bars denote 20 mV, 2 V/s in A and C, and 20 mV, 5 V/s in B. Horizontal bars in A, B and C denote 100 ms.

Figure I-6. Change in the membrane potential response of P. caudatum to an inward current pulse (200 ms, 0.8 nA in A and

C, 1 nA in B) after increasing the Ja ratio from 4 to 20 as in the cases shown in Fig. I-5. Time-changes in the maximum hyperpolarization (open triangles), steady level of the hyperpolarization measured at the end of the pulse (open circles), the amplitude (open squares) and the maximum rate of rise (solid triangles) of the anode break Ca action potential are shown in A', B' and C' (see the inset in A'). Shaded bars in A', B' and C' show approximate duration of the long-lasting ciliary reversal. See the legend of Fig. I-5 for the detailed explanation of the figure. Vertical bars in A, B and C denote 20 mV and 2 V/s. Horizontal bars in A, B and C denote 100 ms.

Figure I-7. Membrane potential response of CNR mutant of P. caudatum to a change in the Ja ratio from 4 to 20. The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca. A; membrane potential change. B; changes in the membrane potential response to an outward current pulse (200 ms, 0.8 nA). B'; time changes in the maximum depolarization (open triangles) in the electrical response and the steady IR shift (open circles) at the end of the test pulse. C; changes in the membrane potential response to an inward current pulse (200 ms, 1 nA). C'; time changes in the maximum hyperpolarization (open triangles) and steady level of the IR shift at the end of the pulse (open circles). See the legends of Fig. I-5 for the detailed explanation of the figure. Vertical bars in B and C denote 20 mV, 2 V/sec. Horizontal bars in B and C denote 100 ms.

Figure I-8. Membrane potential response of P. caudatum (Vm) to

an increase (A) and to a decrease (B) in the external ionic strength. The Ja ratio was kept unchanged at 4 throughout. Broken lines; membrane potential level before changing the ionic strength.

Figure I-9. Change in the membrane potential response of P. caudatum to an outward current pulse (200 ms, 0.8 nA) after changing the external ionic strength. The Ja ratio had been kept constant at 4 throughout. The ionic strength was increased (from 4 K + 1 Ca to 10 K + 6.25 Ca) in A and A', and it was decreased in B and B' (from 10 K + 6.25 Ca to 4 K + 1 Ca). See the legend of Fig. I-5 for the detailed explanation of the figures. Vertical bars denote 20 mV, 5 V/s in A and 20 mV, 4 V/s in B. Horizontal bars in A and B denote 100 ms.

Figure I-10. Change in the membrane potential response of P. caudatum to an inward current pulse (200 ms, 0.8 nA) after increasing (A and A') and decreasing (B and B') the external ionic strength. The Ja ratio had been kept constant at 4 throughout as in the cases shown in Fig. I-9. See the legends of Figs. I-5 and I-6 for the detailed explanation of the figure. Vertical bars denote 20 mV, 2 V/s in A and 20 mV, 4 V/s in B. Horizontal bars in A and B denote 100 ms.

Figure I-11. Membrane potential responses of P. caudatum to a change in the Ja ratio from 20 to 4. The external solution was changed from 10 K + 0.25 Ca to 12 K + 9 Ca in A, from 20 K + 1 Ca

to 10 K + 6.25 Ca in B and from 20 K + 1 Ca to 4 K + 1 Ca in C. Broken lines; membrane potential level before decreasing the Ja ratio.

Figure I-12. Change in the membrane potential response of P. caudatum to an outward current pulse (200 ms; 0.8 nA in A, 0.6 nA in B and C) after decreasing the Ja ratio from 20 to 4. The external solution was changed from 10 K + 0.25 Ca to 12 K + 9 Ca in A and A', from 20 K + 1 Ca to 10 K + 6.25 Ca in B and B' and from 20 K + 1 Ca to 4 K + 1 Ca in C and C'. A, B, C; the membrane potential responses (Vm) and their first order time derivatives ($\dot{V}m$). A', B', C'; time changes in the amplitude (open triangles) and the maximum rate of rise (solid triangles) of the Ca action potential, and in the steady IR shift of the membrane potential level measured at the end of the pulse (open circles). See the legend of Fig. I-5 for the detailed explanation of the figure.

Figure I-13. Change in the membrane potential response of P. caudatum to an inward current pulse (200 ms, 0.8 nA in A, 0.6 nA in B and 0.5 nA in C) after decreasing the Ja ratio from 20 to 4. Time changes in the maximum hyperpolarization (open triangles), steady level of the hyperpolarization measured at the end of the pulse (open circles). See the legend of Fig. I-5 for the detailed explanation of the figure.

Fig. I-1

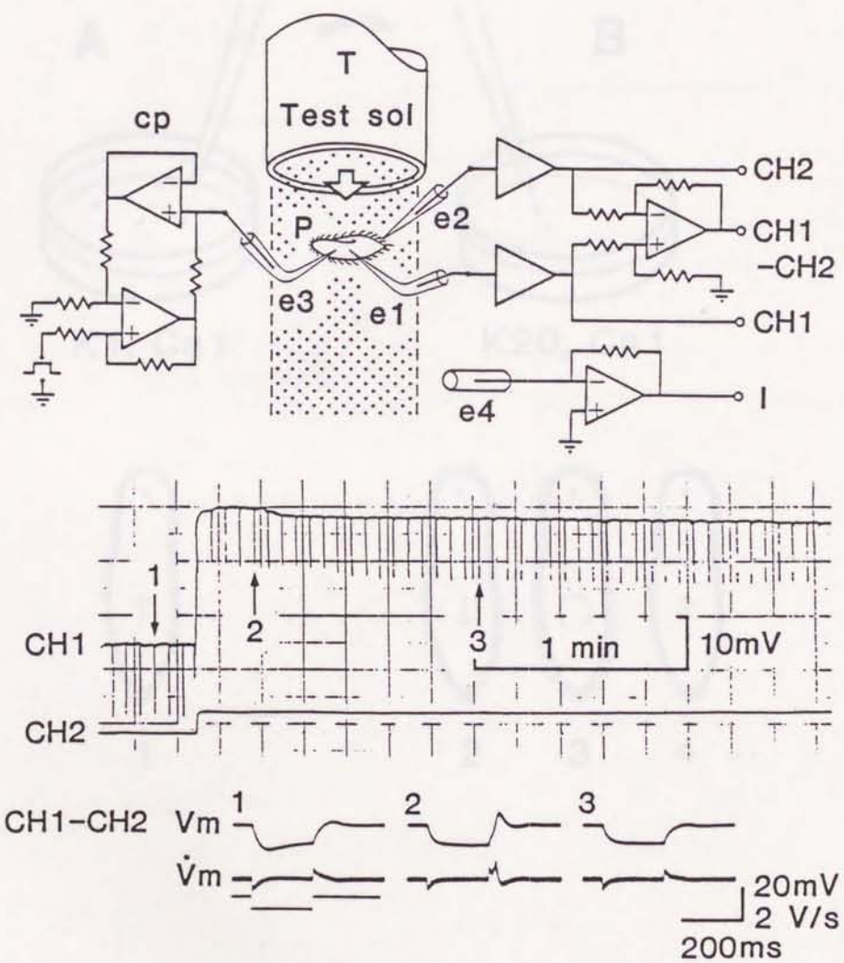

T
Test sol
cp
P
e2
e3
e1
e4
CH2
CH1
-CH2
CH1
I
1
2
3
1 min
10mV
CH1
CH2
CH1-CH2
Vm
1
2
3
V̇m
20mV
2 V/s
200ms

Fig. I–2

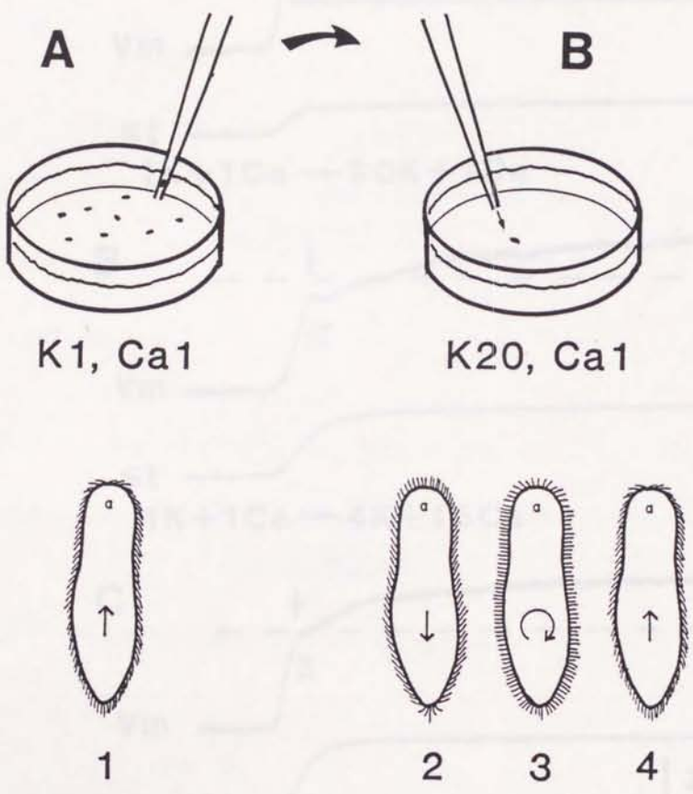


Fig. I-3

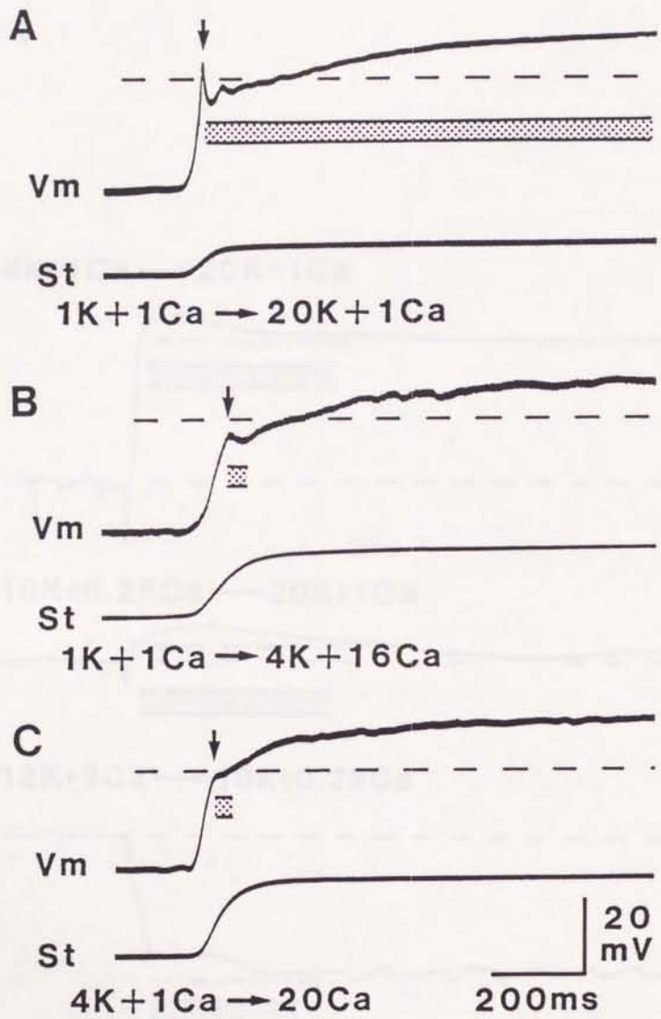

A
Vm
St
1K+1Ca → 20K+1Ca
B
Vm
St
1K+1Ca → 4K+16Ca
C
Vm
St
20
mV
4K+1Ca → 20Ca
200ms

Fig. I-4

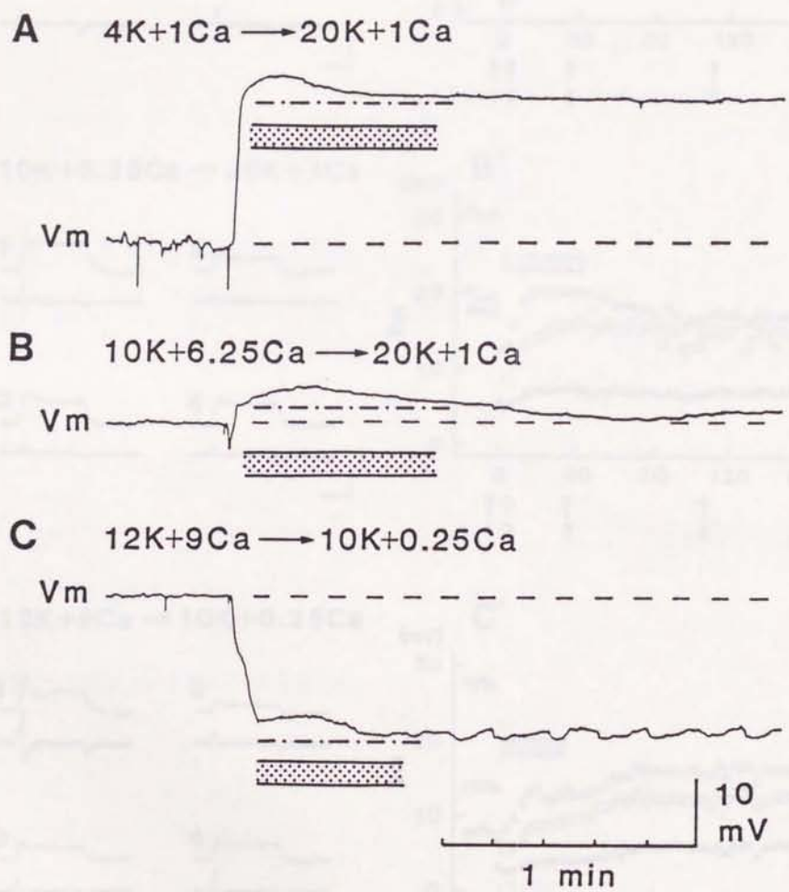

A 4K+1Ca ⟶ 20K+1Ca
Vm
B 10K+6.25Ca ⟶ 20K+1Ca
Vm
C 12K+9Ca ⟶ 10K+0.25Ca
Vm
10
mV
1 min

Fig. I-5

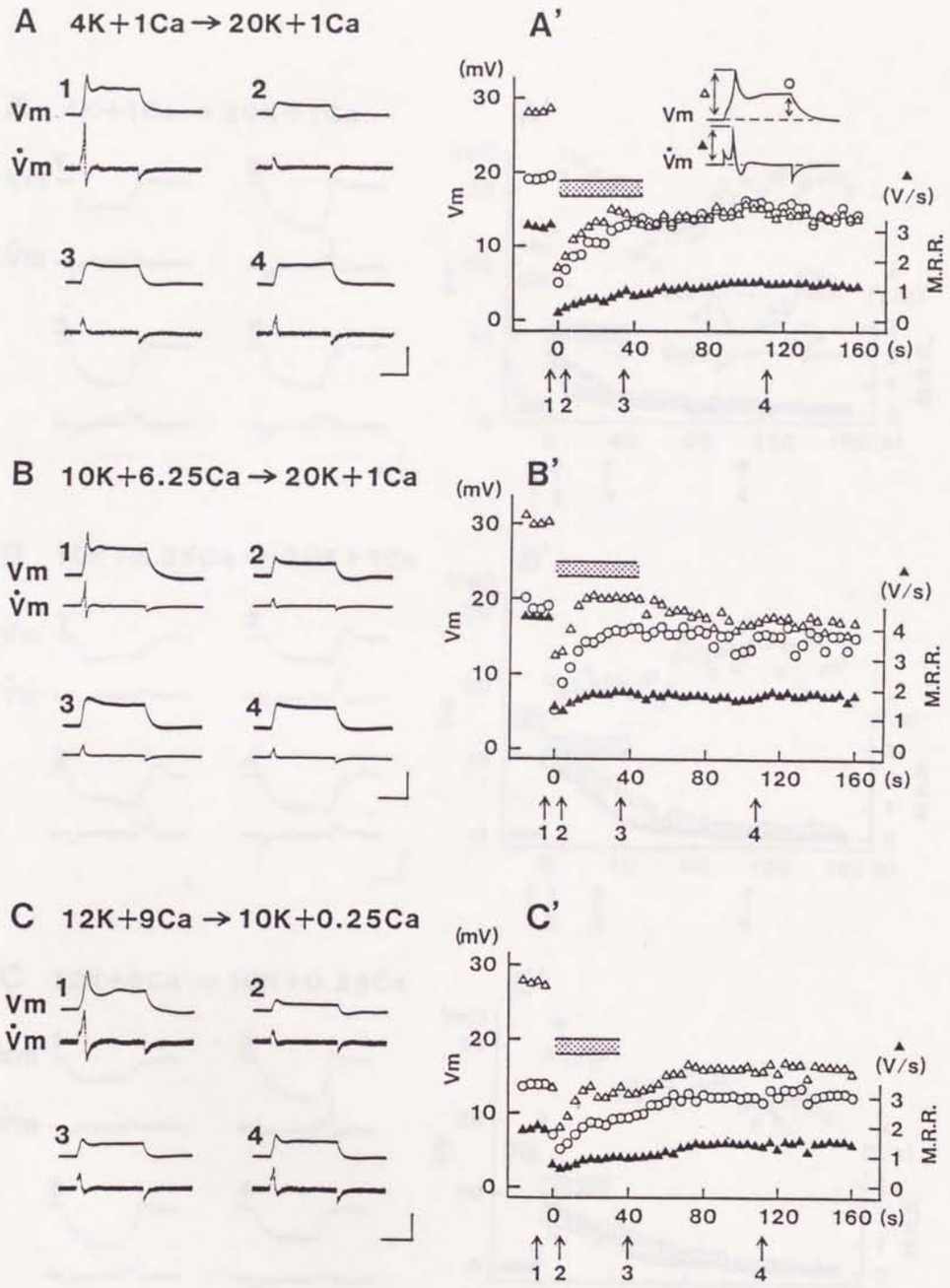

A 4K+1Ca → 20K+1Ca
A'
B 10K+6.25Ca → 20K+1Ca
B'
C 12K+9Ca → 10K+0.25Ca
C'
Vm
V̇m
(mV)
(V/s)
M.R.R.
0
40
80
120
160 (s)
1 2 3 4

Fig. I-6

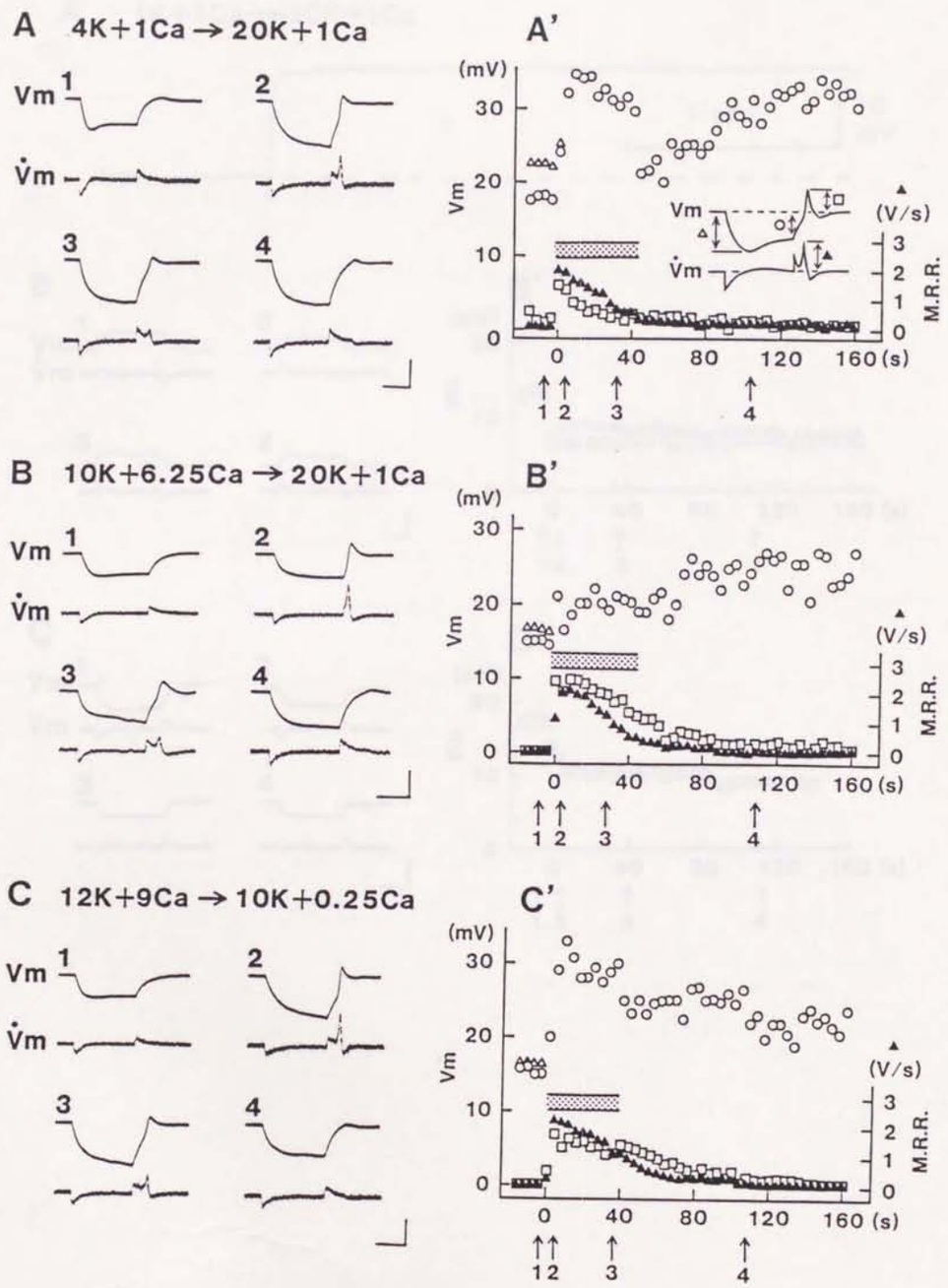

A 4K+1Ca → 20K+1Ca
A'
Vm
V̇m
1
2
3
4
(mV)
30
20
10
0
Vm
(V/s)
3
2
1
0
M.R.R.
0
40
80
120
160 (s)
1 2 3 4
B 10K+6.25Ca → 20K+1Ca
B'
C 12K+9Ca → 10K+0.25Ca
C'

Fig. I-7

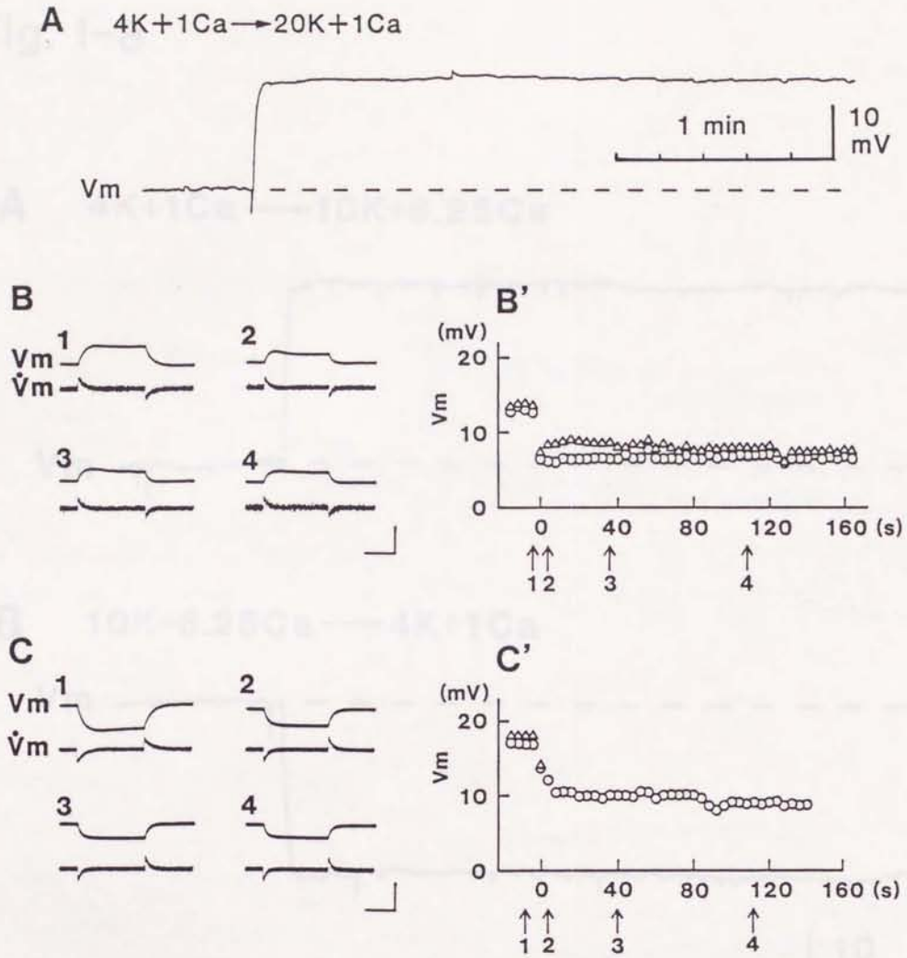

A
4K+1Ca→20K+1Ca
1 min
10
mV
Vm
B
B'
Vm
V̇m
1
2
3
4
(mV)
20
10
0
Vm
0
40
80
120
160 (s)
1 2
3
4
C
C'
Vm
V̇m
1
2
3
4
(mV)
20
10
0
Vm
0
40
80
120
160 (s)
1 2
3
4

Fig. I-8

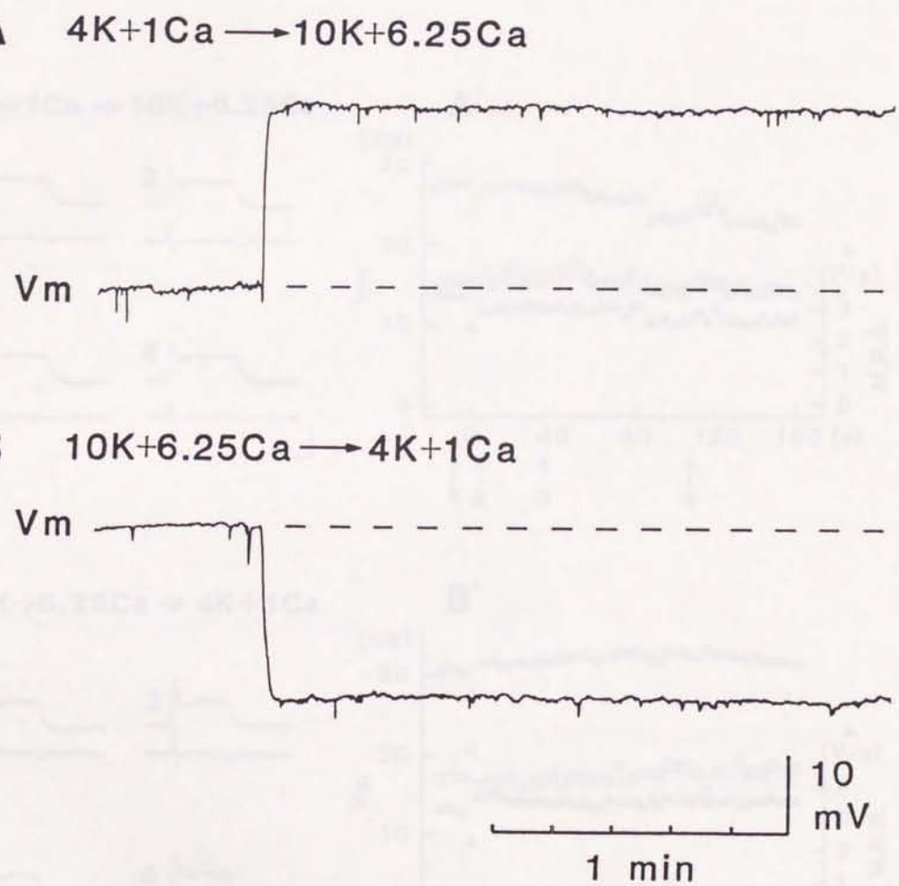


Fig. I-9

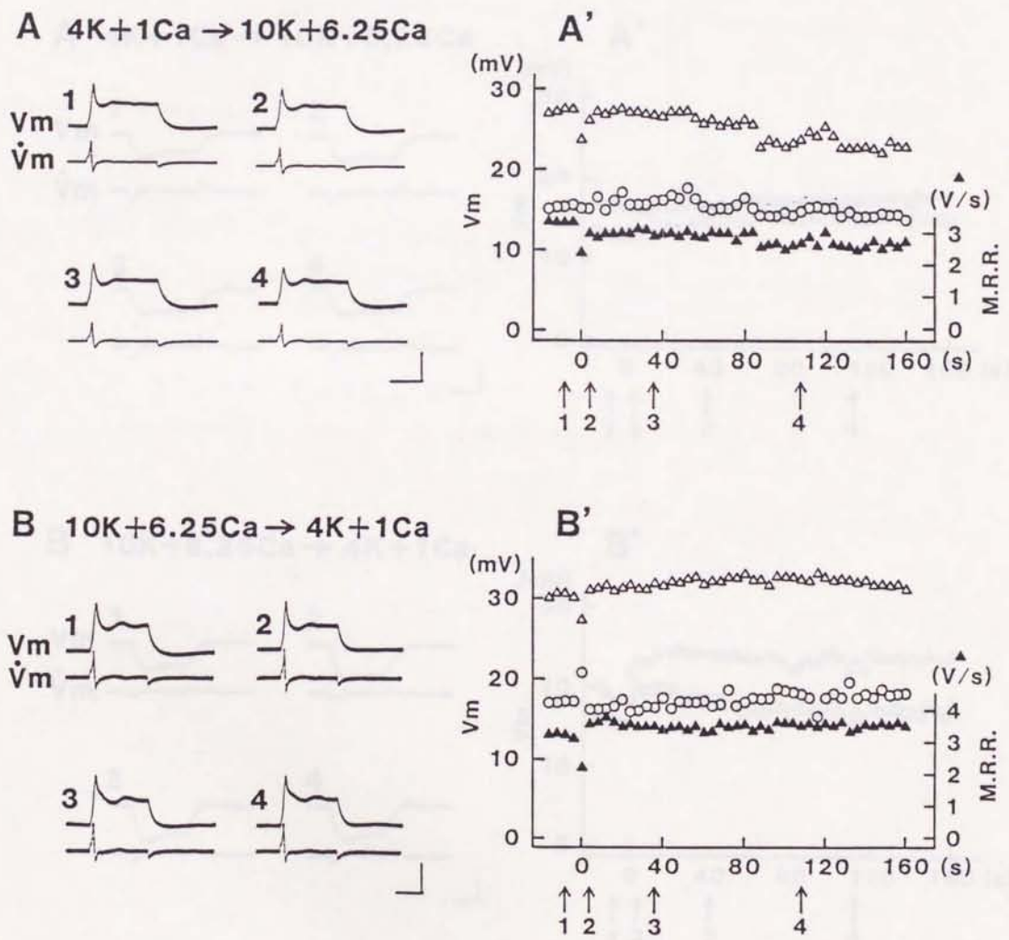

A 4K+1Ca → 10K+6.25Ca
A'
Vm
V̇m
1
2
3
4
(mV)
30
20
10
0
Vm
(V/s)
3
2
1
0
M.R.R.
0
40
80
120
160 (s)
1 2
3
4
B 10K+6.25Ca → 4K+1Ca
B'
Vm
V̇m
1
2
3
4
(mV)
30
20
10
0
Vm
(V/s)
4
3
2
1
0
M.R.R.
0
40
80
120
160 (s)
1 2
3
4

Fig. I-10

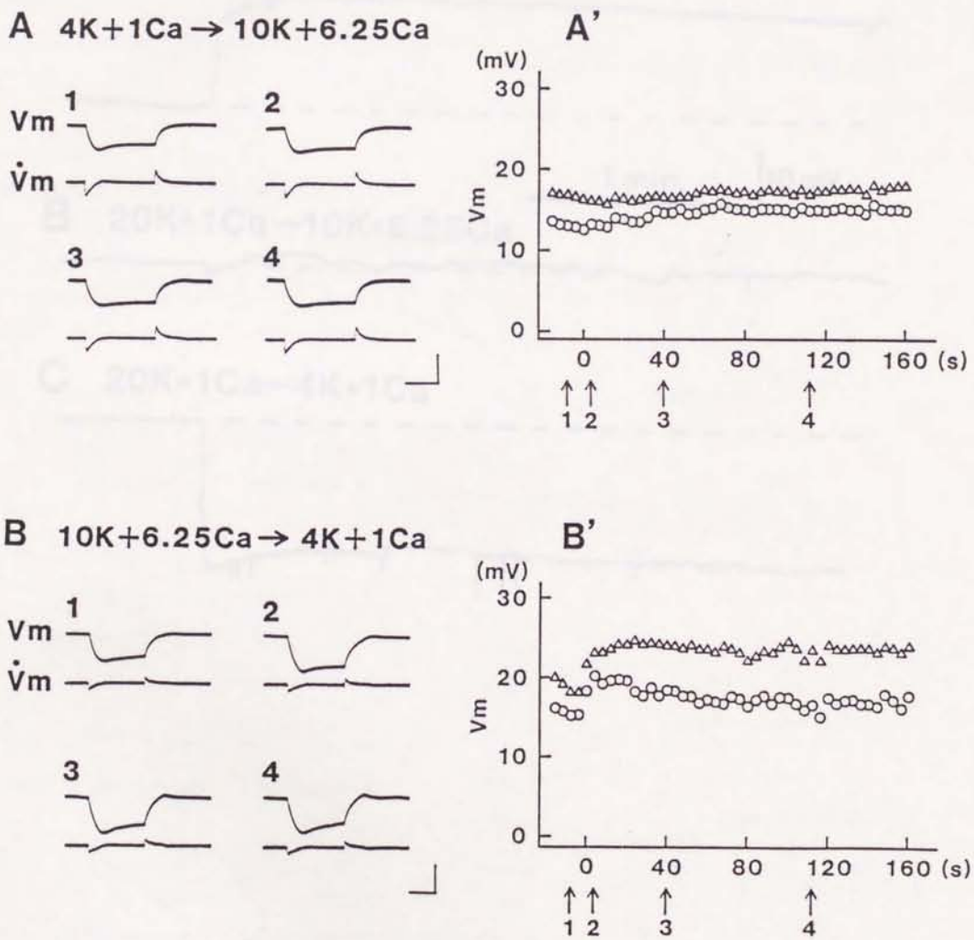

A 4K+1Ca → 10K+6.25Ca
A'
Vm
V̇m
1
2
3
4
(mV)
30
20
10
0
Vm
0
40
80
120
160 (s)
1 2
3
4
B 10K+6.25Ca → 4K+1Ca
B'
Vm
V̇m
1
2
3
4
(mV)
30
20
10
0
Vm
0
40
80
120
160 (s)
1 2
3
4

Fig. I-11

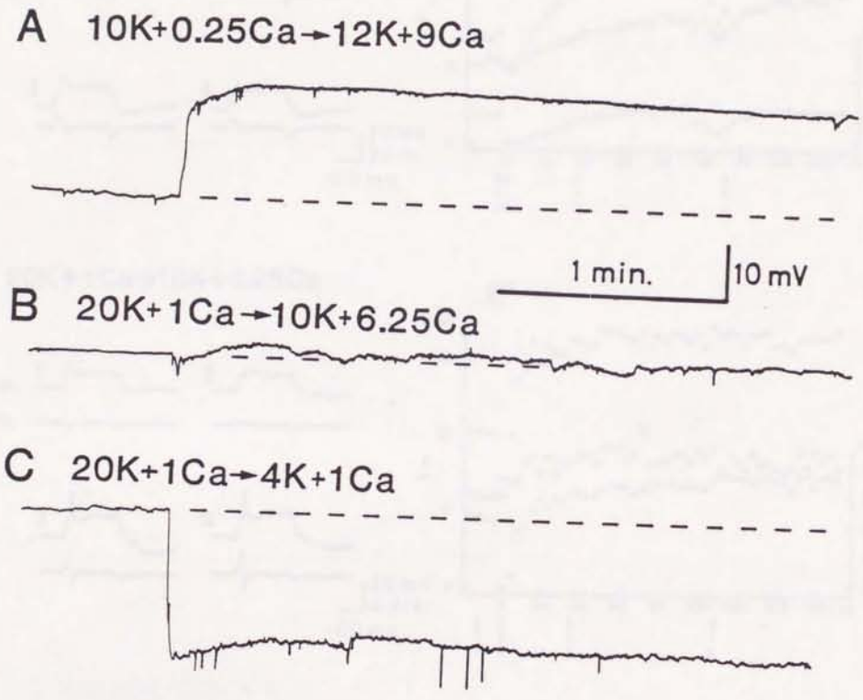

A 10K+0.25Ca→12K+9Ca
1 min.
10 mV
B 20K+1Ca→10K+6.25Ca
C 20K+1Ca→4K+1Ca

Fig. I-12

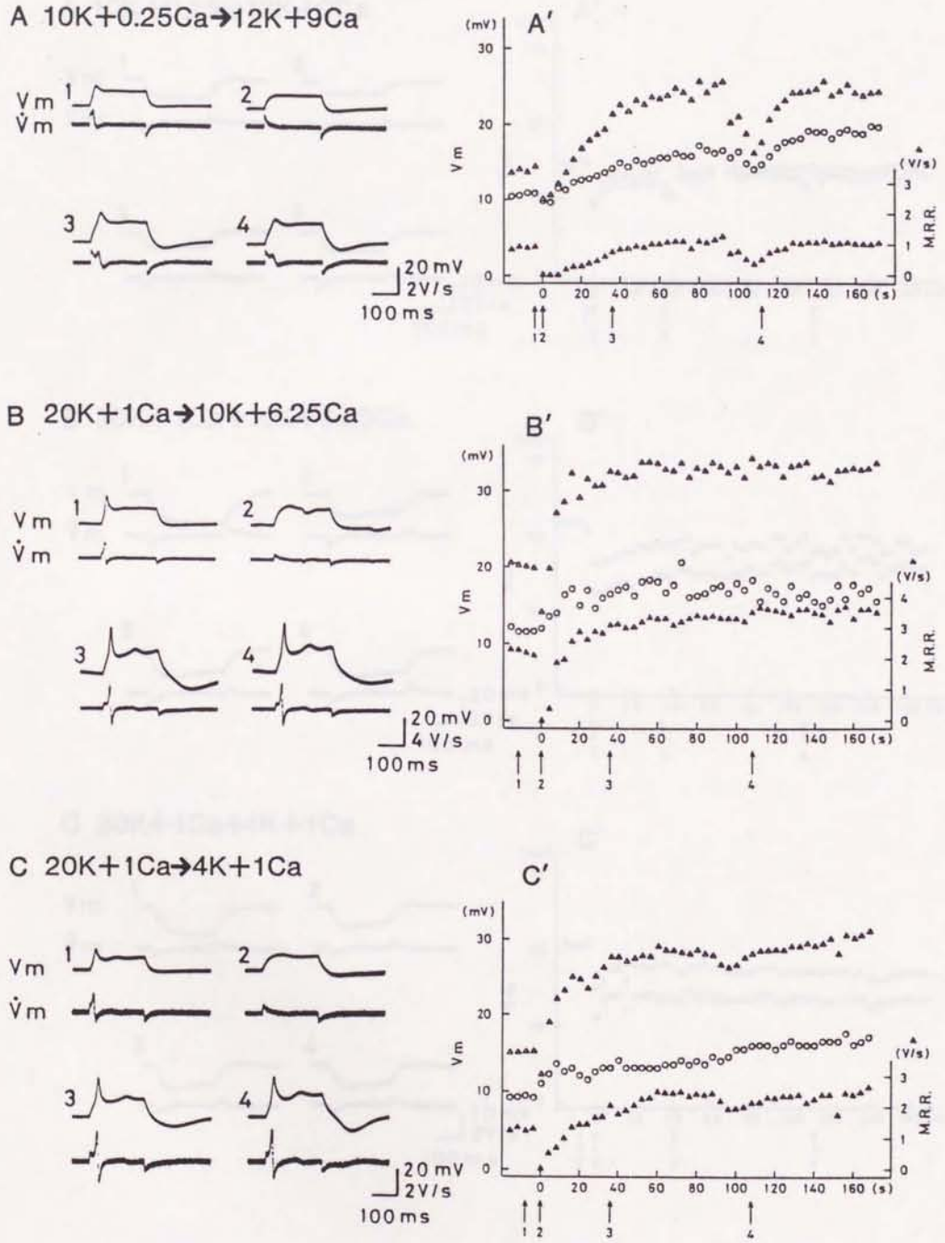
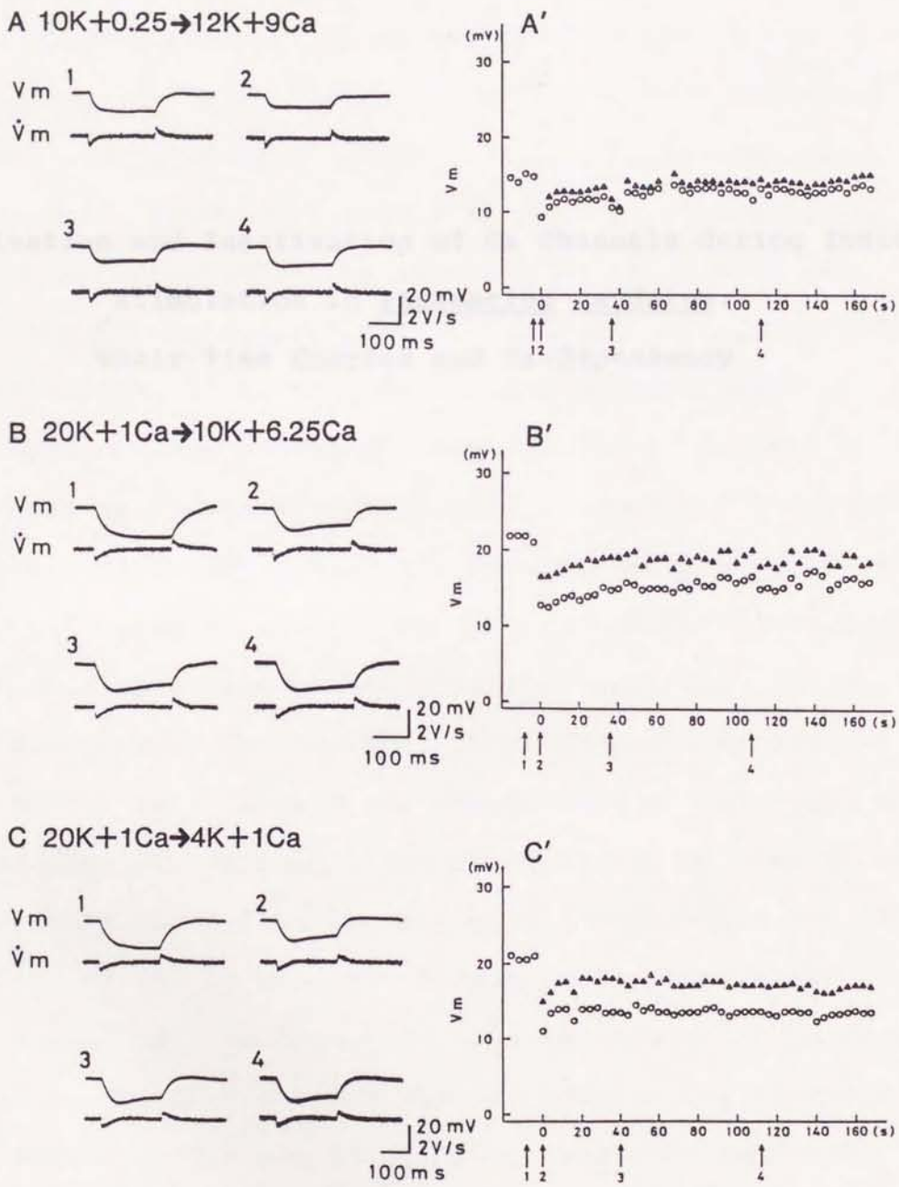

A 10K+0.25Ca→12K+9Ca
A′
B 20K+1Ca→10K+6.25Ca
B′
C 20K+1Ca→4K+1Ca
C′
Vm
V̇m
(mV)
(V/s)
M.R.R.
20 mV
2 V/s
4 V/s
100 ms
0 20 40 60 80 100 120 140 160 (s)

Fig. I-13


A 10K+0.25→12K+9Ca
A′
Vm
V̇m
1
2
3
4
20 mV
2V/s
100 ms
(mV)
30
20
10
0
Vm
0 20 40 60 80 100 120 140 160 (s)
12
3
4
B 20K+1Ca→10K+6.25Ca
B′
C 20K+1Ca→4K+1Ca
C′

Part II

Activation and Inactivation of Ca Channels during Ionic Stimulation in *Paramecium caudatum*: Their Time Courses and Ca-Dependency

INTRODUCTION

Ciliate protozoan Paramecium caudatum shows backward swimming due to reversed beating of cilia (ciliary reversal), when it is transferred into a solution with higher K^+ concentration. Ciliary reversal is caused by increased intraciliary Ca^{2+} concentration by an inflow of Ca^{2+} ions into the cilia through the activated Ca channels in the ciliary membrane. As shown in part I, Ca channel activation by ionic stimulation depended on an increase in the external Ja ratio (the ratio of K^+ concentration to the square root of Ca^{2+} concentration), which caused the binding of K^+ to the membrane in exchange with membrane bound Ca. Theoretical considerations (in part V) revealed that an increase in the Ja-ratio produces a decrease in transmembrane diffusion potential, and thus activates voltage-sensitive Ca channels. It is presumable that Ca channels are activated showing a time course identical with that of the ion-exchange reaction, then they are gradually inactivated with slower time course. In part II, antagonism between ionic stimulation and electric current stimulation in the Ca channel activation was examined. Time courses of the activation and of the inactivation of the Ca channels during ionic stimulation (i.e. Ja ratio increase) will be discussed. Ca-dependence of the slow inactivation, which is responsible for termination of ciliary reversal during continuous stimulation, was also examined.

MATERIALS and METHODS

Specimens of Paramecium caudatum (strain kyk201, wild type) reared in a wheat straw infusion were washed well with a standard saline solution, which contained 4 mM (or 1 mM in some experiments) KCl, 1 mM $CaCl_2$ and 1 mM Tris-HCl buffer (pH 7.2), equilibrated in the solution for more than 30 min prior to experimentation. Experimental procedures were basically equal to those of part I. Glass capillary microelectrodes filled with 1 M KCl (50-100 MOhm) were used for recording the membrane potential and for constant current injection into the cell. The adaptation (standard saline) solution around the inserted specimen was quickly exchanged (within 1 sec) with a given test solution by squirting the solution gently (5 mm/sec) against the specimen from a thick glass capillary pipette (0.5 mm in inner diameter), the opening of which was placed less than 0.5 mm apart from the specimen. The test solution was kept flowing out of the pipette during experiment. PH of the experimental solution was adjusted to 7.2 by 1 mM Tris-HCl buffer. All the experiments were done under room temperature ranging from 20 to 22° C.

RESULTS

Antagonism between Ja ratio increase and inward current injection

When specimens of Paramecium equilibrated in a mixture of 1 mM KCl + 1 mM $CaCl_2$ (in final concentration; hereafter the solution will be described as 1 K + 1 Ca) were transferred into a high K^+-test solution with constant Ca^{2+} concentration (1 mM), they showed ciliary reversal. Duration of ciliary reversal increased with increasing K^+ concentration in the test solution (10 mM K, 12.9±0.8 sec; 15 mM K, 27.6±1.4 sec; 20 mM K, 42.4±1.5 sec: mean±S.E., n=10). These results are consistent with previous results (Naitoh, 1968). The K^+-induced ciliary reversal was inhibited by injecting an inward current into the cell. Intensity of the current to inhibit the ciliary reversal was higher when the K^+ concentration was higher (0.3-0.4 nA for 10 mM, 0.4-0.5 nA for 15 mM and 0.5-0.6 nA for 20 mM).

A specimen of Paramecium equilibrated in 4 K + 1 Ca had been subjected to 0.8 nA inward current for 220 sec. The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca 30 sec after onset of the inward current. Ciliary reversal was not observed upon solution exchange, while it was observed when the inward current was not applied to the cell. When the inward current was turned off during subjection of the specimens into 20 K + 1 Ca, ciliary reversal took place for about 40 sec (Fig. II-1). Duration of the ciliary reversal was almost identical with that in the case without inward current.

Electrical responses of the membrane to an outward current pulse (0.8 nA, 100 msec) were examined in order to find out changes in the membrane electrical properties during the

application of the inward current and K^+ ions. The electric pulse was applied every 4 sec. Electrical response to the pulse before application of inward current consisted of an initial Ca action potential and following passive IR potential shift (Fig. II-1A1). The electrical response during application of the inward current was of more or less passive CR type without Ca action potential (Fig. II-1A2). When the external solution was changed to 20 K + 1 Ca while the inward current was passing through the membrane, the membrane became to show a Ca action potential upon application of the current pulse (Fig. II-1A3). Since intensity of the sustained inward current was 0.8 nA, application of an outward current pulse corresponds to a stoppage of the inward current during the pulse. Amplitude and maximum rate of rise of the action potential tended to increase for about ten seconds after changing the solution, reaching each plateau value (Fig. II-1B). IR shift measured at the end of the pulse also tended to increase after changing the solution (open circles in Fig. II-1B).

These results suggest that activation of Ca-channels by high K^+-stimulation is suppressed by an inward current, and it occurs when the current is turned off. The activation upon stoppage of the sustained inward current with three different durations were examined in the next series of experiments. The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca 16 sec after application of inward current. The inward current was turned off at 24, 44 and 100 sec after the exchange of external solution. Time changes of membrane electrical responses to short inward

current pulses (200 msec, 0.8 nA) applied every for 4 sec were determined. In the case without inward current (Fig. II-2A), anode break responses similar to those described in the previous section were observed after the solution exchange. When a sustained inward current was applied, the anode break responses were not observed till turning off the current. Time changes of the anode break responses after turning off the inward current are shown in Fig. II-2B, C and D. Degree of the response decreased monotonously. In contrast, the degree first increased and subsequently decreased in the case without inward current injection (Fig. II-2A). The period of time when the anode break response had been seen became slightly shorter with longer inward current injection. The period approximated the duration of the ciliary reversal which occurred after turning off the current.

Change of membrane electrical properties during prolonged outward current injection

A prolonged outward current injection induced a long-lasting ciliary reversal as ionic stimulation. The duration of ciliary reversal depended on intensity of the outward current (0.4 nA, 16 sec; 0.6 nA, 32 sec; 0.8 nA, 41 sec). Normalization of ciliary beet direction during continuous injection of an outward current implies inactivation of Ca^{2+} channels similar to that found during high K^{+}-stimulation.

Figure II-3 shows electrical responses to inward current pulses (100 msec, 0.8 nA, every 3 sec) which were superimposed on the prolonged outward current (0.8 nA). When the outward current was injected into the cell, a Ca action potential was elicited

(Fig. II-3-1), then the membrane potential reached a certain depolarizing level in one or two minutes after it showed a temporary repolarization (open circle in Fig. II-3). Ca action potentials were observed upon turning off of each inward current pulse. The maximum rate of rise (M.R.R.) of the action potential decreased gradually with time after onset of the sustained current. Turning off of the inward current pulse corresponds to reapplication of an outward current after its interruption for 100 msec during the inward current pulse. Therefore, the gradual decrease in M.R.R. indicates that degree of recovery for generation of the action potential during 100 msec interruption of the outward current gradually decreases. The application of repetitive pulses did not affect the total duration of ciliary reversal.

Ca^{2+} dependence of inactivation of Ca channels after their activations by high K^+-stimulation

To examine effects of Ca^{2+} on the inactivation of Ca channels after their activation in high K^+ solution, specimens of Paramecium equilibrated in 1 K + 1 Ca first subjected to 20 mM K^+ solution with 5 mM EGTA (20 K + 5 EGTA). The specimens continued to swim forward even after their transfers into 20 K + 5 EGTA. After the specimens had been immersed in the solution for various periods of time, they were transferred into 20 K + 1 Ca. They began to show ciliary reversal immediately after the transfer. Duration of ciliary reversal in 20 K + 1 Ca was plotted against the period of the pre-treatment of the specimens with 20 K + 5 EGTA. The duration was not different from that of control

without the pre-treatment with 20 K + 5 EGTA, and it became slightly shorter with pre-treatment longer than 30 sec. Membrane resistance in 20 K + 5 EGTA decreased below 10% of that in 1 K + 1 Ca (from 30-40 MOhm to 2 MOhm) in 2 sec.

On the other hand, when specimens were pre-treated with 20 K without EGTA, duration of ciliary reversal produced by subsequent stimulation with 20 K + 1 Ca decreased gradually with period of the pre-treatment (open square in Fig. II-4).

DISCUSSION

The K^+-induced ciliary reversal was inhibited by injecting an inward current. Ca channels can be activated by a decrease in the transmembrane diffusion potential (a membrane depolarization) due to an increase in the Ja ratio of external solution (part V). An inward current injection into the cell apparently hyperpolarizes the membrane, thus, antagonizes membrane depolarization by high K^+-stimulation. An inward current, therefore, deactivates the K^+-activated Ca-channels. Generation of Ca action potentials in response to short outward current pulses superimposed on a sustained inward current (corresponding to an anode break responses, Fig. II-1) indicates that Ca channels are ready to be activated upon removal of the inward current. Time course of the change in the maximum rate of rise of the short pulse-activated action potential corresponds to the time course of the Ca channel activation by the high K^+-solution when no inward current is applied. The M.R.R. gradually increased to reach its maximum level after subjection of the specimen to a high K^+-solution. The time course, therefore, corresponds to that of liberation of Ca^{2+} from the membrane in exchange with K^+. Thus, these responses have time constants of several seconds.

The fact that the amplitude of action potential and M.R.R. do not decrease while an inward current is applied indicates that no inactivation of Ca channel occurs in high K^+-solution when inward current is applied, and hence ciliary reversal is not produced. When the inward current was turned off, anode break response to short test pulse and ciliary reversal were elicited (Fig. II-2). There are some differences in the time courses of

the change in the anode break responses during ciliary reversal by a high K^+-stimulus between the cases with sustained inward current and those without the current. In the former cases (Fig. II-2B,C,D), the ion-exchange reaction have accomplished, so that the time course might correspond to the time course of inactivation of Ca^{2+} channels. In the latter cases (Fig. II-2A), the time course is a complexed one which is affected by both activation and inactivation.

Ca channels of Paramecium membrane are activated by a membrane depolarization produced in association with ion-exchange reaction, and are inactivated rather slowly during high K^+-stimulation. Duration of ciliary reversal appears to be correlated with the period of time when the Ca permeability is kept above a certain threshold during high K^+- stimulation. Therefore, the slow inactivation of Ca channels is responsible for termination of ciliary reversal. Two mechanisms of the inactivation of Ca channels in Paramecium membrane, one depends on the Ca ions entered into the cell during membrane depolarization (Brehm et al, 1980), and the other on the membrane depolarization itself (Hennessey and Kung, 1985) have been investigated. Present results indicate that the inactivation during high K^+-stimulation depends on Ca^{2+} and not on the membrane depolarization (Fig. II-4). In the case of prolonged outward current injection (Fig. II-3), the slow inactivation occurred with a time course similar to that in ionic stimulation. Ca dependent mechanism of inactivation seems to be important for regulation of membrane Ca permeability during ciliary reversal.

More detailed discussion on the inactivation will be appear in part III.

FIGURE LEGENDS

Figure II-1. Membrane potential response of P. caudatum to an outward current pulse (100 ms, 0.8 nA) under the influences of both an increased external K^+ concentration and of prolonged application of an inward current (I; 220 sec, 0.8 nA). The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca 30 sec after onset of the inward current. A; the membrane potential responses (Vm) and their first order time derivatives ($\dot{V}m$). B; time changes in the amplitude (open triangles) and the maximum rate of rise (solid triangles) of the Ca action potential, and the steady IR shift measured at the end of the pulse (open circles). Number under each arrow corresponds to number in membrane potential response in A. A shaded bar in B shows approximate duration of ciliary reversal.

Figure II-2. Membrane potential responses of P. caudatum to inward current pulse (200 ms, 0.8 nA) under the influences of both an increased the external K^+ concentration and of prolonged application of an inward current (I; 0.8 nA) of varied duration (0 sec in A, 24 sec in B, 44 sec in C and 100 sec in D). The external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca 16 sec after application of a prolonged inward current (B, C, D). Open squares; time changes in the amplitude and solid triangles; the maximum rate of rise of the anode break Ca-action potential (see the inset). Number under each arrow in D corresponds to the number in each membrane potential response in E.

Figure II-3. Change in the membrane potential responses of P. caudatum to an inward current pulse (100 ms, 0.8 nA) during prolonged outward current injection (0.8 nA) in 1 K + 1 Ca. A; the membrane potential responses (Vm) and their first order time derivatives ($\dot{V}m$). The potential response with number 1 was recorded at the onset of the outward current injection. B; time changes in the amplitude (open circles) and the maximum rate of rise (solid triangles) of the Ca action potential. Number under each arrow corresponds to the number in each membrane potential response in A. Shaded bar in B shows approximate duration of ciliary reversal.

Figure II-4. Effects of the treatment of the cell with a Ca^{2+}-free solution on the duration of ciliary reversal of P. caudatum. Specimens equilibrated in 1 K + 1 Ca were treated with 20 K + 5 EGTA (open circles) or 20 K + 0 Ca (open squares) for various periods of time, and then were transferred into 20 K + 1 Ca. Broken line corresponds to a remaining duration of ciliary reversal which will be exhibited by the specimens after their transfers into 20 K + 1 Ca. Therefore, the line shows usual time course of inactivation of Ca channels during high K^+-stimulation.

Fig. II-1

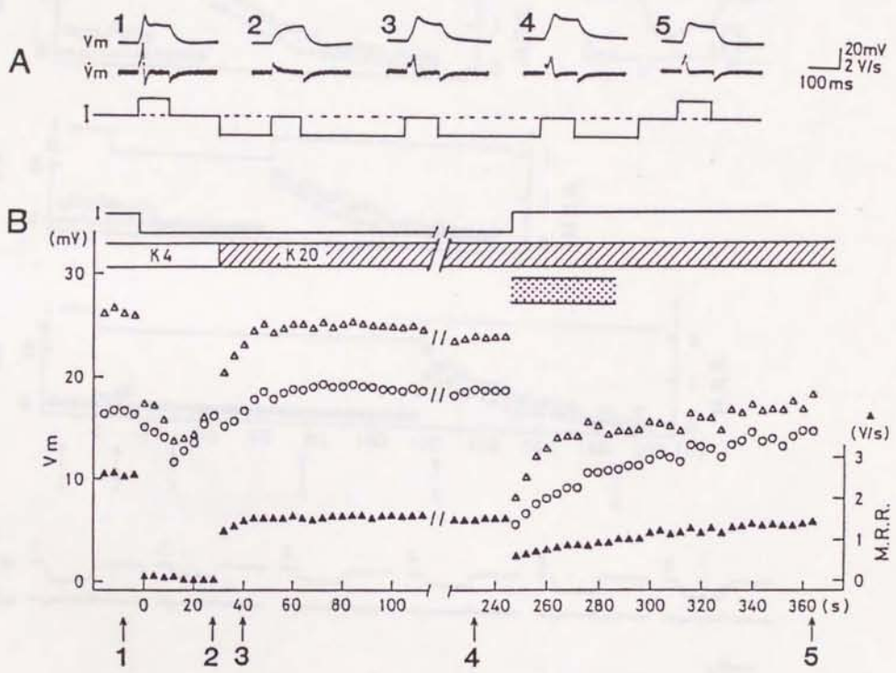

A
Vm
V̇m
I
1
2
3
4
5
20mV
2 V/s
100ms
B
I
(mV)
K4
K20
30
20
10
0
Vm
(V/s)
3
2
1
0
M.R.R.
0
20
40
60
80
100
240
260
280
300
320
340
360 (s)
1
2
3
4
5

Fig. II-2

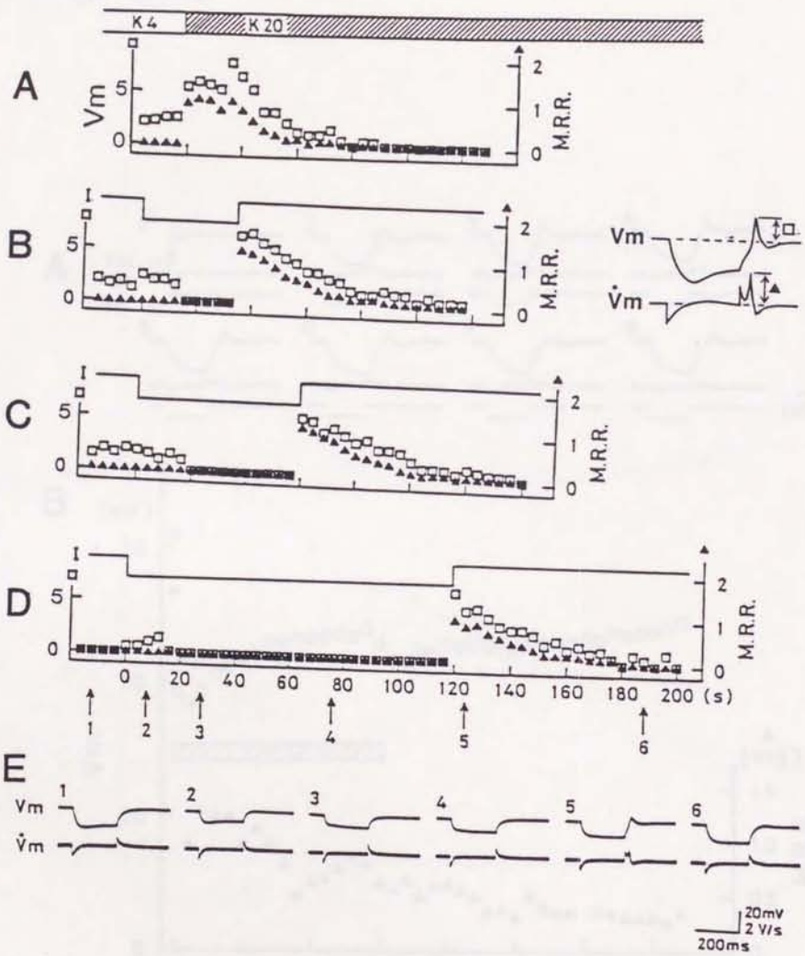

K4
K20
A
Vm
M.R.R.
B
I
Vm
V̇m
C
D
0 20 40 60 80 100 120 140 160 180 200 (s)
1 2 3 4 5 6
E
20mV
2 V/s
200ms

Fig. II-3

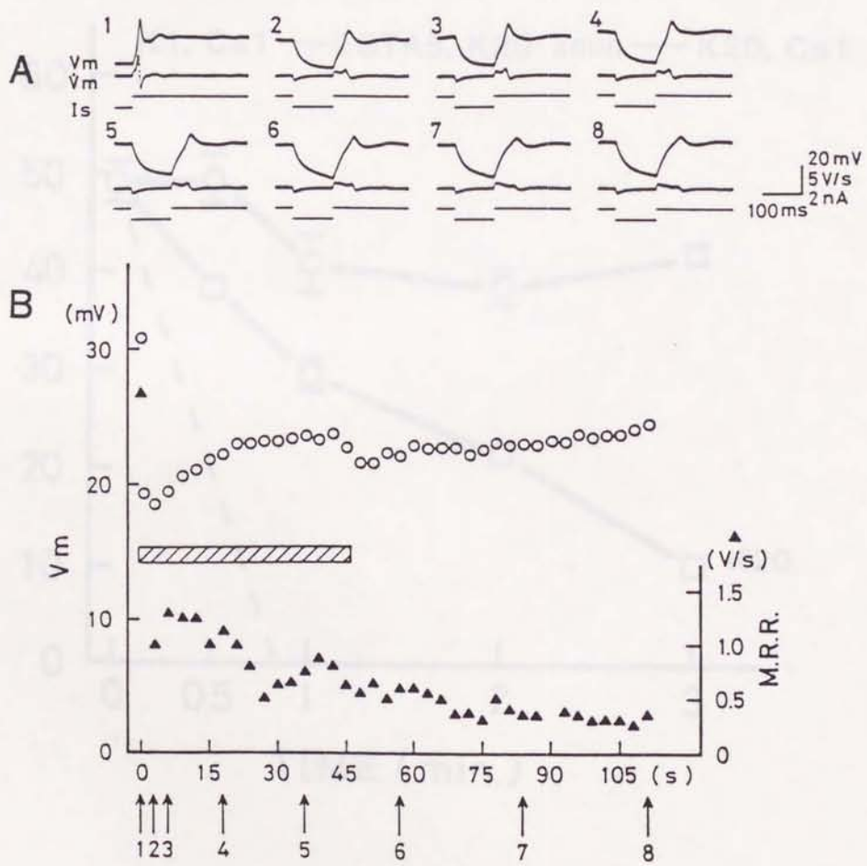
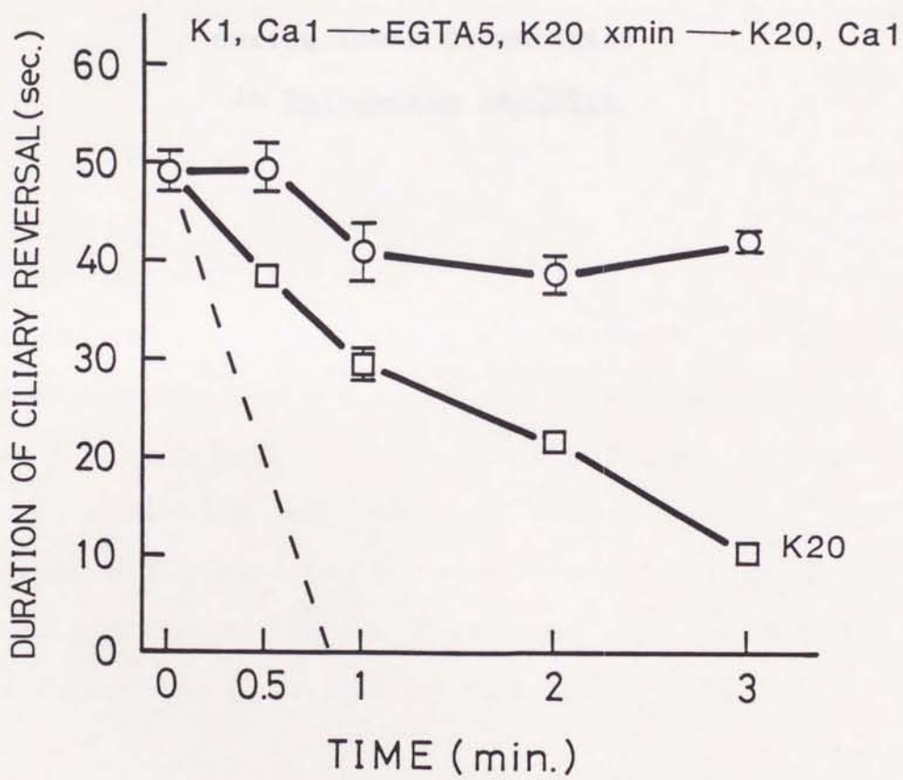

A
Vm
V̇m
Is
1
2
3
4
5
6
7
8
20 mV
5 V/s
2 nA
100 ms
B
(mV)
30
20
10
0
Vm
(V/s)
1.5
1.0
0.5
0
M.R.R.
0
15
30
45
60
75
90
105 (s)
1 2 3
4
5
6
7
8

Fig. II-4


K1, Ca1 → EGTA5, K20 xmin → K20, Ca1
DURATION OF CILIARY REVERSAL (sec.)
60
50
40
30
20
10
0
0
0.5
1
2
3
TIME (min.)
K20

Part III

Mechanism of Inactivation of Ca Channels during Ionic Stimulation in *Paramecium caudatum*

INTRODUCTION

In parts I and II, it was demonstrated that Ca^{2+}-dependent slow inactivation of Ca channels was responsible for termination of ciliary reversal during ionic stimulation. This inactivation can not be removed by reduction of internal Ca^{2+} concentration below 10^{-6} M after the inactivation was once established during sustained stimulation. Part III will deal with removal of the inactivation by inward current injection or membrane hyperpolarization. Mechanism of the inactivation will be discussed.

MATERIALS and METHODS

Specimens of Paramecium caudatum (strain kyk201 or KyKy1, wild type) reared in a wheat straw infusion were washed well with a standard saline solution, which contained 4 mM (or 1 mM in some experiments) KCl, 1 mM $CaCl_2$ and 1 mM Tris-HCl buffer (pH 7.2), equilibrated in the solution for more than 30 min prior to experimentation. Experimental procedures were essentially identical with those described in part I.

RESULTS

Time course of inactivation

The external solution of a specimen of Paramecium impaled by microelectrodes was changed from 1 K + 1 Ca to 20 K + 1 Ca, then membrane electrical responses to an inward current pulse (0.8 nA, 200 msec or 800 msec) were examined every 10 seconds. The membrane produced Ca action potential upon turning off the inward current (anode break response) as described in parts I and II. Figure III-1A shows time change of the maximum rate of rise (M.R.R.) of the anode break response. In the cases of 200 msec-test pulse, the M.R.R. decreased with single exponential time course (time constant; 26 sec, Fig. III-1B). The period of time when the anode break response was observed approximated the duration of ciliary reversal. In the cases of 800 msec-test pulse, time course of decrease in the M.R.R. consisted of two components with fast (32 sec) and slow (260 sec) time constant.

Removal of inactivation by inward current injection

Results shown in Fig. III-1 suggest that inactivation of Ca channels during ionic stimulation is removed by injection of inward current. To examine removal of inactivation by inward current, an prolonged inward current was injected into the cell after ciliary reversal by ionic stimulation (from 4 K + 1 Ca to 20 K + 1 Ca) ceased. As shown in Fig. III-2, duration of ciliary reversal seen after turning off the inward current was almost the same as that of the first ciliary reversal. This result indicates that the inactivation can be removed by prolonged inward current injection. The removal of inactivation was also clearly indicated by appearance of anode break response to short

inward test pulse after turning off the prolonged inward current. As shown in Fig. III-2, time changes in amplitude and the M.R.R. of anode break response after turning off the prolonged inward current were similar to those observed during first ionic stimulation.

To examine time course of the removal of inactivation by prolonged inward current, an inward current (1 nA) was injected into the cell 3 min and 10 min after the subjection of the specimen to 20 K + 1 Ca. The inward current was briefly interrupted (for 100 msec) every 2 sec, and the membrane electrical responses seen at the moment of turning off the inward current (anode break response) were analyzed. The anode break response came to be observed soon after injection of the inward current. As shown in Fig. III-3, the M.R.R. of the anode break response increased with the subjection time, and reached a steady value in about 1 min (Fig. III-3). The time change in the M.R.R. was faster and its steady value was larger when the current was injected sooner after subjection of the specimen into a high K^+-solution (compare 3 min with 10 min).

Relationship between duration of the inward current and that of ciliary reversal seen after cessation of the current was examined. After 10 min subjection of the specimen to 20 K + 1 Ca, inward current (1 nA) was injected into the specimen for various period of time, then duration of ciliary reversal seen upon turning off the current was measured. As shown in Fig. III-4, duration of ciliary reversal increased almost exponentially with the length of the inward current.

Removal of high K^+ induced inactivation of Ca channels by membrane hyperpolarization

In this series of experiments specimens of Paramecium equilibrated in 1 K + 1 Ca were first subjected to 20 K + 1 Ca, then to several solutions containing 0.1 mM EGTA and K^+ of varied concentrations (0.2-3.2 mM; EGTA-K solution) for 20 sec after ciliary reversal subsided in 20 K + 1 Ca. Then the specimens were transferred into 20 K + 1 Ca, and duration of ciliary reversal was measured. As shown in Fig. III-5, duration of ciliary reversal was longer when the specimens were treatment with the EGTA-K solution with lower K^+ concentration. Membrane potential of the specimen was measured in each EGTA-K solution, and plotted in Fig. III-5 as a function of K^+ concentration. The membrane was more hyperpolarized in EGTA-K solution with lower K^+ concentration.

Slower change of state of inactivation

Degree of removal of the inactivation by membrane hyperpolarization was examined at various time after ionic stimulation. Specimens equilibrated in 1 K + 1 Ca were transferred into 20 K + 1 Ca for various periods of time, then they were returned to 1 K + 1 Ca for 30 sec. The specimens were transferred again into 20 + 1 Ca and duration of ciliary reversal was measured. As shown in Fig. III-6, duration of ciliary reversal gradually decreased with increasing the period of time for treatment of the specimens by 20 K + 1 Ca with a time constant of several minutes.

DISCUSSION

Duration of ciliary reversal by ionic stimulation is closely related to the period of time when membrane Ca permeability is kept higher than a certain threshold level. The increased Ca permeability could be detected by the presence of anode break response to short inward current pulse. Termination of ciliary reversal is thought to be caused by inactivation of Ca channels responsible for the Ca permeability. The inactivation was found to be dependent of Ca^{2+} (see part II). Time course of the inactivation during ionic stimulation was single exponential (Fig. III-1). The inactivation could be removed by injecting inward current into the cell (Fig. III-2). Degree of the inactivation was found to increase slowly with time even after termination of ciliary reversal in 20 K + 1 Ca. This inactivation with longer time constant is thought to be different from the inactivation with shorter time constant seen earlier after transfer of the specimen into a high K^+-solution in their characteristics. Removal of the inactivation was dependent on degree of membrane hyperpolarization (Fig. III-5).

Hennessey and Kung (1985) demonstrated that slow inactivation, the time constant of which was tens of seconds, depended on voltage but not internal Ca^{2+}. They suggested that this inactivation was responsible for renormalization of the ciliary motion during maintained stimulation. On the other hand, as indicated in part II, the inactivation during ionic stimulation, which also has time constant of tens of seconds, depended on Ca^{2+} and was never caused by depolarization itself. However, this does not mean that Ca^{2+}-dependent slow inactivation does not

require membrane depolarization. This inactivation is not removed even if internal Ca^{2+} concentration became below 10^{-6} M, once it was accomplished and removal of the inactivation depends only on membrane hyperpolarization.

I propose here a hypothesis for the mechanism of Ca-channel inactivation. The inactivation consists of following three separable processes: 1) Ca-dependent closure of the channels activated by a membrane depolarization, 2) fixation of the inactivated Ca channels, 3) slow adaptation of fixed inactivated channels. The first process had been reported by Brehm and Eckert (1978) and Brehm et al. (1980). Intracellular Ca^{2+} ions, whose concentration is elevated beyond 10^{-6} M due to Ca^{2+} inflow through Ca channels activated by a depolarization, bind to inactivation sites of inner surface of the membrane. This leads Ca channels to close and its time constant is several ten milliseconds. The second process is most important for renormalization of ciliary reversal during sustained membrane depolarization. The inactivated channels changes their characteristics to be kept closed even if intracellular Ca^{2+} concentration is decreased below 10^{-6} M. This process depends on the membrane depolarization and its time constant is several ten seconds (Fig. III-1). Voltage dependent inactivation demonstrated by Hennessey and Kung (1985) seems to correspond to this process. Development of inactivation in EGTA-injected cell reported by Hennessey and Kung (1985) does not mean that this inactivation is independent on intracellular Ca^{2+}, because injection of EGTA into the cell can not decrease intracellular

Ca^{2+} concentration below 10^{-6} M. This was demonstrated by the fact that ionophoretic application of EGTA never blocked ciliary reversal during depolarization (Brehm et al, 1980), indicating intracellular Ca^{2+} concentration above 10^{-6} M. Furthermore, they reported that membrane depolarization in the cells treated with W-7, which blocks Ca channels, also caused slow inactivation. Concentration of W-7 they employed blocked the inward Ca current by more than 90%. There is, however, a possibility that internal Ca^{2+} concentration was higher than 10^{-6} M due to small remnant Ca current similarly to the case of EGTA-injected cells. The mechanism of action of W-7 is unknown (Hennessey and Kung, 1984). A possibility that W-7 acts to inactivation site is remained too. Third process corresponds to an inactivation reported by Hildebrand (1978). Inactivated channels changes their characteristics progressively so as to become difficult in removing the inactivation by hyperpolarization. This process has time constant of several minutes (Fig. III-6). This may be concerned with long term adaptation of the cell.

Mechanism of fixation of inactivation is unknown. Involvement of membrane depolarization is probable. However, other explanations are also possible; for example, enzymatic reaction, such as dephosphorylation (i.e. the removal of the phosphate group) inactivates Ca channels and plays a role in determining the functional sate of the channel (Eckert and Chad, 1984; Chad and Eckert, 1986). The mechanism of fixation of inactivation is an interesting subject for future study.

FIGURE LEGENDS

Figure III-1. A: Time change in the maximum rate of rise (M.R.R.) of the anode break response to an inward current pulse during high K^+-stimulation in P. caudatum. The external solution was changed from 1 K + 1 Ca to 20 K + 1 Ca at time 0. Intensity of the inward current pulse was 0.8 nA, and duration of the pulse was 200 ms (solid circles) or 800 ms (open circles). Inset is an example of membrane potential response (Vm) to an inward current pulse (200 ms), first order time derivative of Vm ($\dot{V}m$) and the inward current pulse (Is) recorded immediately after exchange of the external solution. The potential response corresponds to a solid circle pointed by an arrow.
B: Semilogarithmic plots of the maximum rate of rise of the anode break response (all the data are shown in A) against time after exchange of the external solution.

Figure III-2. Removal of the inactivation of Ca channels by prolonged inward current injection in P. caudatum. The inward current (20 sec, 0.8 nA) was injected 40 sec after the external solution was changed from 4 K + 1 Ca to 20 K + 1 Ca. Time changes in the amplitude (Vm; open squares) and the maximum rate of rise (M.R.R.; solid triangles) of the anode break response to an inward current pulse (200 ms, 0.8 nA, applied every 4 sec) are shown to indicate time change in the degree of activation of Ca^{2+} channels. Shaded bars show approximate duration of the ciliary reversal.

Figure III-3. Time course of removal of the inactivation by inward current injection in P. caudatum. Prolonged inward current (1 nA) was injected 3 min (open circles) or 10 min (solid circles) after the external solution was exchanged from 1 K + 1 Ca to 20 K + 1 Ca. Maximum rate of rise of anode break Ca action potential seen at the moment of interruption of the inward current for 100 ms every 2 sec was plotted against duration of inward current.

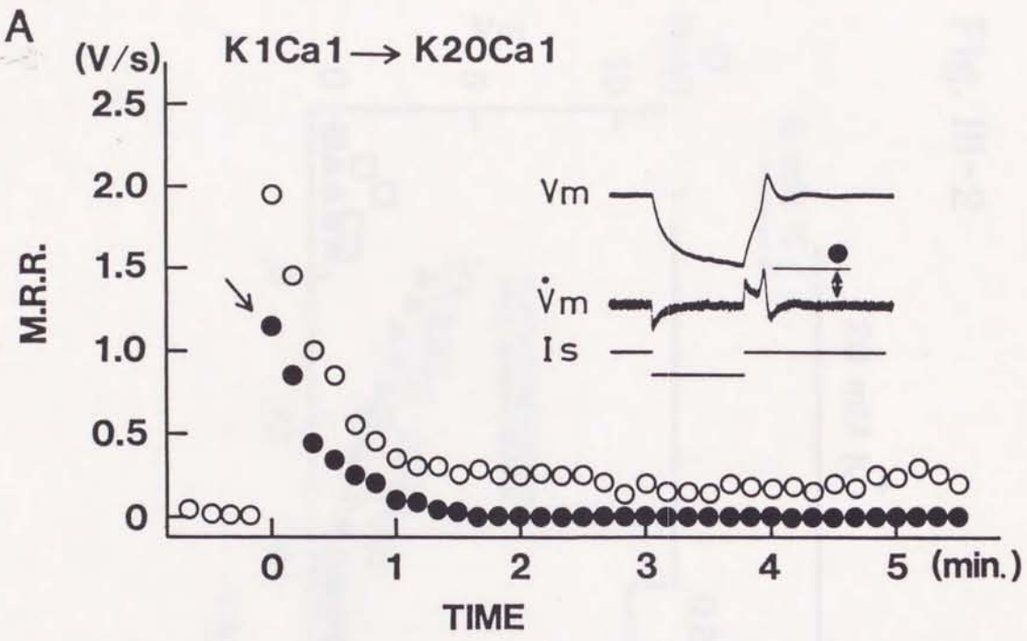
Figure III-4. Relationship between ciliary reversal and removal of inactivation. Inward currents (1 nA) with varied lengths of time were injected about 10 min after exchange of the external solution from 1 K + 1 Ca to 20 K + 1 Ca. Duration of ciliary reversal elicited upon turning off the inward current was plotted against the length of the inward current.

Figure III-5. Relationship between removal of inactivation and hyperpolarization of the membrane. A specimen of Paramecium equilibrated in 1 K + 1 Ca was transferred into 20 K + 1 Ca. The specimen was then treated with 0.1 mM EGTA + 0.2-3.2 mM K^+ (EGTA-K) solution for 20 sec after the ciliary reversal (about 60 sec) seen in the stimulation medium. The EGTA-K treated specimen was transferred again into the 20 K + 1 Ca. Duration of ciliary reversal seen in 20 K + 1 Ca (open circles) was measured. The duration is regarded as degree of the removal of inactivation and plotted against K^+ concentration of the EGTA-K solution. Resting membrane potential (open triangles) in each EGTA-K solutions with

varied K^+ concentrations was plotted against the K^+ concentration.

Figure III-6. Slower time change in the inactivation in high K^+ solution. A specimen of P. caudatum equilibrated in 1 K + 1 Ca was transferred into a stimulation medium (20 K + 1 Ca). After various periods of time in the stimulation medium, the specimen was returned to 1 K + 1 Ca for 30 sec. Then specimen was transferred again into the stimulation medium and the duration of ciliary reversal was measured. Broken line shows the remaining duration of ciliary reversal which will exhibited by the specimens if the inactivation was not removed (control condition).

Fig. III-1


A
(V/s)
K1Ca1 → K20Ca1
2.5
2.0
1.5
1.0
0.5
0
M.R.R.
Vm
V̇m
Is
0
1
2
3
4
5 (min.)
TIME

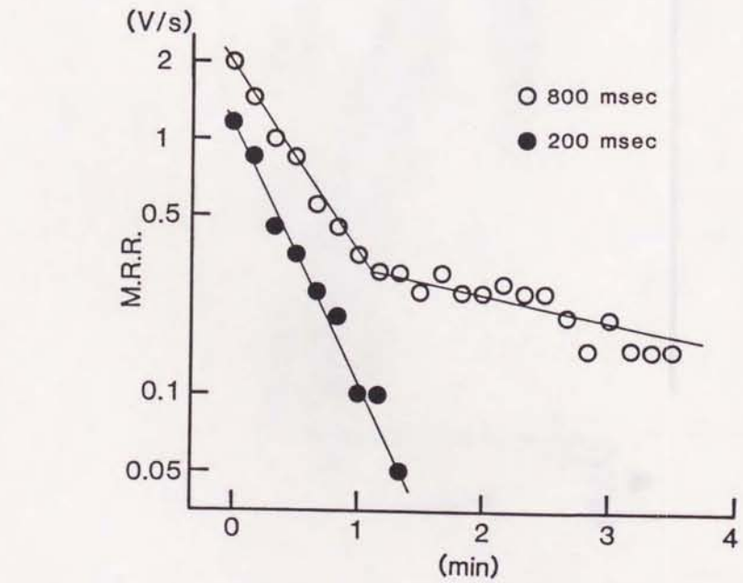

B
(V/s)
2
1
0.5
0.1
0.05
M.R.R.
800 msec
200 msec
0
1
2
3
4
(min)

Fig. III-2

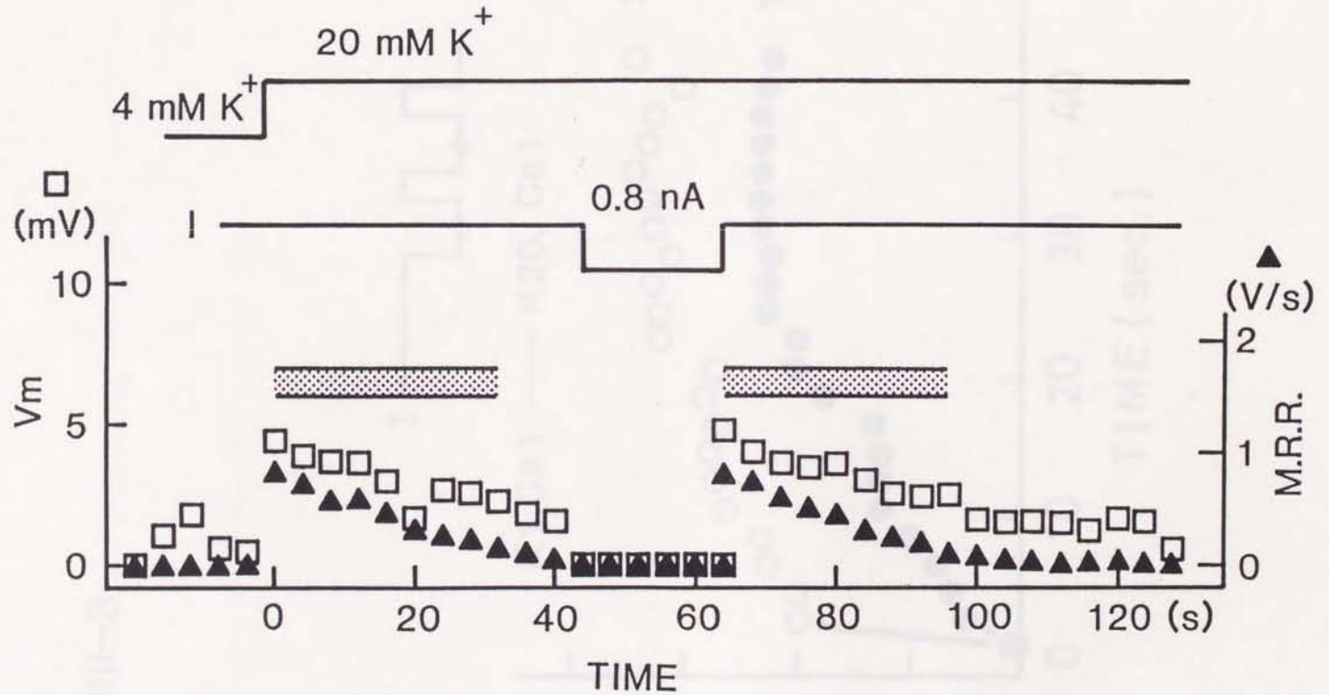

20 mM K+
4 mM K+
0.8 nA
I
(mV)
10
5
0
Vm
(V/s)
2
1
0
M.R.R.
0
20
40
60
80
100
120 (s)
TIME

Fig. III-3

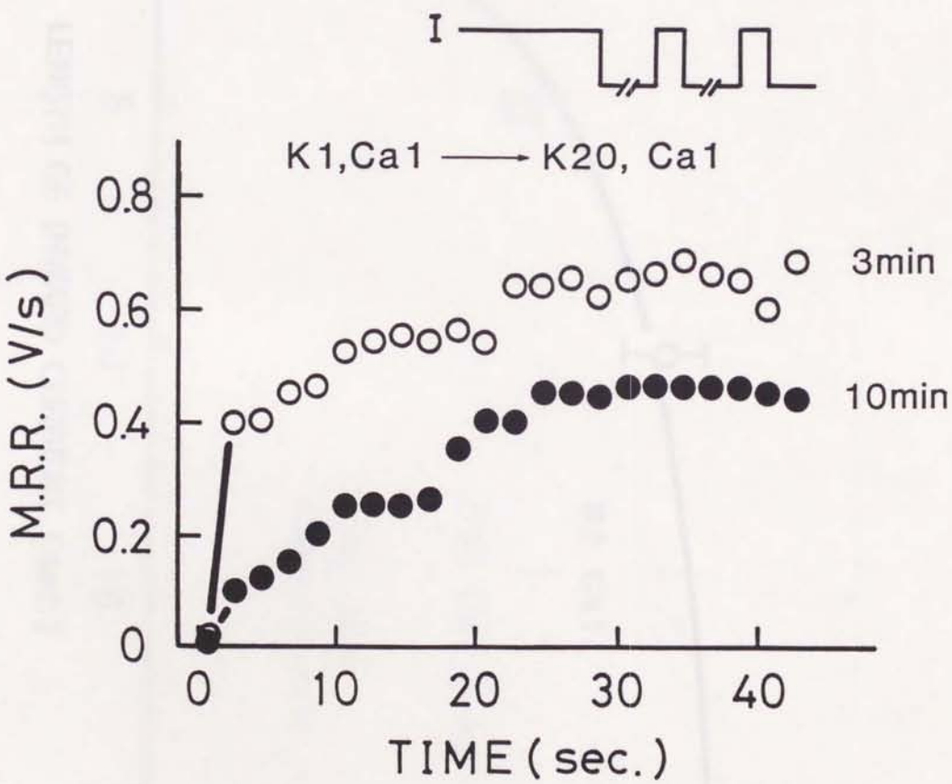

I
K1,Ca1 ⟶ K20, Ca1
0.8
0.6
0.4
0.2
0
M.R.R. (V/s)
3min
10min
0
10
20
30
40
TIME (sec.)

Fig. III-4

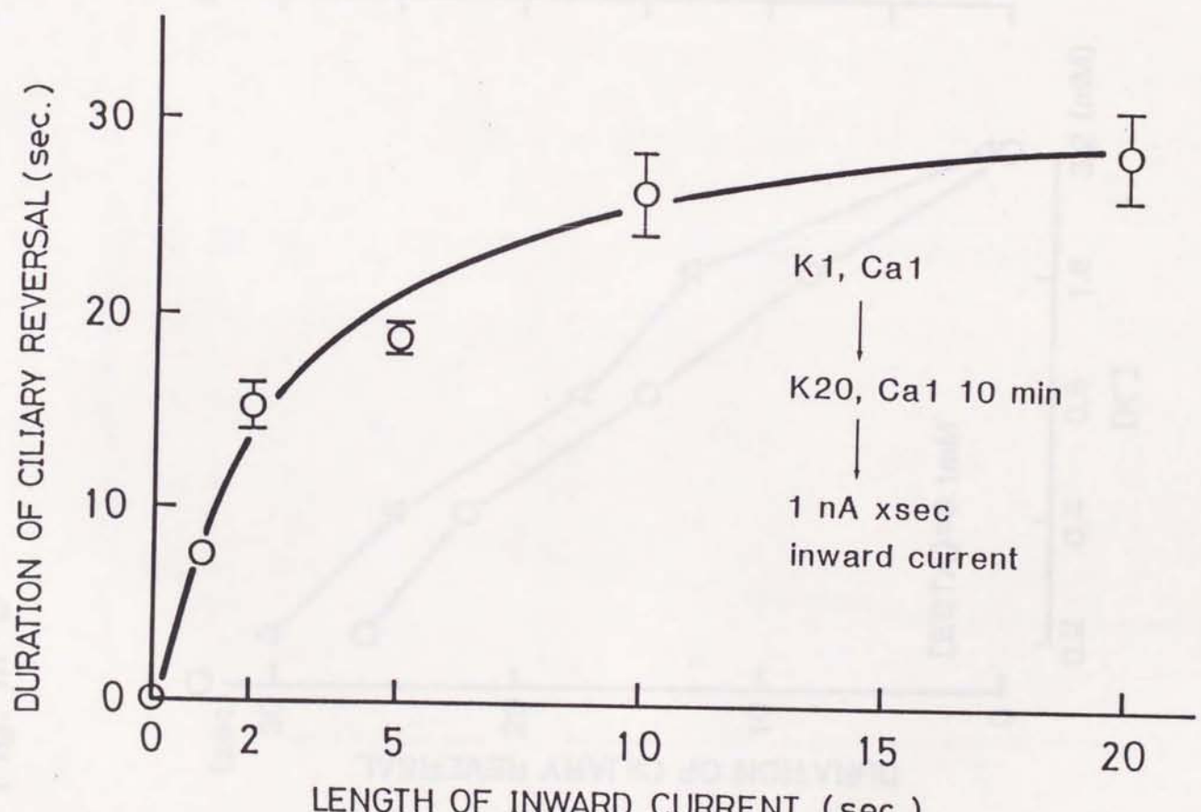

DURATION OF CILIARY REVERSAL (sec.)
30
20
10
0
0
2
5
10
15
20
LENGTH OF INWARD CURRENT (sec.)
K1, Ca1
K20, Ca1 10 min
1 nA xsec
inward current

Fig. III-5

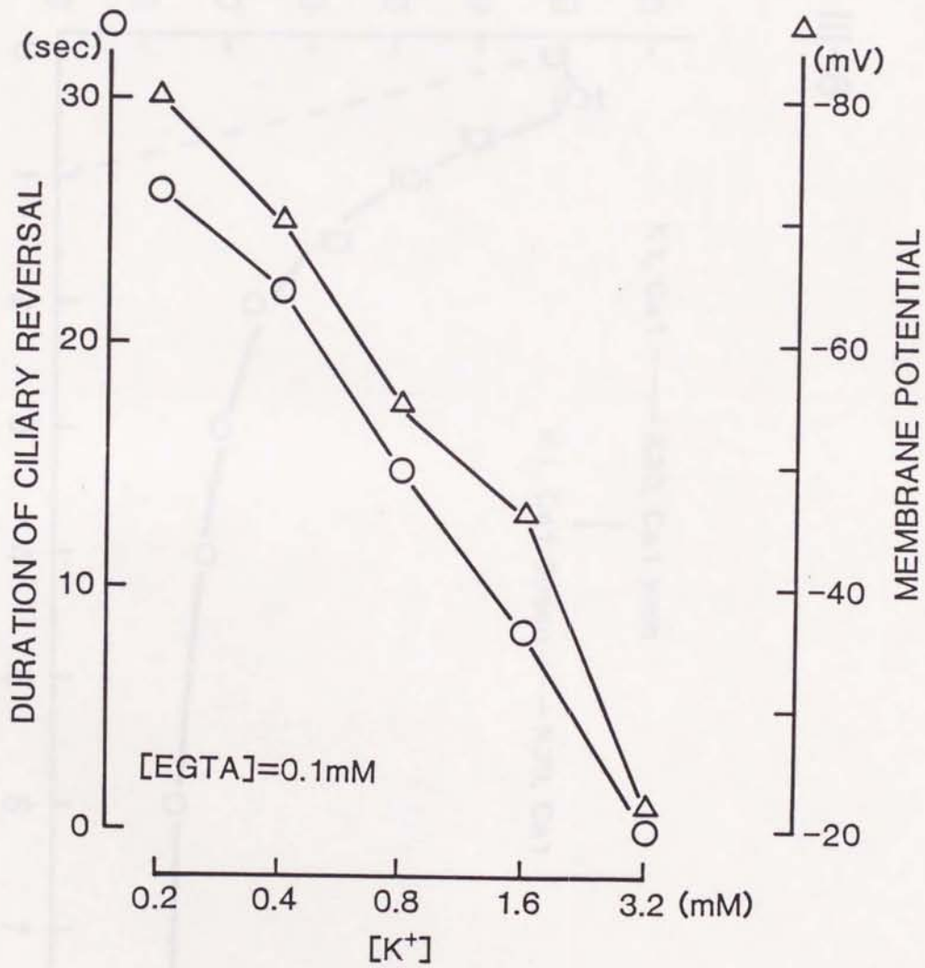
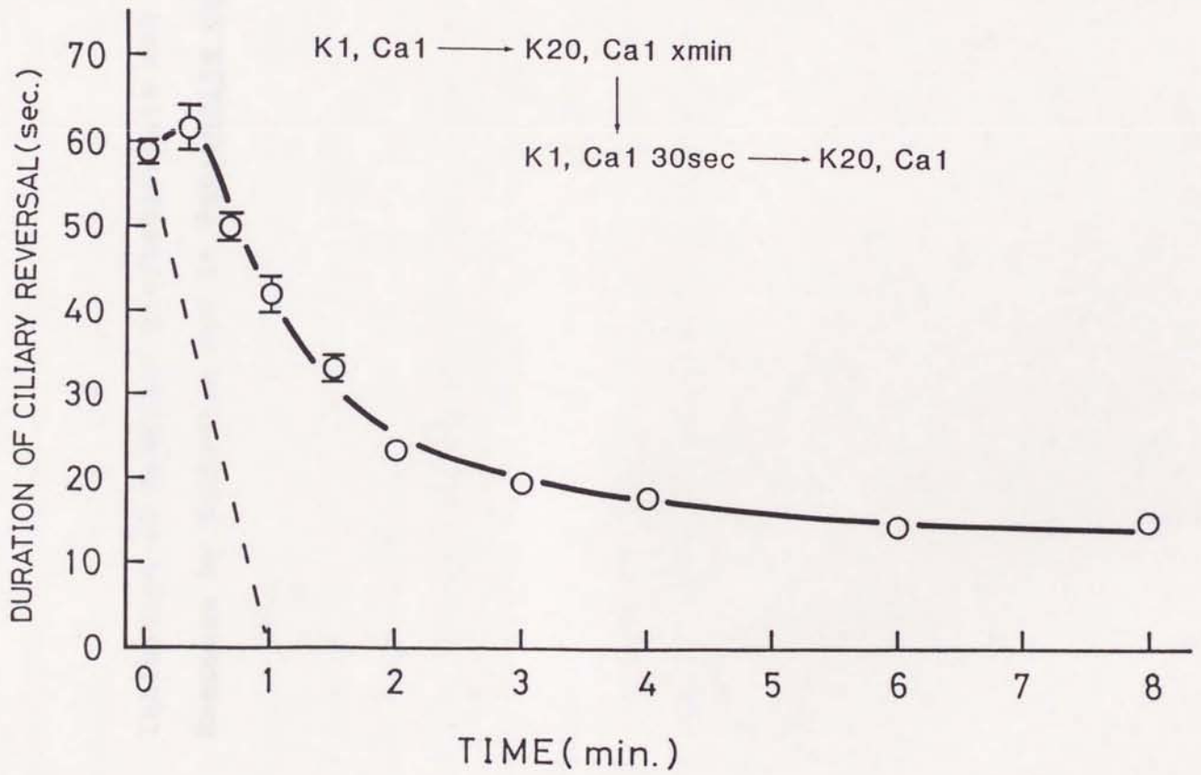

(sec)
30
20
10
0
DURATION OF CILIARY REVERSAL
[EGTA]=0.1mM
0.2
0.4
0.8
1.6
3.2 (mM)
[K+]
(mV)
-80
-60
-40
-20
MEMBRANE POTENTIAL

Fig. III-6


K1, Ca1 ⟶ K20, Ca1 xmin
K1, Ca1 30sec ⟶ K20, Ca1
DURATION OF CILIARY REVERSAL (sec.)
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
TIME (min.)

Part IV

Inhibition of Membrane Electrogenesis and Ciliary Response by Ruthenium Red in *Paramecium caudatum*

INTRODUCTION

Paramecium membrane behaves as an ion-exchanger membrane with negative surface charges (Naitoh and Yasumasu, 1967). This property seems to play an important role in generation of membrane potential and gating of ion channels (part V). It had been reported that there was a layer of acidic mucopolysaccharides, which were stained with Ruthenium Red (R.R.), in the external surface of the membrane (Wyroba and Przelecka, 1973). R.R., a poly-cationic dye (Luft, 1971 a,b), binds to the sites with negative charge in sialic acid occupying the terminal region of polysaccharides and produces many effects on membrane functions such as transmitter release (Baux et al, 1979), muscle contraction (Dörrsheidt-Käfer, 1979; Robertson and Wann, 1987) and membrane excitability (Bhattacharyya et al 1981). Sialic acid residues are possible to be main negative charges of membrane surface in Paramecium. In this part (IV), effects of R.R. on physiological functions of Paramecium membrane will be described.

MATERIALS and METHODS

Specimens of Paramecium caudatum (strain KyKy1, wild type) reared in a wheat straw infusion were washed well with a standard saline solution, which contained 1 mM KCl, 1 mM $CaCl_2$ and 1 mM Tris-HCl buffer (pH 7.2), equilibrated in the solution for more than 30 min prior to experimentation. Experimental procedures were essentially similar to those described in parts I, II and III.

Ruthenium Red (R.R., $[Ru(NH_3)_4OHCl]2H_2O$, MW 858.5; Luft, 1971) was obtained from K&K Laboratories and was purified to remove a violet complex present in commercial R.R. (Brooks, 1969). Commercial R.R. was dissolved in 0.5 M NH_4OH. The supernatant was recrystallized in Acetone and desiccated.

RESULTS

I. Effects of R.R. on the electrical characteristics of the membrane.

Resting membrane potential

Since the resting membrane of Paramecium is more or less permeable to both K^+ and Ca^{2+} (more permeable to K^+ than Ca^{2+}), the external application of these ions results in a depolarization of the membrane (Kamada, 1934; Yamaguchi, 1960; Naitoh and Eckert, 1968). Concentration effects of these ions on the membrane potential were compared in the presence and absence of R.R. in the external solutions. As shown Fig. IV-1A, no appreciable effects of R.R. on the concentration effect of K^+ was observable. Concentration effect of Ca^{2+} was also not much affected by the presence of R.R. in the external solution (Fig. IV-1B).

Calcium action potential

A membrane depolarization by an outward current produces a transient regenerative increase in Ca conductance (Ca-action potential) of the membrane of Paramecium, which is followed by an increase in K conductance. Application of 0.5 μM R.R. in the external solution containing 1 mM KCl and 1 mM $CaCl_2$ (1 K + 1 Ca) brought about rapid inhibition of the action potential. A decrease in the amplitude of action potential and of its rate of rise (dV/dt) began to take place in 8 sec after application of R.R. (Fig. IV-2B), and the membrane became to behave as a passive parallel R-C network in response to an electric current (Fig. IV-2C,D,E). The recovery of the membrane electrical activities after removal of R.R. needed long time and was imperfect (Fig.

IV-2H). Figure IV-3A shows the time change in the membrane electrical response to outward current pulse during long exposure of the specimen in R.R.-containing solution.

Membrane response to hyperpolarization

In response to a hyperpolarization by an inward current, K conductance of the membrane increases rather slowly, resulting in a slow decrease in IR potential (inward going rectification, Fig. IV-2I). Application of 0.5 μM R.R. brought about a quick disappearance of the decrease in I-R potential. The general shape of the potential response, therefore, became more or less passive R-C type (Fig. IV-2J,K,L). The recovery of the inhibition of inward going rectification after removal of R.R. from the external solution was also slow and imperfect as shown in Fig. IV-2N.

Time change in the membrane resistance to inward current during exposure of the specimen in the R.R.-containing solution is illustrated in Fig. IV-3B. The membrane resistance was calculated from the potential value at the end of the inward current pulse (150 msec) and the current intensity (0.8 nA). Long exposure of the specimen in the R.R.-containing solution resulted in an marked increase in the membrane resistance.

Mechanoreceptor potential

A brief tap of the membrane of the anterior part of Paramecium by a glass microstylus mounted on a piezo-electric phonocartrige results in generation of a depolarizing mechanoreceptor potential which induces regenerative Ca-action potential in similar way to an outward current stimulation (Fig.

IV-4A). While a brief tap of the posterior membrane produces a hyperpolarizing mechanoreceptor potential (Fig. IV-4D) (Naitoh and Eckert, 1969). External application of R.R. resulted in dramatic retardation of mechanosensitivity of Paramecium membrane. As shown in Fig. IV-4C and F, both anterior (depolarizing) and posterior (hyperpolarizing) responses disappeared in 1 min after the application of 0.5 μM R.R. The anterior receptor membrane was found to be more sensitive to R.R. than the posterior membrane. The anterior receptor potential diminished in a solution containing R.R. of lower concentration (0.2 μM) than that effective to inhibit the posterior receptor potential (Fig. IV-4B). It is noteworthy that the peak level of the hyperpolarizing posterior response significantly increased in a solution containing low concentration of R.R. (0.2 μM; Fig. IV-4E).

II. Inhibition of K^+-induced ciliary reversal by R.R.

When a specimen of Paramecium is transferred into K^+-rich medium (stimulation medium) it shows long-lasting backward swimming due to its ciliary reversal, whose duration is depending on the ionic composition of both equilibration medium, in which the specimen is equilibrated prior transfer, and stimulation medium (Naitoh, 1968). Equilibration medium was 1 K + 1 Ca through out the present experiment. As shown in Fig. IV-5, the duration of ciliary reversal was longer in stimulation medium with higher K^+-concentration. It increased almost linearly with increasing K^+ concentration from 22 to 38 while at constant (1 mM) Ca-concentration (open circle in Fig. IV-5). The duration of

ciliary reversal became shorter by the presence of R.R. in stimulation medium. The degree of inhibition of ciliary reversal by R.R. was greater in the medium with higher concentration of R.R.

In order to find competition of R.R. with cations in its inhibitory effect on the ciliary reversal was examined in four series of test solutions with 4 kinds of R.R. concentration. The Ja ratio ($K^+/\sqrt{Ca^{2+}}$) was kept constant at 30 in all the test solutions, but absolute concentrations of K^+ and Ca^{2+} were widely changed (Ca 0.01-2.56 mM, K 3.0-48 mM). As shown in Fig. IV-6, the inhibitory effect of R.R. on ciliary reversal decreased with increasing the K^+ and Ca^{2+} concentrations of the stimulation medium, and even R.R. of the highest concentration (1 μM) showed no inhibitory effect in the most concentrated solutions (48 K + 2.56 Ca).

The competition between stimulant cations and R.R. was further examined in the following experiment. A pair of stimulation medium with different ionic concentration was first determined so as the specimen of Paramecium to show ciliary reversal of the same duration, when they are transferred into each solutions. Then the concentration effect of R.R. on the duration of ciliary reversal was measured. The duration of ciliary reversal was much more decreased with increasing R.R. concentrations of in the stimulation solution with lower ionic concentrations of K^+ and Ca^{2+}. The results with two pair of experimental conditions are presented in Fig. IV-7.

DISCUSSION

R.R. exhibited no appreciable effects on the resting membrane potential of Paramecium, but it strongly inhibited generation of Ca action potential. R.R. also brought about a marked increase in the membrane resistance. The inhibition of action potential appeared faster than the increase in the membrane resistance. R.R. also inhibited ciliary reversal by high K^+ solution. Inhibitory effect of R.R. on ciliary reversal was antagonized by coexisting K^+ and Ca^{2+}. The antagonizing effects of K^+ + Ca^{2+} were stronger when concentration of these ions were higher. Therefore, it is thought that R.R. competes the same anionic sites of the membrane surface with K^+ and Ca^{2+}.

The mechanism by which R.R. inhibits Ca-channel activation may be explained by a marked increase in the transmembrane diffusion potential (hyperpolarization) caused by binding of R.R. to membrane negative charges. According to theoretical analysis of Paramecium membrane described in the next part, the transmembrane diffusion potential increases without changing the membrane potential difference across the membrane (resting potential actually measured), when the cations bind to the negative charges, if the cation is impermeable to the membrane. Gradual increase in the amount of membrane bound R.R. is followed by gradual increase in membrane resistance as a result of decrease in resting conductances of K^+ and Ca^{2+}.

Competition between R.R. and Ca^{2+} and/or K^+ to the same anionic sites leads to a conclusion that these negative charges are probably originate from sialic acid residues of mucopolysaccharide layer composing surface coat of the membrane.

It was suggested that this layer was important in the membrane electrogenesis of Paramecium.

FIGURE LEGENDS

Figure IV-1. Effects of R.R. on resting membrane potential of P. caudatum. A: Concentration effects of Ca^{2+} on the resting potential in the absence (open circles) and presence (solid circles) of R.R. (0.5 μM). The K^+ concentration was constant at 1 mM. B: Concentration effects of K^+ on the resting potential in the absence (open circles) and presence (solid circles) of R.R. (0.5 μM). The Ca^{2+} concentration was constant at 1 mM.

Figure IV-2. Effects of R.R. on the electrical responses to current injection. A-H; responses to outward current pulses (150 ms, 0.6 nA). I-N; responses to inward current pulses. A and I; responses in 1 K + 1 Ca without R.R. B and J; C and K; and D are responses 8 sec, 16 sec and 24 sec after an addition of 0.5 μM R.R. respectively. E and L; F; G and M; and H and N are electrical responses at 24 sec, 104 sec, 15 min and 25 min after remove of R.R. from the external solution respectively. V; membrane potentials. $\dot{V}$; first order time derivatives of Vm. I; currents.

Figure IV-3. Time changes in membrane electrical responses to outward (A) and inward (B) current pulses (150 ms, 0.8 nA) in 1 K + 1 Ca solution containing 0.5 μM R.R. Solid triangles (P); the peak value of the electrical response. Solid circles (S); steady IR shift of the membrane potential level measured at the end of the pulse.

Figure IV-4. Effects of R.R. on mechanoreceptor potential in P. caudatum. A-C; depolarizing mechanoreceptor potentials. D-F; hyperpolarizing potentials. A and D; mechanoreceptor potentials in 1 K + 1 Ca without R.R. B and D; mechanoreceptor potentials in 1 K + 1 Ca containing 0.2 μM R.R. C and F; mechanoreceptor potentials in 1 K + 1 Ca containing 0.5 μM R.R. V; membrane potentials. V; first order time derivatives of Vm. MS; mechanical stimulation indicated as voltage of the pulses applied to drive piezoelectric element.

Figure IV-5. Effects of R.R. on the duration of ciliary reversal by high K^+ stimulation. Specimens of P. caudatum equilibrated in 1 K + 1 Ca were transferred into the stimulation media with various K^+ concentrations at constant Ca^{2+} concentration (1 mM) in the absence (open circles) and presence (solid circles) of R.R. of various concentrations (0.25-8.0 μM).

Figure IV-6. Effects of ionic strength of K^+ and Ca^{2+} on inhibitory effects of R.R. on ciliary reversal by high K^+ stimulation. Specimens of P. caudatum equilibrated in 1 K + 1 Ca were transferred into stimulation media with various ionic strength at constant Ja ratio (30) in the absence (open circles) and presence (solid circles) of R.R. (0.25-1.0 μM).

Figure IV-7. Competition between external cations (K^+, Ca^{2+}) and R.R. Specimens of P. caudatum equilibrated in 1 K + 1 Ca were transferred into stimulation media with various concentrations of

R.R. (0-4.0 μM). Two pairs of stimulation media with different ionic strength; one pair is 48 K + 2.56 Ca (open circles) and 32 K + 0.64 Ca (open squares), and another is 38 K + 2.56 Ca (solid circles) and 24 K + 0.64 Ca (solid squares) were employed. Duration of ciliary reversal was identical in each solution of each pair.

Fig. IV-1

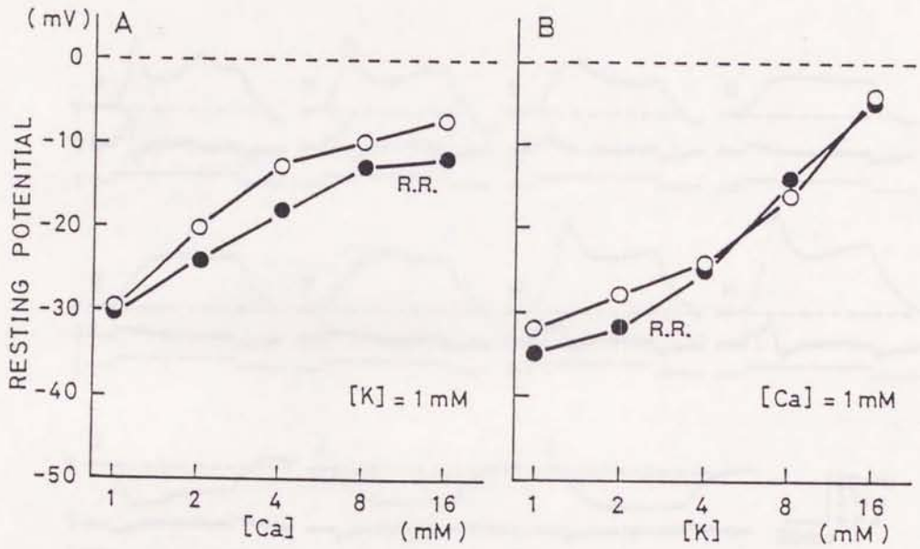

(mV)
A
0
-10
-20
-30
-40
-50
RESTING POTENTIAL
R.R.
[K] = 1 mM
1
2
4
8
16
[Ca]
(mM)
B
R.R.
[Ca] = 1 mM
1
2
4
8
16
[K]
(mM)

Fig. IV-2

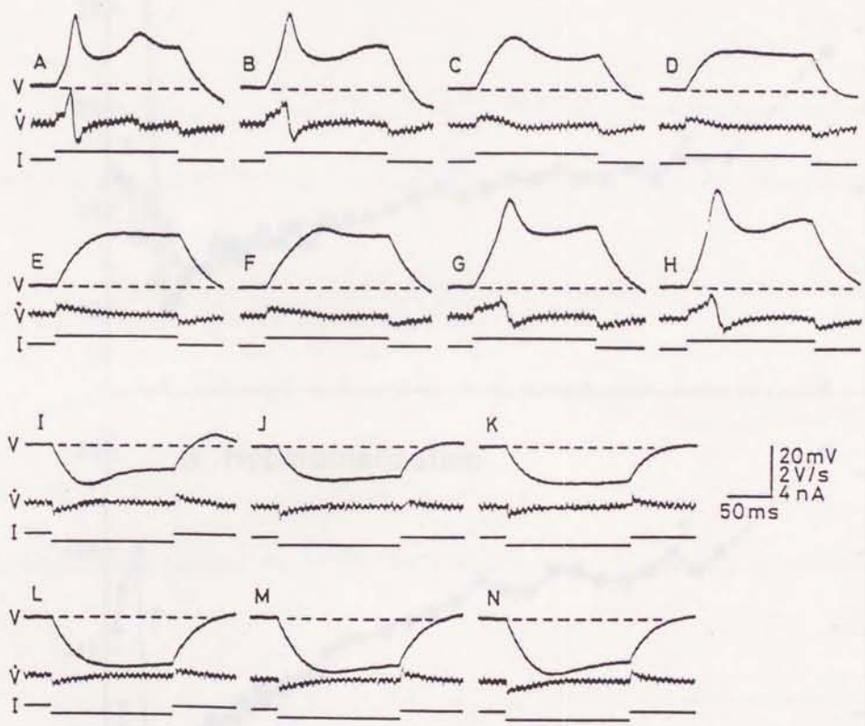


Fig. IV-3

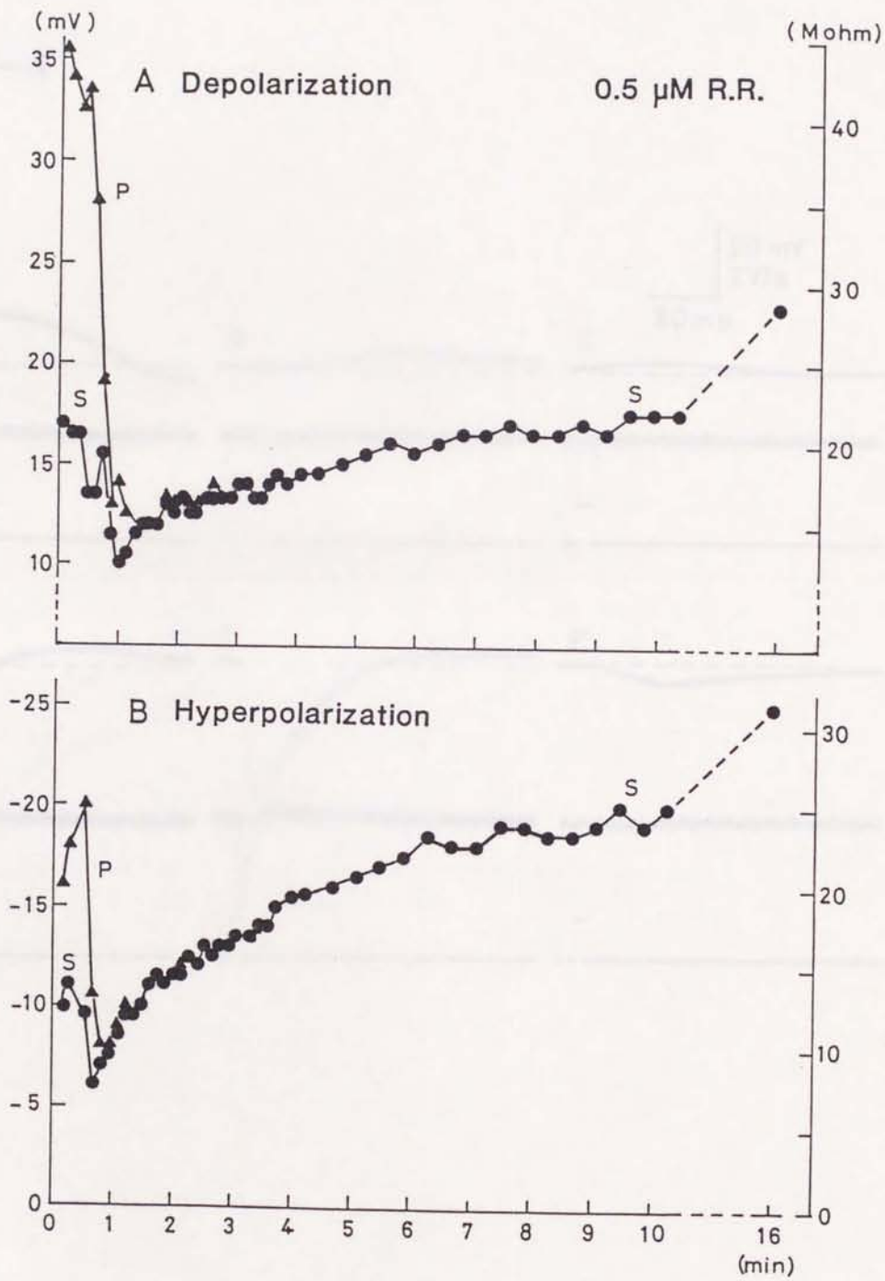

(mV)
(Mohm)
A Depolarization
0.5 μM R.R.
35
30
25
20
15
10
40
30
20
P
S
S
B Hyperpolarization
-25
-20
-15
-10
-5
0
30
20
10
0
P
S
S
0 1 2 3 4 5 6 7 8 9 10 16
(min)

Fig. IV-4

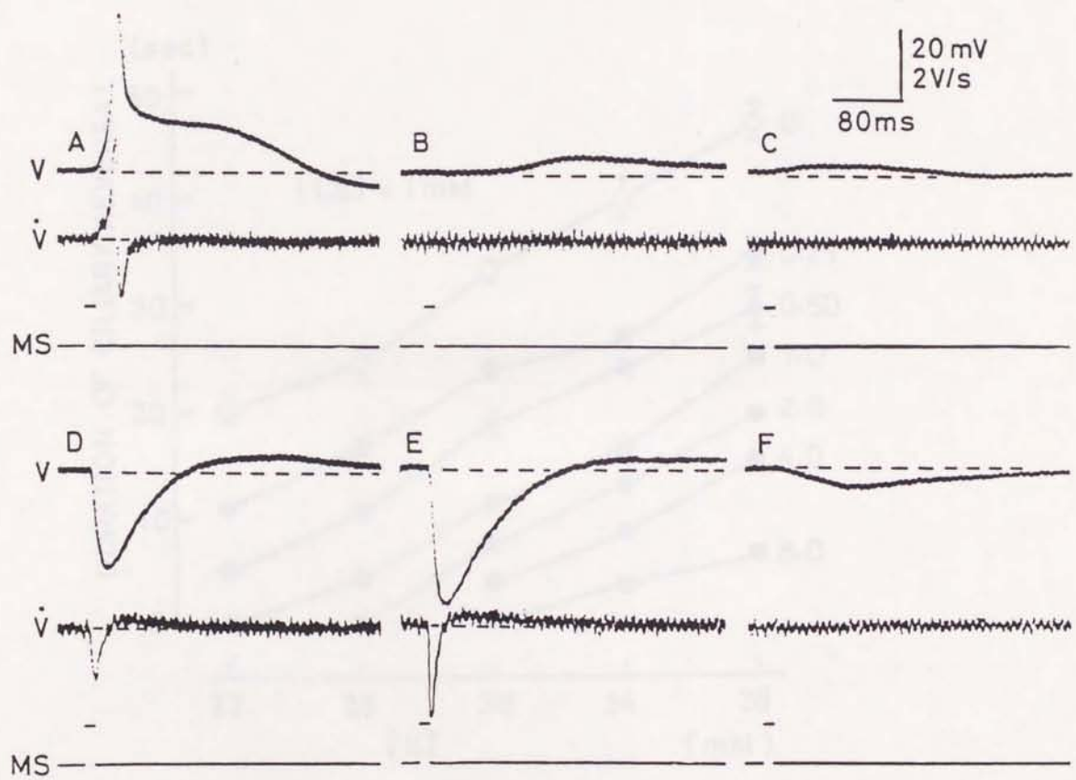


Fig. IV-5

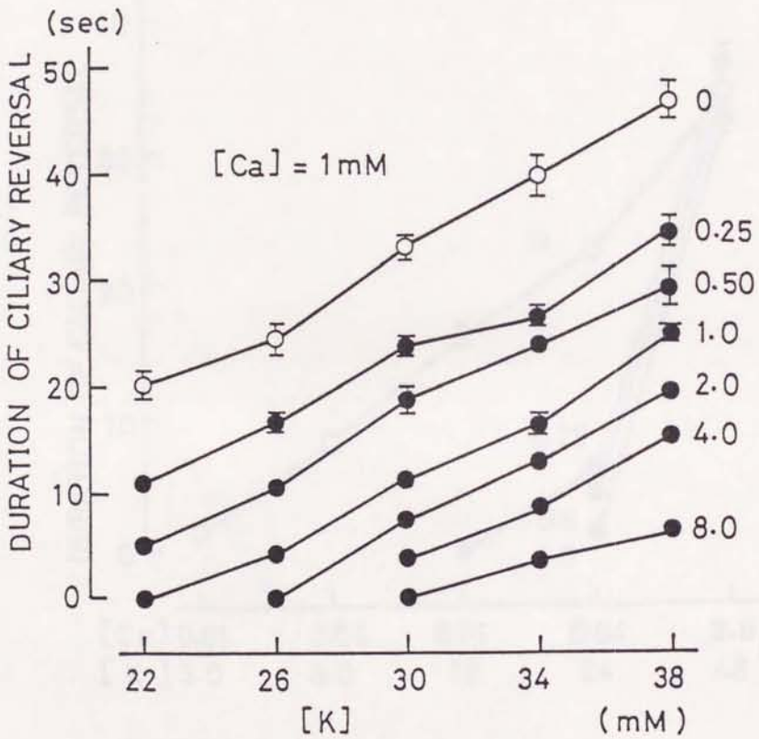

(sec)
DURATION OF CILIARY REVERSAL
50
40
30
20
10
0
[Ca] = 1mM
0
0.25
0.50
1.0
2.0
4.0
8.0
22
26
30
34
38
[K]
(mM)

Fig. IV-6

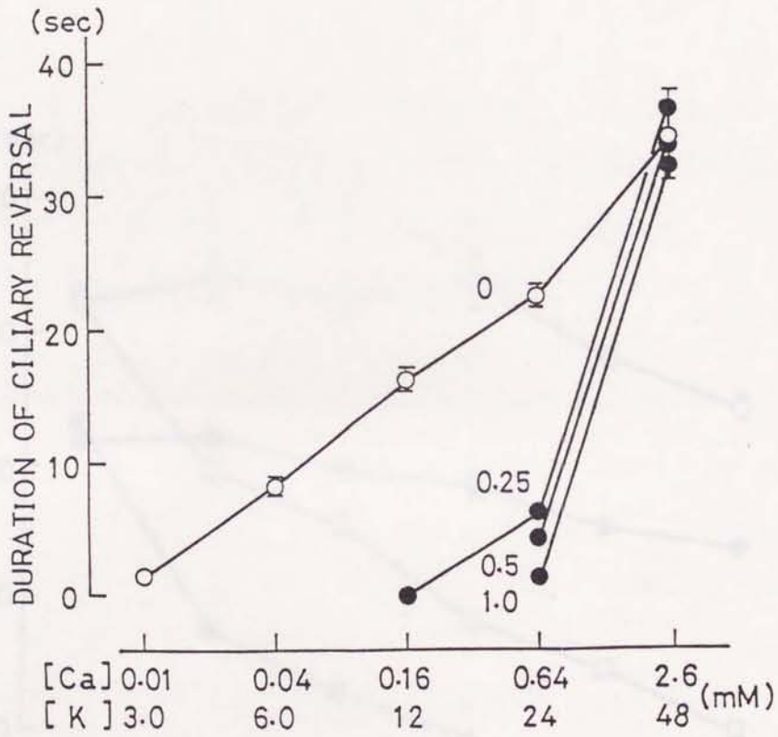
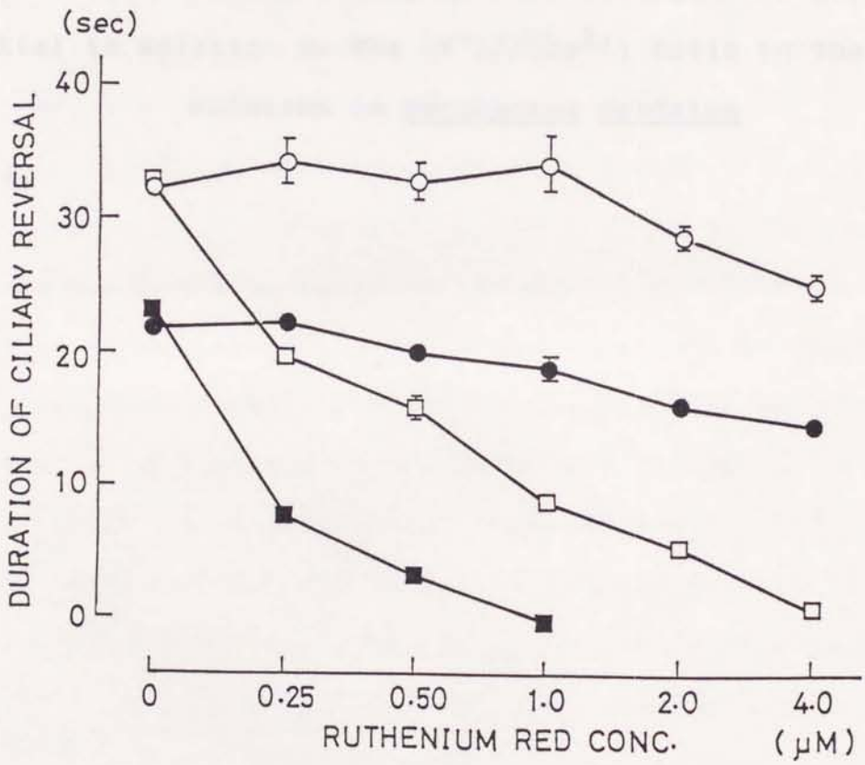

(sec)
40
30
20
10
0
DURATION OF CILIARY REVERSAL
0
0.25
0.5
1.0
[Ca] 0.01 0.04 0.16 0.64 2.6 (mM)
[K] 3.0 6.0 12 24 48

Fig. IV-7


(sec)
40
30
20
10
0
DURATION OF CILIARY REVERSAL
0
0·25
0·50
1·0
2·0
4·0
RUTHENIUM RED CONC.
(μM)

Part V

Theoretical Considerations of The Transmembrane Diffusion Potential in Relation to The $[K^+]/\sqrt{[Ca^{2+}]}$ Ratio in The External Solution in *Paramecium caudatum*

1. Theory.

According to theoretical analyses of membrane potential across an ion-exchanger membrane by Teorell (1935) and Meyer and Sievers (1936), the electrical potential difference between the external bulk solution and the cell interior (E_m) consists of a diffusion potential across the membrane (E_{diff}) and phase boundary potentials (Donnan potentials) at both surfaces of the membrane (E_{donn}). Thus E_m is written as;

$$E_m = E_{diff} + Eo_{donn} - Ei_{donn} \qquad (1),$$

where Eo_{donn} (Ei_{donn}) is the Donnan potential of the outer (inner) surface of the membrane (Fig. V-1).

E_{donn} and E_{diff} were formulated as a function of K^+ and Ca^{2+} concentrations in the solutions of both sides of the membrane to propose a mathematical model of the membrane potential of Paramecium according to the calculation methods by Watanabe et al. (1977) (see also Tasaki, 1968). The membrane of Paramecium behaves as a cation-exchanger (Naitoh and Yasumasu, 1967). The external solution as well as the internal solution of Paramecium consists mainly of KCl and $CaCl_2$ (Naitoh and Eckert, 1972). In this ionic condition E_{diff} and E_{donn} can be formulated as;

$$E_{donn} = \frac{RT}{F} \ln \frac{[K]_o (r_o + 1)}{[K]_i (r_i + 1)} \qquad (2),$$

and

$$E_{diff} = \frac{RT}{F} \frac{s - 1}{2s - 1} \ln \frac{(r_i + 1)(s r_o - s + 1)}{(r_o + 1)(s r_i - s + 1)} \qquad (3),$$

where R, T and F have conventional meanings. $[K]_o$ and $[K]_i$ are K^+ concentration in the external solution and in the cytoplasm respectively. s is the ratio of the mobility of Ca^{2+}(U_{Ca}) to

that of $K^+(U_K)$ in the membrane ($s=U_{Ca}/U_K$). r_o and r_i can be written as;

$$r_o = 1 + 8\ k\ X\ n_o \qquad (4),$$

and

$$r_i = 1 + 8\ k\ X\ n_i \qquad (4'),$$

where k is the equilibrium selectivity coefficient, X is the fixed charge density of the membrane, and n_o and n_i are the ratio of Ca^{2+} concentration to the square of K^+ concentration in the external solution and in the cytoplasm respectively.

k can be written as;

$$k = \frac{[K]_o^2\ (Ca)_o}{(K)_o^2\ [Ca]_o} = \frac{[K]_i^2\ (Ca)_i}{(K)_i^2\ [Ca]_i} \qquad (5),$$

where $[Ca]_o$ and $[Ca]_i$ are Ca^{2+} concentration in the external solution and in the cytoplasm respectively. $(K)_o$ and $(Ca)_o$ are K^+ and Ca^{2+} concentrations at the outer surface of the membrane respectively, and $(K)_i$ and $(Ca)_i$ are those at the inner surface of the membrane.

X, therefore, can be written as;

$$X = (K)_o + 2(Ca)_o = (K)_i + 2(Ca)_i \qquad (6).$$

and n_o and n_i can be written as;

$$n_o = \frac{[Ca]_o}{[K]_o^2} = \frac{1}{Ja^2} \qquad (7),$$

and

$$n_i = \frac{[Ca]_i}{[K]_i^2} \qquad (7').$$

Since $[K]_i$ and $[Ca]_i$ are assumed to be constant, r_i is constant. It is, therefore, obvious from the Eq. (3) that E_{diff} is a function of r_o, thus of n_o. This means that E_{diff} is

determined by Ja ratio ($[K]_o/\sqrt{[Ca]_o}$) independently of the absolute concentration of each ion in the external solution. It should be noted that the equation obtained by addition of the Eq. (2) with the Eq. (3) is identical with the equation of Tasaki and Kobatake (1968).

A change in the E_{diff} (dE_{diff}) produced by a change in Ja can be formulated from Eq. (3) as;

$$dE_{diff} = \frac{RT}{F} \frac{s - 1}{2s - 1} \ln \frac{(r_o1 + 1)(s r_o2 - s + 1)}{(r_o2 + 1)(s r_o1 - s + 1)} \quad (8),$$

where r_o1 and r_o2 are the values of r_o before and after a change in Ja respectively. s was assumed to be less than 1, since U_{Ca} is smaller than U_K in an aqueous solution (Robinson and Stokes, 1959). Actually, the value estimated so as theoretical calculation to fit to experimental results was 0.14 (see below). Since r_o decreases with an increase in Ja, dE_{diff} becomes positive when Ja is increased, and negative when Ja is decreased. A positive dE_{diff} corresponds to a membrane depolarization, and a negative dE_{diff} to a membrane hyperpolarization. It should be emphasized that these conclusion is dependent on only value of s (i.e. $s<1$) and independent of other parameters.

2. Voltage sensitivity of Ca channel.

As clearly shown in Table 1 (see also Naitoh et al., 1972), resting potential of Paramecium changes in association with a change in K^+ and/or Ca^{2+} concentration in the external solution. Threshold depolarization for producing a Ca action potential also changes in accordance with the change in the resting potential

(Naitoh et al., 1972). This means that the threshold depolarization for activation of voltage-sensitive Ca channels to a certain degree changes in accordance with the change in the resting potential.

Since resting membrane potential of Paramecium, V_{rest}, is presumable to consist of a surface potential and a diffusion potential, it can be written as;

$$V_{rest} = \Phi_s + V_c \qquad (9),$$

where Φ_s is a sum of the surface (Donnan) potentials at the both surfaces of the membrane. Therefore, Φ_s corresponds to E_{donn} and V_c to E_{diff} in Eq. (1). V_c is a voltage to which Ca channels in the membrane are subjected.

In the two-step voltage clamp from V_{rest} to a depolarized level, let V_{max} be a depolarized level, where maximum inward Ca current, I_{max}, was evoked, V_{max} can be written as;

$$V_{max} = \Phi_s + V_{cmax} \qquad (10),$$

where V_{cmax} is a transmembrane potential corresponding to I_{max} (Fig. V-2).

Determination of kX: Since V_{cmax} is thought to be a constant, it is clear from Eq. (10) that a change in V_{max} caused by a change in the external ionic environment is attributable to a change in Φ_s caused by that ionic change. From Eq. (2), the change in Φ_s ($d\Phi_s$) can be led as;

$$d\Phi_s = dE_{donn} = \frac{RT}{F} \frac{[K]_o1\ (r_o1 + 1)}{[K]_o2\ (r_o2 + 1)} \qquad (11),$$

where $[K]_o1$ and r_o1 are $[K]_o$ and r_o before the change in the ionic environment respectively, and $[K]_o2$ and r_o2 are those after the change.

When $[Ca]_o$ is changed, while $[K]_o$ is kept constant, Eq. (11) can be written as;

$$d\Phi_s = dE_{donn} = \frac{RT}{F} \frac{(r_o1 + 1)}{(r_o2 + 1)} \quad (12).$$

According to Eqs. (4) and (12), the value for kX was determined so as the calculated dE_{donn} to fit best to the actual data of dV_{max} shown in Table 1 (Fig. V-3E). The value was 120 mM.

Determination of s: Magnitude of depolarization corresponding to I_{max}, ∇_{max}, is;

$$\nabla_{max} = V_{max} - V_{rest} \quad (13),$$

From Eqs. (9) and (10), Eq. (13) is written as;

$$\nabla_{max} = V_{cmax} - V_c \quad (14).$$

A change in ∇_{max} ($d\nabla_{max}$) caused by a change in the external ionic environment is attributable to a change in V_c (dV_c) caused by that ionic change. Since V_c corresponds to E_{diff} in Eq. (3), dV_c can be written as;

$$dV_c = dE_{diff} = \frac{RT}{F} \frac{s - 1}{2s - 1} \ln \frac{(r_o1 + 1)(s\, r_o2 - s + 1)}{(r_o2 + 1)(s\, r_o1 - s + 1)} \quad (15),$$

According to Eqs. (4) and (15), and using 120 mM for kX, the value for s was determined so as the calculated dV_{diff} to fit best to the actual data of $d\nabla_{max}$ shown in Table 1 (Fig. V-3B). The value was 0.14.

Using these values for kX and s, calculated dV_{donn} and dV_{diff} were compared with the actual data of dV_{max} and $d\nabla_{max}$ respectively in other two series shown in Table 1; 1) K^+-varied at constant Ca^{2+} and 2) ionic strength varied at constant Ja. Each calculated dV_{donn} and dV_{diff} fitted fairly well to actual data (Fig. V-3).

3. Calculation of E_m.

E_m in various mixtures of K^+ and Ca^{2+} was calculated according to the Eqs. (1), (2) and (3), and the calculated E_ms were compared with the membrane potential of Paramecium measured in each corresponding mixture by Naitoh and Eckert (1968), Naitoh et al (1972) and Naitoh (unpublished data, Table 1). $[K]_i$ is assumed to be 18 mM (Naitoh and Eckert, 1973). $[Ca]_i$ is negligible (Naitoh and Kaneko, 1972). s = 0.14 and kX = 120 mM were used. Fig. V-4 shows the calculated E_m and E_{diff} together with the corresponding observed E_m. Each calculated E_m fits fairly well to the observed E_m. Deviation of the observed E_m from calculated E_m is seen in a lower Ca^{2+} concentration range in a series of varied Ca^{2+} at constant K^+ (Fig. V-4B). This might be attributable to some leakage of K^+ ions from the cell and resultant increase in the Ja ratio near the outer surface of the cell.

4. Relation between diffusion potential and membrane bound Ca.

According to Naitoh (1968), the percentage of membrane bound Ca (PCa) to total binding sites is

$$PCa = \frac{100}{k_N Ja + 1} \quad \text{or} \quad Ja = \frac{100 - PCa}{k_N PCa} \qquad (16),$$

where k_N is the equilibrium constant. Relationship between diffusion potential and membrane bound Ca was calculated from Eqs. (3), (4), (7) and (16) by introducing the value of k_N (0.35) (Naitoh and Yasumasu, 1967). The result is shown in Fig. V-5.

5. Some phenomena expectable from the theory.

In the case that s is larger than 1

s (U_{Ca}/U_K) is assumed to be less than 1 in the usual ionic condition in Paramecium membrane. If s is more than 1, it is expected that a change in Ja ratio produces a change in the diffusion potential toward a direction opposite to that in the case of s<1. It is obvious from Eq. (8) that membrane is hyperpolarized by an increase in Ja and depolarized by a decrease in Ja.

It is assumed that s might be more than 1 if we introduce Cs^+ ions instead of K^+ ions, since U_{Cs} is thought to be smaller than U_{Ca}. Therefore, specimens of Paramecium were equilibrated in 1 Cs + 1 Ca for various periods of time, then transferred into 20 Cs + 1 Ca. When equilibration time was less than 60 min, the specimen showed ciliary reversal for 18.4 $\pm$ 2.5 sec upon their transfer into 20 Cs + 1 Ca. However, when equilibration time was more than three hours, they did not show ciliary reversal. When the specimens were first equilibrated in 20 Cs + 1 Ca for more than 90 min, they showed ciliary reversal for 60.1 $\pm$ 11.7 sec upon their transferring into 1 Cs + 1 Ca.

Cs^+ ion is known to reduce membrane permeability remarkably to monovalent cation (Hennessey and Kung, 1984). When Paramecium is adapted in a Cs-Ca solution, K^+ ions bound by the membrane and intracellular K^+ ions are exchanged by Cs^+ ions with time. Thus the mobility of monovalent cation in the membrane might decrease, and s, therefore, becomes more than 1.

In the case that s equals 0

In excitable membranes such as nerve and muscle, an increase

in the external Ca^{2+} ions produced a shift of activation curve of Na channels toward positive direction along voltage axis with little change in resting membrane potential, and, therefore, made the membrane less excitable (Frunkenhaeuser and Hodgkin, 1957). This phenomenon which was called a 'Ca-stabilizing effect' was attributed to an increase in the diffusion potential (hyper-polarization) associated with a decrease in surface potential by binding of Ca^{2+} to the negative surface charges of the membrane. Change in the surface potential was analyzed by Gouy-Chapman equation for the diffuse double layer (Ohmori and Yoshii, 1977; Cota and Stefani, 1984; Byerly et al., 1985).

This phenomenon is well understood also based on the present theory. When the mobility of Ca^{2+} in the membrane is assumed to be negligible (s=0), in other words, Ca permeability is very low as in nerves and muscles, Eq. (3) can be written as;

$$E_{diff} = \frac{RT}{F} \ln \frac{r_i + 1}{r_o + 1} \qquad (17).$$

From Eqs. (2) and (17), total membrane potential is represented as;

$$E_m = E_{donn} + E_{diff} = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i} \qquad (18).$$

It is obvious from Eq. (18) that, when external Ca^{2+} concentration is changed, E_m does not change (showing equilibrium potential of K), while diffusion potential changes to an extent identical with that in the surface potential but in opposite direction.

6. Discussion.

Hook and Hildebrand (1980) analyzed transmembrane potential (V_c) in Paramecium according to Gouy-Chapman theory for the diffuse double layer. In their analysis, V_c was not represented as a simple function of Ja ratio. However they demonstrated that, when ionic strength was increased so as to keep Ja ratio constant, V_c was kept almost constant, because the surface potential (Φ_s) and the resting potential (V_{rest}) decreased concomitantly. There are still some difficulties in explaining the Ja dependency in membrane excitation by ionic stimulation, because their calculation depends on too many parameters. In contrast, Ja-ratio dependency of V_c can be explained by the present theory independently of parameters in the equation (8) with only an assumption that s is less than 1. It should be noted that V_c can be formulated and analyzed independently of Φ_s in the present theory, while V_c is given as a difference between the V_{rest} and the theoretically calculated Φ_s in the analysis using Gouy-Chapman theory.

The duration of the ciliary reversal in response to ionic stimulation is affected by 1) the initial amount of Ca bound to the membrane, 2) the amount of Ca^{2+} released from binding sites in the stimulation medium and 3) the absolute concentration of Ca^{2+} in the stimulation medium (Naitoh, 1968). The first factor may be related to the threshold level of activation of Ca channels, because it determines the depth of transmembrane diffusion potential (i.e. V_c). The second factor corresponds to the amplitude of depolarization by a Ja-ratio increase. The third factor affects the electromotive force acting on Ca^{2+} ions which

flow into the cell through activated Ca channels.

In contrast, transient inward current (Ca-current) induced by depolarizing voltage clump was dependent on the Ja ratio (therefore, the amount of bound Ca) but not absolute Ca^{2+} concentration in the external solution (Table 1, I_{max} in the condition of Ja ratio constant). Amplitude of inward Ca current depends on Ca^{2+} concentration in just outer side of Ca channels. Therefore, the actual electromotive force for the transient inward current depends on the amount of membrane bound Ca. In this case, equilibration between external bulk solution and membrane is disordered transiently in association with inflow of Ca^{2+} due to its fairly fast reaction. On the other hand, in the case of ionic stimulation, the equilibration is scarcely disordered during long-lasting activation of Ca channels because of its slower reaction. Therefore, the actual electromotive force for the long-lasting inflow of Ca^{2+} depends on Ca^{2+} concentration of bulk solution.

Ja ratio-dependency of Ca-channel activation by ionic stimulation in Paramecium membrane was clearly interpreted with the present theory; an increase in Ja ratio in the external solution causes a decrease in the diffusion potential. Present theory can simulate fairly well the resting membrane potential and voltage sensitivity of Ca channels in Paramecium. This theory is applicable to general excitable membranes other than Paramecium membrane.

FIGURE LEGENDS

Figure V-1. Schematic presentation of the distribution of electrical potentials near and across the membrane of Paramecium. OUT; external solution. IN; internal solution. $[K]_o$ and $[Ca]_o$; K^+ and Ca^{2+} concentrations in the external solution respectively. $[K]_i$ and $[Ca]_i$; K^+ and Ca^{2+} concentrations are those in the internal solution. $(K)_o$ and $(Ca)_o$; K^+ and Ca^{2+} concentrations at the outer surface of the membrane respectively. $(K)_i$ and $(Ca)_i$; K^+ and Ca^{2+} concentrations at the inner surface of the membrane. E_m; observed membrane potential. Eo_{donn} and Ei_{donn}; Donnan potential of the outer and the inner surfaces of the membrane respectively. E_{diff}; diffusion potential across the membrane.

Figure V-2. Schematic presentation of the relationship between the surface potential and the transmembrane potentials relating to activation of Ca channels. Φ_s; a sum of the surface potentials at the both surfaces of the membrane. V_{rest}; observed resting membrane potential. V_c; a voltage to which Ca channels in the membrane are subjected in resting condition. V_{max}; a depolarized level of membrane potential where maximum inward Ca current (I_{max}) was evoked. V_{cmax}; a transmembrane potential corresponding to I_{max}. ∇_{max}; depolarization corresponding to I_{max}.

Figure V-3. Comparison between calculated E_{diff} and measured shifts in ∇_{max} ($d\nabla_{max}$; upper) and V_{max} (dV_{max}; lower) in three different ionic conditions. $d\nabla_{max}$ and dV_{max} are measured

relative to those in 2 mM K + 1 mM Ca solution. Experimental data (open circles) come from Naitoh (unpublished) (Table 1). Solid lines are shifts in calculated transmembrane potentials (dE_{diff}). A and D; varied K^+ at constant Ca^{2+} (1 mM). B and E; varied Ca^{2+} at constant K^+ (2 mM). C and F; varied ionic strength at constant Ja ratio (2).

Figure V-4. Comparison between calculated and measured membrane potentials of P. caudatum. Experimental data are from Naitoh and Eckert (1968) (open circles), Naitoh et al. (1972) (solid circles) and Naitoh (unpublished) (Table 1) (open squares). Solid lines are calculated membrane potentials (E_m). Broken lines are calculated transmembrane diffusion potentials (E_{diff}). A; varied K^+ at constant Ca^{2+} (1 mM). B; varied Ca^{2+} at constant K^+ (2 mM). C; varied Ca^{2+} without K^+. D, E and F; varied ionic strength at constant Ja ratio, 1, 2 and 8 respectively.

Figure V-5. Relationship between the amount of bound Ca (PCa) and transmembrane diffusion potential (E_{diff}). E_{diff} was calculated from Eqs. (3), (4), (7) and (16) in the text as a function of PCa. Ja ratios which correspond to PCa are indicated on the abscissae.

Table 1. Concentration effects of K^+ and Ca^{2+} in the external solution on the membrane characteristics in voltage-clamped Paramecium caudatum.

$[K^+]_o$	$[Ca^{2+}]_o$	Ja	V_{rest} (mV)	I_{max} (%)	∇_{max} (mV)	$d\nabla_{max}$	V_{max} (mV)	dV_{max}
1	1	1	-32.0	109	46.5	4.2	14.5	1.0
2	1	2	-28.8	100	42.3	0	13.5	0
4	1	4	-20.6	86	38.4	- 3.9	17.8	4.3
8	1	8	-11.0	72	31.5	-10.8	20.5	7.0
16	1	16	+ 1.0	23	21.0	-21.3	22.0	8.5
2	10	0.63	-10.3	106	50.0	6.7	39.7	25.2
2	1	2	-28.8	100	43.3	0	14.5	0
2	0.1	6.3	-35.7	67	33.3	-10.0	- 2.4	-16.9
2	0.01	20	-46.0	54	19.5	-23.8	-26.5	-41.0
0.5	0.063	2	-60.5	98	46.0	- 0.5	-14.5	-32.0
1	0.25	2	-45.5	99	46.5	0	1.0	-16.5
2	1	2	-29.0	100	46.5	0	17.5	0
4	4	2	- 9.5	101	46.5	0	37.0	19.5

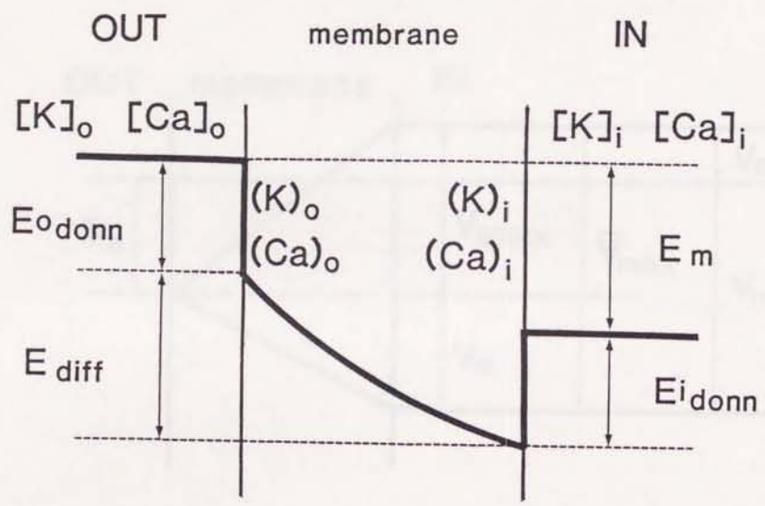
I_{max}; the largest peak value of the early inward current determined in the I-V relationships for the peak inward current.

∇_{max}; magnitude of depolarization corresponding to I_{max}.

V_{max}; depolarization level of membrane potential where the I_{max} is evoked, equal to $V_{rest} + \nabla_{max}$.

I_{max}, $d\nabla_{max}$ and dV_{max} are relative to values for 2 mM K + 1 mM Ca solution. (from Naitoh unpublished data).

Fig. V-1



$$E_{donn} = E^{o}_{donn} + E^{i}_{donn}$$

$$E_{m} = E_{donn} + E_{diff}$$

Fig. V-2

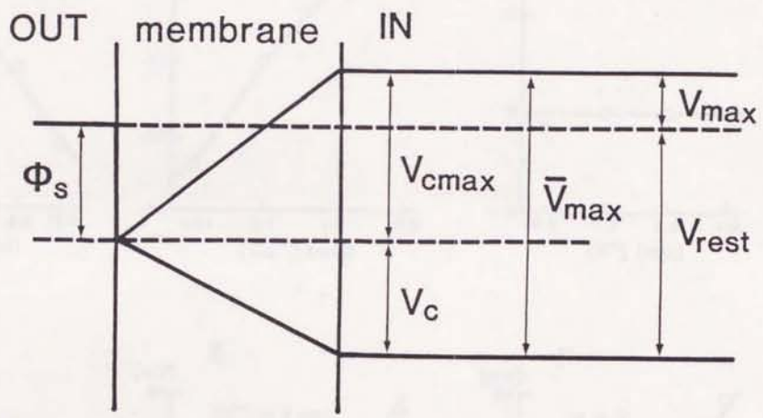


Fig. V-3

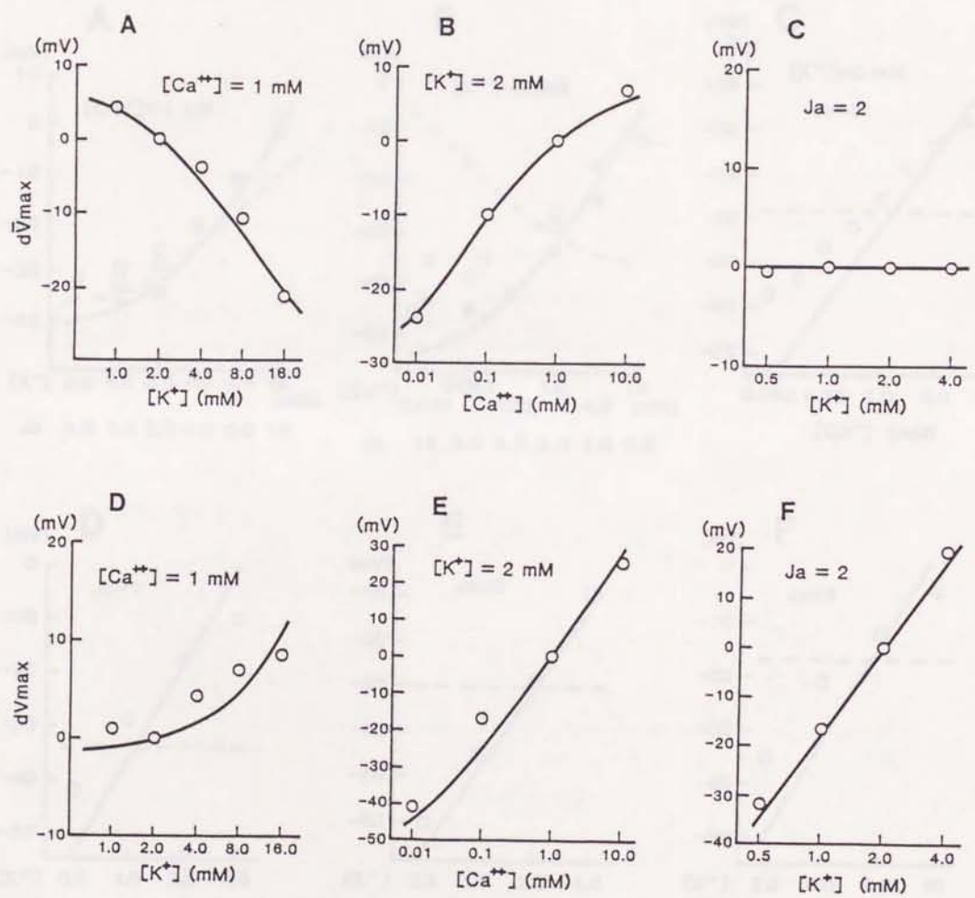

A
(mV)
10
0
-10
-20
dV̄max
[Ca++] = 1 mM
1.0 2.0 4.0 8.0 16.0
[K+] (mM)
B
(mV)
10
0
-10
-20
-30
[K+] = 2 mM
0.01 0.1 1.0 10.0
[Ca++] (mM)
C
(mV)
20
10
0
-10
Ja = 2
0.5 1.0 2.0 4.0
[K+] (mM)
D
(mV)
20
10
0
-10
dVmax
[Ca++] = 1 mM
1.0 2.0 4.0 8.0 16.0
[K+] (mM)
E
(mV)
30
20
10
0
-10
-20
-30
-40
-50
[K+] = 2 mM
0.01 0.1 1.0 10.0
[Ca++] (mM)
F
(mV)
20
10
0
-10
-20
-30
-40
Ja = 2
0.5 1.0 2.0 4.0
[K+] (mM)

Fig. V-4

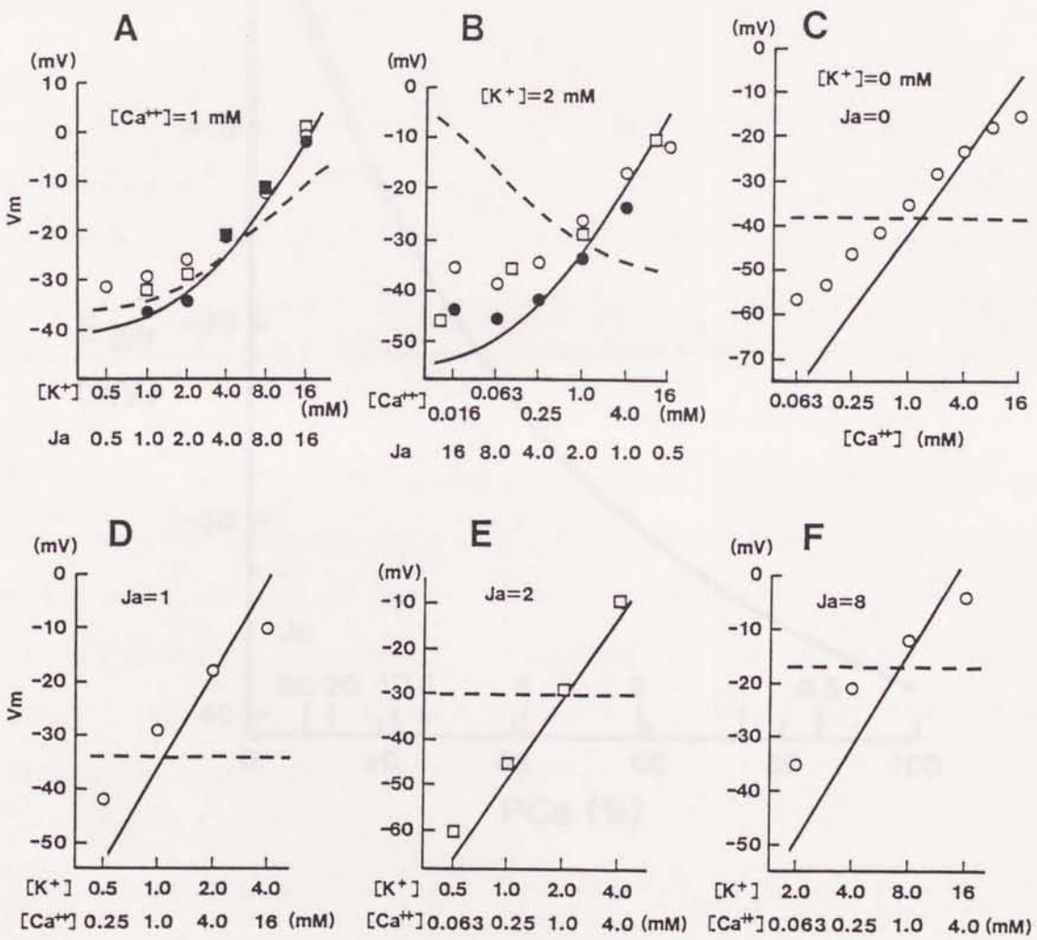
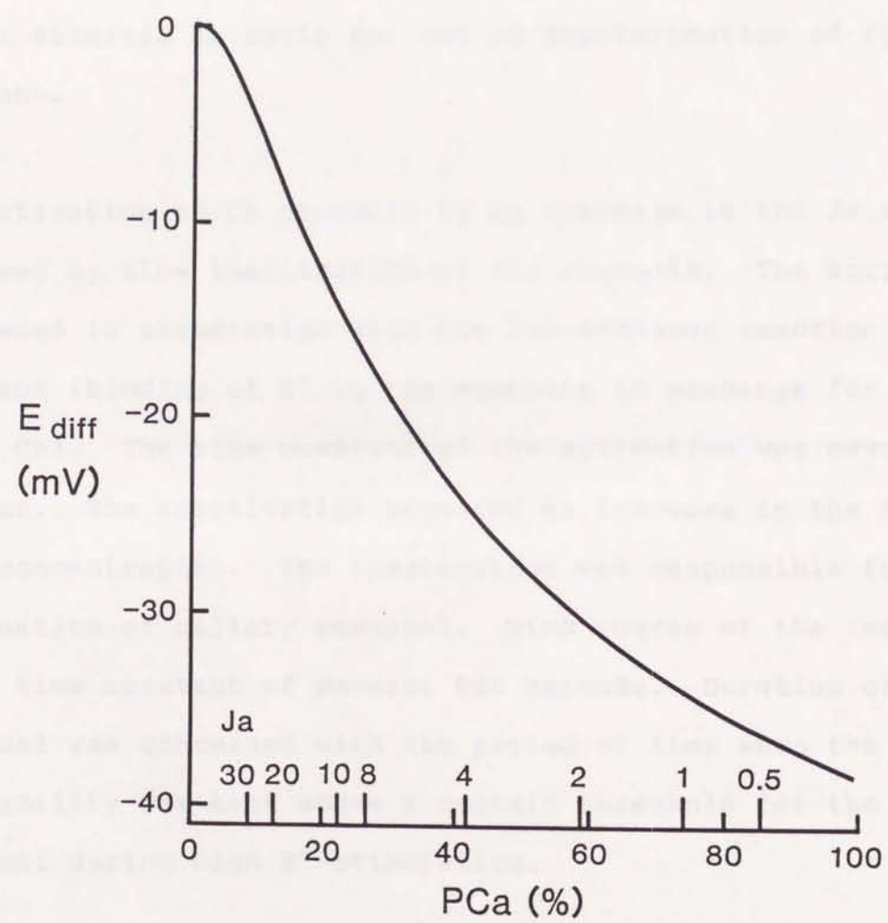

A
(mV)
[Ca++]=1 mM
Vm
[K+] 0.5 1.0 2.0 4.0 8.0 16 (mM)
Ja 0.5 1.0 2.0 4.0 8.0 16
B
(mV)
[K+]=2 mM
[Ca++] 0.016 0.063 0.25 1.0 4.0 16 (mM)
Ja 16 8.0 4.0 2.0 1.0 0.5
C
(mV)
[K+]=0 mM
Ja=0
0.063 0.25 1.0 4.0 16
[Ca++] (mM)
D
(mV)
Ja=1
Vm
[K+] 0.5 1.0 2.0 4.0
[Ca++] 0.25 1.0 4.0 16 (mM)
E
(mV)
Ja=2
[K+] 0.5 1.0 2.0 4.0
[Ca++] 0.063 0.25 1.0 4.0 (mM)
F
(mV)
Ja=8
[K+] 2.0 4.0 8.0 16
[Ca++] 0.063 0.25 1.0 4.0 (mM)

Fig. V-5



SUMMARY

1. Electrophysiological evidences for increase in Ca conductance of Paramecium membrane during ciliary reversal elicited by ionic stimulation were presented. Activation of depolarization-sensitive Ca channels of Paramecium was dependent on an increase in the external Ja ratio but not on depolarization of the membrane.

2. Activation of Ca channels by an increase in the Ja ratio was followed by slow inactivation of the channels. The activation proceeded in association with the ion-exchange reaction on the membrane (binding of K^+ to the membrane in exchange for membrane bound Ca). The time constant of the activation was several seconds. The inactivation required an increase in the internal Ca^{2+} concentration. The inactivation was responsible for termination of ciliary reversal. Time course of the inactivation had a time constant of several ten seconds. Duration of ciliary reversal was concerned with the period of time when the Ca permeability was kept above a certain threshold for the ciliary reversal during high K^+-stimulation.

3. The inactivation was not removed even if internal Ca^{2+} concentration was reduced below 10^{-6} M, when it was once accomplished with the Ca dependent mechanism. The change in the state of inactivation was termed "fixation" of inactivated Ca channels. The removal of the inactivation depends on membrane hyperpolarization.

4. Ruthenium Red (R.R.), which binds to the anionic sites of the membrane in competition with K^+ or Ca^{2+}, inhibited activation of Ca channels and ciliary reversal. It is suggested that negative charges of mucopolysaccharide layer composing surface coat of the membrane are important in the electrogenesis and the property as an ion-exchanger of Paramecium membrane.

5. Electrogenesis in Paramecium membrane was analyzed according to a theory for the potential generation across an ion-exchange membrane. The transmembrane diffusion potential could be presented as a function of the external Ja ratio. The theory clearly interpreted Ja ratio-dependency of Ca-channel activation by ionic stimulation. The theory could simulate fairly well the resting membrane potential and the voltage sensitivity of Ca channels in Paramecium. The theory is applicable to general excitable membranes.

REFERENCES

Armstrong, C.M. and Bezanilla, F. (1973). Currents related to movement of the gating particles of the sodium channels. Nature 242:459-451.

Armstrong, C.M. and Bezanilla, F. (1974). Charge movement associated with the opening and closing of the activation gates of the Na channels. J. Gen. Physiol. 63:533-552.

Bancroft, F.W. (1906). The control of galvanotropism in Paramecium by chemical substances. Univ. Calif. Publ. (Berkeley) Physiol. 3:21-31.

Baux, G., Simonneau, M. and Tauc, L. (1979). Transmitter release: Ruthenium red used to demonstrate a possible role of sialic acid containing substrates. J. Physiol. (Lond.) 291:161-178.

Bezanilla, F. and Armstrong, C.M. (1974). Gating currents of the sodium channels: Three ways to block them. Science 183:753-754.

Bhattacharyya, M.L., Nathan, R.D. and Shelton, R.D. (1981). Release of sialic acid alters the stability of the membrane potential cardiac muscle. Life Sciences 29:1071-1078.

Brehm, P., Dunlap, K. and Eckert, R. (1978). Calcium-dependent repolarization in Paramecium. J. Physiol. (Lond.) 274:639-654.

Brehm, P. and Eckert, R. (1978). Calcium entry leads to inactivation of calcium channel in Paramecium. Science 202:1203-1206.

Brehm, P., Eckert, R. and Tillotson, D. (1980). Calcium-mediated inactivation of calcium current in Paramecium. J. Physiol. (Lond.) 306:193-203.

Brooks, R.E. (1969). Ruthenium red stainable surface layer on lung alveolar cells; electron microscopic interpretation. Stain Tech. 44:173-177.

Byerly, L., Chase, B. and Stimers, J.R. (1985). Permeation and interaction of divalent cations in calcium channels of snail neurons. J. Gen. Physiol. 85:491-518.

Chad, J.E. and Eckert, R. (1986). An enzymatic mechanism for calcium current inactivation in dialysed Helix neurones. J. Physiol. (Lond.) 378:31-51.

Cota, G. and Stefani, E. (1984). Saturation of calcium channels and surface charge effects in skeletal muscle fibers of the frog. J. Physiol. (Lond.) 351:135-154.

Dörrscheidt-Käfer, M. (1979). Excitation-contraction coupling in frog sartorius and the role of the surface charge due to the carboxyl group of sialic acid. Pflügers Arch. 380:171-179.

Dunlap, K. (1976). Calcium channels in Paramecium confined to ciliary membrane. Am. Zool. 16:185.

Dunlap, K. (1977). Localization of calcium channels in Paramecium caudatum. J. Physiol. (Lond.) 271:119-133.

Eckert, R. (1972). Bioelectric control of ciliary activity. Science 176:473-481.

Eckert, R. and Brehm, P. (1979). Ionic mechanisms of excitation in Paramecium. Ann. Rev. Biophys. Bioeng. 8:353-383.

Eckert, R. and Chad, J.E. (1984). Inactivation of Ca channels. Prog. Biophys. molec. Biol. 44:215-267.

Eckert, R. and Naitoh, Y. (1970). Passive electrical properties of Paramecium and problems of ciliary coordination. J. Gen.

Physiol. 55:467-483.

Eckert, R. and Naitoh Y. (1972). Bioelectric control of locomotion in the ciliates. J. Protozool. 19:237-243.

Eckert, R., Naitoh, Y. and Friedman, K. (1972). Sensory mechanisms in Paramecium. I. Two components of electric response to mechanical stimulation of the anterior surface. J. exp. Biol. 56:683-694.

Eckert, R., Naitoh, Y. and Machemer, H. (1976). Calcium in the bioelectric and motor functions of Paramecium. Symp. Soc. exp. Biol. 30:233-255.

Frankenhaeuser, B. (1957). The effect of calcium on the myelinated nerve fibre. J. Physiol. (Lond.) 137:245-260.

Frankenhaeuser, B. and Hodgkin, A.L. (1957). The action of calcium on the electrical properties of squid axons. J. Physiol. (Lond.) 137:218-244.

Hennessey, T.M. and Kung, C. (1984). An anticalmodulin drug, W-7, inhibits the voltage-dependent calcium current in Paramecium caudatum. J. exp. Biol. 110:169-181.

Hennessey, T.M. and Kung, C. (1985). Slow inactivation of the calcium current of Paramecium is dependent on voltage and not internal calcium. J. Physiol. (Lond). 365:165-179.

Hildebrand, E. (1978). Ciliary reversal in Paramecium: Temperature dependence of K^+-induced excitability decrease and of recovery. J. comp. Physiol. 127:39-44.

Hildebrand, E. and Dryl, S. (1976). Significance of Ca^{2+} and K^+ ions for the excitation of the protozoan membrane. Bioelectrochem. Bioenerg. 3:543-544.

Hill, S.E. and Osterhout, W.J.V. (1938). Calculations of bioelectric potentials. II. The concentration potential of KCl in Nitella. J. Gen. Physiol. 21:541-556.

Hille, B. (1968). Charges and potentials at the nerve surface divalent ions and pH. J. Gen. Physiol. 51:221-236.

Hodgkin, A.L. and Huxley, A.F. (1952). Current carried by sodium and potassium ions through the membrane of the giant axon of Loligo. J. Physiol. (Lond.) 116:449-472.

Hodgkin, A.L. and Katz, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. J. Physiol. (Lond.) 108:37-77.

Hook, C. and Hildebrand, E. (1980). Excitability of Paramecium and the significance of negative surface charges. J. Math. Biology 9:347-360.

Jahn, T.L. (1962). The mechanism of ciliary movement. II. Ion antagonism and ciliary reversal. J. cell. comp. Physiol. 60:217-218.

Jennings, H.S. (1906). Behavior of the lower organisms. Columbia University Press.

Kamada, T. (1934). Some observations on potential differences across the ectoplasm membrane on Paramecium. J. exp. Biol. 11:94-102.

Kamada, T. and Kinosita, H. (1940). Calcium-potassium factor in ciliary reversal of Paramecium. Jap. Acad. Proc. 16:125-130.

Keynes, R.D. and Rojas, E. (1974). Kinetics and steady state properties of the charge system controlling sodium conductance in the squid giant axon. J. Physiol. (Lond.) 239:393-434.

Kinosita, H., Dryl, S. and Naitoh, Y. (1964). Changes in the

membrane potential and the response to stimuli in Paramecium. J. Fac. Sci. Univ. Tokyo, IV. 10:291-301.

Kinosita, H., Murakami, A. and Yasuda, M. (1965). Interval between membrane potential change and ciliary reversal in Paramecium immersed in Ba-Ca mixture. J. Fac. Sci. Tokyo Univ. IV, 10:421-425.

Kung, C. (1971). Genic mutants with altered system of excitation in Paramecium aurelia. Z. vergl. Physiol. 71:142-164.

Kung, C. and Eckert, R. (1972). Genetic modification of electric properties in an excitable membrane. Proc. natn. Acad. Sci. U.S.A. 69:93-97.

Kung, C. and Naitoh, Y. (1973). Calcium-induced ciliary reversal in the extracted models of 'pawn' a behavioral mutant of Paramecium. Science 179:195-196.

Kung, C. and Saimi, Y. (1982). The physiological basis of taxes in Paramecium. Ann. Rev. Physiol. 44:519-534.

Luft, J.H. (1971a). Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anatomical Record 171:347-368.

Luft, J.H. (1971b). Ruthenium red and violet. II. Fine structural localisation in animal tissues. Anatomical Record 171:369-416.

Machemer, H. and Eckert, R. (1975). Ciliary frequency and orientational responses to clamped voltage steps in Paramecium. J. comp. Physiol. 104:247-260.

Machemer, H. and Ogura, A. (1979). Ionic conductances of membranes in ciliated and deciliated Paramecium. J. Physiol. 296:49-60.

Mast, S.O. and Nadler, J.E. (1926). Reversal of ciliary action in Paramecium caudatum. J. Morphol. Physiol. 43:105-117.

Meyer, K.H. and Sievers, J.F. (1936). La perméabilité des membranes I. Théorie de la perméabilité ionique. Helv. Chim. Acta. 19:649-665.

Naitoh, Y. (1968). Ionic control of the reversal response of cilia in Paramecium caudatum. J. Gen. Physiol. 51:85-103.

Naitoh, Y. (1969). Control of the orientation of cilia by adenosine-triphosphate, calcium and zing in glycerol extracted Paramecium caudatum. J. gen. Physiol. 53:517-529.

Naitoh, Y. (1979). Membrane currents in voltage-clamped Paramecium and their relations to ciliary motion. Acta protozool. 18:1-6.

Naitoh, Y. and Eckert, R. (1968a). Electrical properties of Paramecium caudatum: modification by bound and free cations. Z. vergl. Physiol. 61:427-452.

Naitoh, Y. and Eckert, R. (1968b). Electrical properties of Paramecium caudatum: all or none electrogenesis. Z. vergl. Physiol. 61:453-472.

Naitoh, Y. and Eckert, R. (1969). Ionic mechanisms controlling behavioral responses of Paramecium to mechanical stimulation. Science 164:963-965.

Naitoh, Y. and Eckert, R. (1972). Electrophysiology of the ciliate protozoa. In Experiments and Physiology and Biochemistry, Vol. V. Ed. G.A. Kerkut. London: Academic Press. 17-38.

Naitoh, Y. and Eckert, R. (1973). Sensory mechanisms in Paramecium. II. Ionic basis of the hyperpolarizing

mechanoreceptor potential. J. exp. Biol. 59:53-65.

Naitoh, Y. and Eckert, R. (1974). The control of ciliary activity in protozoa. In cilia and flagella. Ed. M.A. Sleigh. Academic Press, London. 305-352.

Naitoh, Y., Eckert, R. and Friedman, K. (1972). A regenerative calcium response in Paramecium. J. exp. Biol. 56:667-681.

Naitoh, Y. and Kaneko, H. (1972). Reactivated triton-extracted models of Paramecium: modification of ciliary movement by calcium ions. Science 176:523-524.

Naitoh, Y. and Kaneko, H. (1973). Control of ciliary activities by adenosine triphosphate and divalent cations in Triton-extracted models of Paramecium caudatum. J. exp. Biol. 58:657-676.

Naitoh, Y. and Yasumasu, I. (1967). Binding of Ca ions by Paramecium caudatum. J. Gen. Physiol. 50:1303-1310.

Onimaru, H. and Naitoh, Y. (1982). The liberation of bound calcium and the opening of calcium channels in Paramecium membrane. Zool. Mag. (Tokyo). 91:422.

Oertel, D., Schein, S.J. and Kung, C. (1977). Separation of membrane currents using a Paramecium mutant. Nature 268:120-124.

Oertel, D., Schein, S.J. and Kung, C. (1978). A potassium conductance activated by hyperpolarization in Paramecium. J. Membrane Biol. 43:169-185.

Ogura, A. and Machemer, H. (1980). Distribution of mechanoreceptor channels in the Paramecium surface membrane. J. comp. Physiol. 135:223-242.

Ogura, A. and Takahashi, K. (1976). Artificial deciliation causes loss of calcium-dependent responses in Paramecium. Nature 264:170-172.

Ohmori, H. and Yoshii, M. (1977). Surface potential reflected in both gating and permeation mechanisms of sodium and calcium channels of the tunicate egg cell membrane. J. Physiol. (Lond.) 267:429-463.

Robertson, B. and Wann, K.T. (1987). On the action of Ruthenium red and neuraminidase at the frog neuromuscular junction. J. Physiol. (Lond.) 382:411-423.

Robinson, R.A. and Stokes, R.H. (1959). Electrolyte Solution, Butterworths. p463.

Satow, Y. (1978). Internal calcium concentration and potassium permeability in Paramecium. J. Neurobiol. 9:81-91.

Satow, Y. and Kung, C. (1979). Voltage sensitive Ca-channels and the transient inward current in Paramecium tetraurelia. J. exp. Biol. 78:149-161.

Satow, Y. and Kung, C. (1980). Ca-induced K^+-outward current in Paramecium tetraurelia. J. exp. Biol. 88:293-303.

Satow, Y. and Kung, C. (1981). Possible reduction of surface charge by a mutation in Paramecium tetraurelia. J. Membrane Biol. 59:179-190.

Schein, S.J., Bennett, M.V.L. and Katz, G.M. (1976). Altered calcium conductance in Pawns, behavioural mutants of Paramecium aurelia. J. exp. Biol. 65:699-724.

Takahashi, M. and Naitoh, Y. (1978). Behavioural mutants of Paramecium caudatum with defective membrane electrogenesis. Nature 271:656-659.

Tasaki, I. (1959). Demonstration of two stable states of the nerve membrane in potassium-rich media. J. Physiol. (Lond.) 148:306-331.

Tasaki, I. (1968). Nerve excitation, a macromolecular approach. Charles C. Thomas, Springfield, I11.

Tasaki, I. and Kobatake, Y. (1968). A theory of membrane potential. In Nerve excitation. Charles C. Thomas, Springfield, I11. 167-180.

Tasaki, I., Singer, I. and Watanabe, A. (1966). Observations of excitation of squid giant axons in sodium-free external media. Amer. J. Physiol. 211:746-754.

Tasaki, I., Takenaka, T. and Yamagishi, S. (1968). Abrupt depolarization and bi-ionic action potentials in internally perfused squid giant axons. Am. J. Physiol. 215:152-159.

Teorell, T. (1935). An attempt to formulate a quantitative theory of membrane permeability. Proc. Soc. Exp. Biol. Med. 33:282-285.

Tobias, J.M. (1964). A chemically specified molecular mechanism underlying excitation in nerve: A hypothesis. 203:13-17.

Verworn, M. (1889). Die polare Erregung der Protisten durch den galvanischen Strom. Pflüger Arch. ges. Physiol. 46:281-303.

Wyroba, E. and Przelecka, A. (1973). Studies on the surface coat of Paramecium aurelia. 1. Ruthenium Red staining and enzyme treatment. Z. Zellforsch. 143:343-353.

Yamaguchi, T. (1960). Studies on the modes of ionic behavior across the ectoplasmic membrane of Paramecium. I. Electric potential differeces measured by the intracellular

microelectrode. J. Fac. Sci. Univ. Tokyo, IV. 8:573-591.

Watanabe, A., Terakawa, S. and Nagano, M. (1977). Excited and resting states of the axonal membrane. Membrane. 2:326-341.

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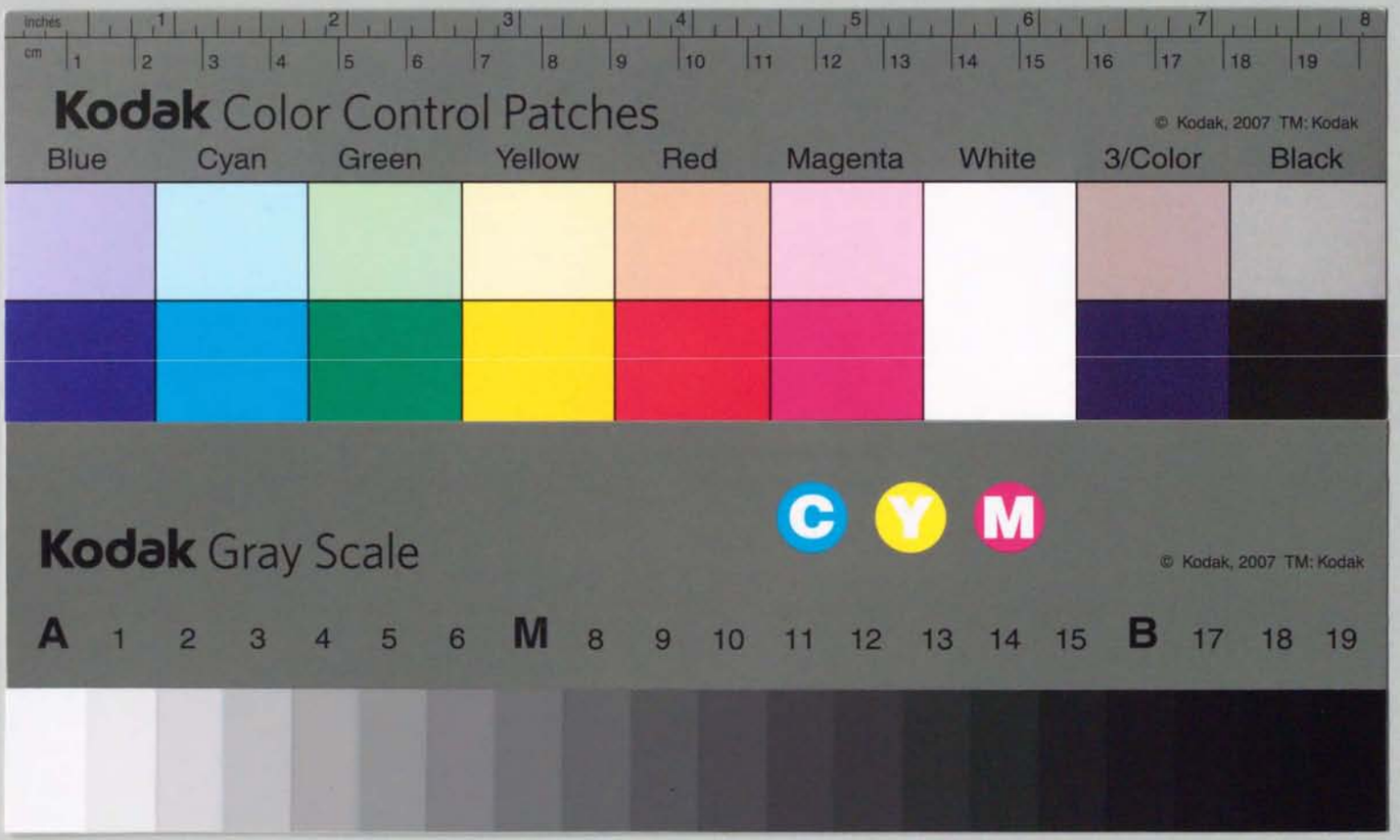
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