Electrophysiological Control of Reversed Ciliary Beating in Paramecium

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ABSTRACT Quantitative relations between ciliary reversal and membrane responses were examined in electrically stimulated paramecia. Specimens bathed in 1 mM CaCl₂, 1 mM KCl, and 1 mM Tris-HCl, pH 7.2, were filmed at 250 frames per second while depolarizing current pulses were injected. At current intensities producing only electrotonic shifts the cilia failed to respond. Stimuli which elicited a regenerative response were followed by a period of reversed ciliary beating. With increasing stimulus intensities the latency of ciliary reversal dropped from 30 to 4 ms or less, and the duration of reversal increased from 50 ms to 2.4 s or more; the corresponding regenerative responses increased in amplitude and rate of rise. With progressively larger intracellular positive pulses, electric stimulation became less effective, producing responses with a progressive increase in latency and decrease in duration of reversed beating of the cilia. When 100-ms pulses shifted the membrane potential to +70 mV or more, ciliary reversal was suppressed until the end of the pulse. “Off” responses then occurred with a latency of 2–4 ms independent of further increases in positive potential displacement. These results suggest that ciliary reversal is coupled to membrane depolarization by the influx of ions which produces the regenerative depolarization of the surface membrane. According to this view suppression of the ciliary response during stimulation occurs when the membrane potential approaches the equilibrium potential of the coupling ion, thereby retarding its influx. Previous data together with the present findings suggest that this ion is Ca²⁺.

INTRODUCTION

The electrical activity of the cell membrane in Paramecium and other ciliates plays an important role in the regulation of cell motility (Kinosita and Murakami, 1967; Eckert, 1972). Depolarization, either by sensory transduction (Naitoh and Eckert, 1969; Eckert et al., 1972) or by current injected with a microelectrode (Eckert and Naitoh, 1969), produces a graded, spike-like response. The maximum peak attained by this regenerative response varies with the concentration of Ca²⁺ (Ba²⁺ or Sr²⁺) in a manner approaching the
slope predicted by the Nernst relation for a pure Ca\textsuperscript{2+} (Ba\textsuperscript{2+}, Sr\textsuperscript{2+}) electrode (Eckert and Naitoh, 1969; Naitoh et al., 1972). This regenerative behavior is therefore believed to result from an increased conductance of the surface membrane to calcium ions when the membrane is depolarized, and has been termed a “calcium response.” It is similar in a number of respects to the graded regenerative calcium responses produced by crustacean muscle (Fatt and Katz, 1953; Fatt and Ginsborg, 1958) and nerve terminals of the squid after treatment with tetrodotoxin and tetraethylammonium (Katz and Miledi, 1969).

Depolarizations of the surface membrane of *Paramecium* also produce a transient modification in the behavior of the cilia so that the direction of the power stroke is shifted from the posterior toward the cell anterior (Kinosita, 1954; Kinosita et al., 1965). This mechanical response of the cilia, which causes the ciliate to swim in reverse, is termed ciliary “reversal.”

Several lines of evidence have led to the hypothesis (Eckert, 1972) that the mechanical response (i.e. reversal) of the cilia is coupled to membrane depolarization by the same inward ionic current which underlies the graded regenerative calcium response. Calcium ions are presumed to enter the interior of the cilium through an increased calcium permeability of the cell membrane (which is continuous over the surface of each cilium) and accumulate transiently within the cilium. This is thought to bring the intraciliary calcium concentration into the range which activates the mechanism for reversed beating. The proposed coupling sequence is: membrane depolarization → increased calcium permeability → Ca\textsuperscript{2+} influx → increased intracellular [Ca] → ciliary reversal.

This scheme implies, firstly, that the injection of depolarizing current into the cell will produce reversal only if it produces a sufficient net influx of calcium through the surface membrane. Secondly, some parameters of the mechanical response (i.e. the latency, intensity and/or duration of ciliary reversal) should be graded with the extent of the transient rise in the intracellular calcium concentration which results from a transient increase in the rate of Ca\textsuperscript{2+} influx. Finally, the present hypothesis predicts a “suppression potential” at which the stimulus is no longer accompanied by the mechanical response, since the calcium current will be reduced, in spite of a high calcium conductance, as the membrane potential approaches the calcium equilibrium potential.

To test these predictions, we have examined the relations between ciliary activity in *Paramecium* and the electrical responses of the surface membrane.

**METHODS**

Specimens of *Paramecium caudatum* grown in hay infusion were isolated for experimentation in a solution of 1 mM CaCl\textsubscript{2}, 1 mM KCl, and 1 mM Tris-HCl at pH 7.2.
High-speed cinematography, together with electrical measurements, was used to document the behavior of the cilia in response to bioelectric events. The experimental system (Fig. 1) permitted simultaneous photographic registration of electric parameters together with images of beating cilia on 16 mm film (Kodak 2496 RAR, Eastman Kodak Co., Rochester, N. Y.) advanced at 250 frames per second (fps). A X 40 water immersion objective was used with a Zeiss (Carl Zeiss, Inc., New York) interference contrast (Nomarski) system to visualize the cilia in profile with the speci-

![Diagram of experimental setup](image)

FIGURE 1. Method of simultaneous electric and photographic registration. Paramecium on underside of cover glass was impaled by current-passing and recording electrodes under a X40 water-immersion objective. Stimulus current (I) and membrane potential (Vm) were displayed without time base on a cathode-ray-tube (CRT) the image of which was projected onto 16 mm cine film along with the image of cilia. A strobe lamp (bottom) was synchronized with the film advance of the cine camera. Stimulus current pulses were injected with an intracellular capillary electrode similar to that used to record membrane potential. Stray capacitance in the recording system was neutralized by positive feedback.

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image was provided by a strobe lamp discharged in synchrony with the film advance and shutter of the cine camera. The sequence of filming and stimulation was programmed so that the camera and strobe lamp operated just long enough to film at full speed for periods up to 2.8 s. These methods eliminated deleterious effects on the specimen from exposure to steady high intensity illumination. Experiments were conducted at temperatures of 18° and 8.5°C. The temperature of the bath was regulated with a cooled platform under the experimental chamber. Bath temperature was monitored within 5 mm of the specimen.

The latency and duration of ciliary reversal were determined at one point of observation on the cell surface of each specimen by a frame-by-frame analysis of the cine films. The reversal of the ciliary beat occurred as a rapid conversion from posteriorly to anteriorly directed power strokes. Cilia “caught” in different positions of their cyclic activity start this reorientation within the cine resolution of 4 m, and complete the initial reversed power stroke with all cilia momentarily positioned nearly flat against the cell surface and directed toward the anterior. This stage is seen in Fig. 2 B in the 48 ms frame. Criteria for reversed beating of the cilia are (a) the portion of the cycle devoted to anteriorly directed movement is shorter in duration than the posteriorly directed movement, and (b) this movement toward the anterior is made with a form characteristic of the power stroke (i.e., ciliary curvature of long radius in a plane relatively perpendicular to the cell surface). The first detectable transition from normal to reversed beating in a group of cilia around the point of observation was defined as the beginning of ciliary reversal. A detailed analysis of temporal and spatial properties of ciliary movement in response to electric stimulation will be presented elsewhere (Machemer, in preparation).

RESULTS

Quantitative Relations of Electrical and Mechanical Responses

Depolarizing current pulses lasting 10 ms, and ranging upward from $10^{-10}$ A were passed from the tip of the intracellular current electrode across the cell membrane to the indifferent electrode immersed in the bath. A potential shift was judged to be purely ohmic (i.e. electrotonic, without a regenerative response) if the rising and falling phases had the appearance of simple exponentials. Such behavior is generally ascribed to the time constant of the leaky capacitance of an unexcited membrane. With injected currents below about $10^{-9}$ A the membrane usually exhibited simple electrotonic shifts in potential (Fig. 2 A, top). With higher depolarizing currents the response of the membrane showed signs of graded regenerative behavior (Naitoh, Eckert, and Friedman, 1972). A calcium response is shown in Fig. 2 B, top.

In the absence of stimulation or with a purely electrotonic potential shift the cilia beat with the power stroke directed diagonally toward the right and rear of the Paramecium (Párducz, 1954; Machemer, 1972), so as to drive the ciliate forward on helical course. Such “normally” beating cilia can be seen in an optical section parallel to the longitudinal axis of the cell in Fig.
2 A in a sequence of frames selected at 8-ms intervals. The stimulus in this sequence produced no calcium response (Fig. 2 A, top), and the cilia show no interruption of their normal rhythm of beating. Cilia in the central part of the image can be seen first performing the power stroke toward the rear (0–24 ms), then swinging anteriad during the recovery phase (32–72 ms), and again starting a power stroke (80 ms).

As the stimulating current strength was increased so as to evoke progressively larger electric responses, the cilia filmed in the image plane underwent a transient reversal in direction of beating, lasting several beats. This only occurred if the stimulus also evoked a membrane calcium response. A large calcium response is seen as an inflection and peak riding on the electrotonic component of the electric response in Fig. 2 B, top. Correlated with this the cilia exhibited an initial quasi-synchronous stroke of the organelles toward the anterior end of the *Paramecium* between the 32- and 40-ms frames, after which there followed several cycles of beating in which the power stroke was directed toward the anterior (i.e. "reversed" beating). Reversed beating always begins with a high frequency which then steadily declines until the cilia exhibit slow, poorly directed movements of small amplitude and frequency while gradually returning to the prestimulus orientation toward the rear. This phase of inactivation occurs before vigorous normally directed beating resumes.

There was no abrupt transition between purely electrotonic potentials and those which also include a regenerative component. That is to say, there was no obvious threshold for the calcium response. Likewise, there was no distinct threshold for ciliary reversal. The first signs of reversal were seen with very weak calcium responses, and the reversal was stronger (i.e. more beats and longer period of reversed beating) with larger calcium responses.

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**Figure 2.** Ciliary activity recorded with a purely electrotonic potential (column A) and a calcium response (column B). *Top*, oscilloscope traces of electric responses of cell membrane (upper trace) to depolarizing current pulse (lower trace). Stimulus in (A), \(10^{-9}\) A for 10 ms; in (B), \(2 \times 10^{-9}\) A for 10 ms. Dashed line gives reference potential. Calibration pulses for potential and electrode resistance occur at far right of voltage trace. Small artifacts on voltage trace indicate 250 Hz discharge of synchronized strobe lamp. Multiple arrows indicate those artifacts which correspond to cine frames reproduced in sequence below. The numbers under the first and last arrows (0, 80) and the numbers on the cine frames indicate time in milliseconds starting with the first frame taken during the CRO sweep. *Bottom*, cine frames at 8-ms intervals corresponding to arrows in CRO traces. The dark band at the bottom of each frame resulted from additional exposure during printing to enhance the contrast of the superimposed CRO beams (white spots) displaying stimulating current (\(I\)) and membrane potential (\(V_m\)) (see Fig. 1). Between these is a fixed beam for positional reference. Outward stimulating current and membrane depolarization are both shown as deflections of the respective beams toward the right. The anterior end of the *Paramecium* is toward the left.
Duration of reversed beating is plotted for one cell as a function of the maximum rate of rise of the calcium response in Fig. 3. The duration of reversed beating was taken from the first signs of the power stroke of an individual cilium toward the cell anterior to the transient perpendicular orientation of the cilium during completion of the last power stroke in reverse, i.e., before inactivation occurred. This duration is plotted in Fig. 4 for single cilia in five specimens as a function of the potential difference between resting potential and the peak of the calcium response. In all cases the duration of re-

![Graph](image)

**Figure 3.** Duration of the period of reversed beating as a function of the maximum rate of rise of the calcium response in one specimen. Representative recordings are shown corresponding to five points along the plot. In these the upper trace is $dV_m/dt$, the middle trace is $V_m$ and the lower trace indicates a 20 ms current pulse. The vertical calibration marks equal 2 V/s. Note the change in scale for the fourth and fifth recordings.

versed beating (and number of beats in reverse) increased with the rate of rise and amplitude of the calcium response. The scattering of data points in Figs. 3 and 4 is due to a combination of two factors. First, there appear to be some intrinsic variations in behavior between individual cilia, which are suppressed at higher beating frequencies by the hydrodynamic coupling which facilitates neighbor-to-neighbor synchronization. At reduced ciliary velocities, such as occur toward the end of reversed beating, hydrodynamic coupling is reduced and individual differences between cilia (e.g. duration of reversed beating) are expressed. Second, because of minor movements of the specimen with respect to the optic axis it was not always feasible to keep track of an individual cilium from one sequence to another.
Reduction of Responsiveness by Lowered Temperature

Further evidence associating ciliary reversal with the calcium response of the surface membrane was obtained by comparing responses in one specimen at two temperatures, 18.0° and 8.5°C (Fig. 5). No ciliary reversal occurred with the responses shown in the upper row of Fig. 5. The first signs of reversed beating occurred with the electric responses shown in the lower records. As the temperature was reduced, the amount of electrotonic depolarization required to evoke a detectable regenerative component increased. At 8.5°C this was more than twice the depolarization required at 18°C. In addition, the onset of reversed beating was delayed at reduced temperature (Fig. 5 B). These effects were reversed as the temperature was raised again to 18°C. Regardless of temperature, ciliary reversal occurred only in conjunction with a calcium response. It was never observed after purely electrotonic depolarizations in any one of over 20 specimens filmed.

**Figure 4.** Duration of reversed beating related to amplitude of the calcium response (resting potential to peak). Symbols represent five different specimens. Each point indicates measurement of one film sequence.
Electrical Suppression of Ciliary Reversal

For this group of experiments the duration of depolarizing current pulses was increased to 100 ms, and current pulses were varied from intensities below those which produced a calcium response up to intensities sufficient to produce steady-state (plateau) shifts in potential of up to +230 mV with respect to the resting potential, which ranged from -25 to -35 mV. An overview of the electrical and mechanical responses is provided by Fig. 6. With a series of stimulus currents of increasing intensity progressively larger positive-going shifts in membrane potential occurred (Fig. 6, bottom). A calcium response can be seen at the beginning of the potential displacement to the lowest current (trace a and perhaps b). The peak at the beginning of the largest displacements (i.e. trace e) is probably due largely to a rapid ohmic upstroke followed by delayed increase in membrane conductance. In this graded series of positive potential displacements, the onset of ciliary reversal (arrows) first decreased (a, b) and then increased in latency (b-e) as the internal positive potential increased. Along with the increase in latency there was a reduction in the duration of the period of reversed beating.

The relation between latency of ciliary reversal and positive-going steady potential shifts is plotted in Fig. 7. With depolarizations below 40 mV the
FIGURE 6. Course of membrane potential and ciliary movements in response to 100-ms pulses of outward current (black bars). Stimulus intensities were (a), $8 \times 10^{-10}$; (b), $10^{-8}$; (c), $2 \times 10^{-8}$; (d), $3 \times 10^{-8}$; (e), $4 \times 10^{-8}$ A. Corresponding membrane responses are reproduced at bottom. Zig-zag plots show alternating anterior-posterior excursions of a single cilium; actual positions are not indicated (see box). Heavy segments indicate power stroke, light segments indicate recovery stroke. Note: conversion of recovery stroke to power stroke after onset of reversed beating; increased frequency during reversed beating; changes in latency of onset (arrows) of reversed beating. “Inactivation” in (d) and (e) is a period of erratic, low-frequency, low-amplitude movements of cilia after the period of reversed beating and preceding the resumption of beating in the normal direction. In records (a)-(c) inactivation occurred beyond the period plotted.

FIGURE 7. Latency of ciliary reversal as a function of steady-state depolarization in three specimens. Stimuli consisted of 100-ms outward current pulses of $4 \times 10^{-10}$ to $1.4 \times 10^{-7}$ A. The latency, measured from onset of stimulus to onset of reversed beating, is minimal for steady-state depolarizations between 30 and 60 mV. Compare similar delay between end of stimulus pulse and start of ciliary reversal at depolarizations above 100 mV. Latencies of less than 4 ms could not be resolved at the framing rate of 250/s. Each symbol represents a different specimen.
latency dropped progressively from above 30 to 4 ms or less. For greater positive-going potentials the latency increased with a positive exponential relation to increasing displacement. With pulses of about 100 mV or more ciliary reversal was delayed until after the end of the 100 ms pulse. The value of the steady-state potential just sufficient to delay reversed beating until after the end of the pulse will be termed the “suppression potential.”

The duration of reversed beating also shows a drop at large amplitudes of positive displacement (Fig. 8). Duration grew steeply with increasing positive potential displacement, increasing to a maximum greater than the period (2.4 s) of filming between 40 and 60 mV. At higher (>60 mV) displacements the duration of reversed beating dropped progressively until it reached a minimum at displacements greater than 100 mV.

The “Off” Response

Whenever ciliary reversal was suppressed by a strong intracellular positive potential pulse it appeared several milliseconds after the stimulus current
ended and the membrane potential had begun its return to the resting level (Fig. 6 d and e and Fig. 7). This delayed onset of ciliary reversal will be termed the “off” response. The horizontal plateau of “off” responses in Fig. 7 starting 2–4 ms after the end of the pulse provides convincing evidence that most or all of these occurred directly in response to termination of the pulse.

**DISCUSSION**

We have shown that ciliary reversal in response to depolarization is correlated quantitatively with the regenerative component of the membrane response. This agrees with the finding that membrane mutants of *Paramecium* which fail to produce regenerative responses to depolarization also fail to respond to depolarization by reversed beating of their cilia (Kung and Eckert, 1972; Kung, 1971). Suppression of the mechanical response to depolarization by strong intracellular positivity (Figs. 6 and 7) suggests that the coupling agent is an extracellular cation with a positive equilibrium potential. The only extracellular cations present in our experiments were Tris, H+, K+, and Ca2+. Ciliary reversal occurs in the absence of Tris or K+ (Jennings, 1906; Naitoh, 1968); moreover, the equilibrium potential for K+ under our conditions is negative (Naitoh and Eckert, 1973). This rules out Tris and K+. Neither these ions nor H+ are effective in evoking mechanical responses in extracted models of *Paramecium*, whereas Ca2+ causes reactivated cilia to beat in reverse (Naitoh and Kaneko, 1972). In living paramecia ciliary reversal does not occur if extracellular Ca2+ is greatly reduced (Kinoshita, 1954; Naitoh, 1968). Finally, there is evidence that the regenerative response of the *Paramecium* membrane is due to an influx of calcium ions (Eckert and Naitoh, 1969; Naitoh et al., 1972). These lines of evidence all support the view that an influx of Ca2+ leads to reversed ciliary beating, and that the duration of reversed beating is related to the amount of Ca2+ entering the cilia through the surface membrane during regenerative depolarizations of the membrane.

It can be assumed as a first approximation that the amplitude and rate of rise of the graded regenerative component (i.e. calcium response) reflect the transient net influx of Ca2+ in response to depolarizing stimuli (Naitoh et al., 1972). At low and moderate stimulus depolarizations (Figs. 2–5) a higher rate of rise and greater amplitude will be taken to indicate a stronger calcium influx. Thus, the relations between ciliary reactions and the electric behavior of the membrane can be interpreted as follows.

Depolarizations too small to produce an adequate increment in calcium conductance to produce an adequate net calcium influx have no effect on ciliary beating. As the intensity of depolarizing stimulation is increased, and the increment in calcium conductance increases, a regenerative influx of calcium ions takes place; the cilia then respond by reversed beating. With
calcium responses of increasing rate of rise and amplitude, the intraciliary Ca\(^{2+}\) concentration rises more quickly and to higher levels. As a result, the cilia respond with a shorter latent period. In addition, longer periods of time are required for the active removal and/or passive dispersion of the accumulated calcium ions from the cilia, and so the duration of reversed beating increases with increased amplitude of calcium response.

When the temperature is reduced, the voltage-sensitive calcium conductance of the membrane becomes less sensitive to depolarization, so that larger stimulating currents and larger electrotonic depolarizations are required to increase the calcium conductance. Therefore greater depolarizations are required at reduced temperature to produce an increment in calcium concentration sufficient to initiate reversed beating (Fig. 5). It is significant that the correlation between the calcium response of the surface membrane and the reversal of direction of ciliary beating is maintained even when the excitability of the membrane is altered.

The increase in latent period which accompanies potential shifts greater than 60 mV (Fig. 7) can also be interpreted in terms of the ionic hypothesis (Hodgkin, 1957; Katz and Miledi, 1967). Thus calcium influx will become smaller as the membrane potential approaches the calcium equilibrium potential. As calcium influx is reduced more time is required for Ca\(^{2+}\) to accumulate to an intracellular concentration high enough to induce ciliary reversal. With further increase in stimulus current a potential is reached, the suppression potential, at which the onset of reversed beating is delayed until the end of the pulse. Adding the resting potential (−25 mV) to the effective potential displacement (about +100 mV) gives a value of about +75 mV for the suppression potential in the experiments of Fig. 7.

The equilibrium potential for calcium has not been accurately established in Paramecium, but can be estimated. Chemically “skinned” models of Paramecium begin to swim in reverse if the ambient Ca\(^{2+}\) levels exceed 10\(^{-6}\) M (Naitoh and Kaneko, 1972). This suggests that the calcium concentration in forward swimming live paramecia is below 10\(^{-8}\) M. In muscle and nerve free intracellular Ca\(^{2+}\) activities range between 10\(^{-6}\) and 10\(^{-7}\) M (Portzehl et al., 1964; Baker et al., 1970). Assuming this range for Paramecium, we obtain an estimated calcium equilibrium potential of +90 to +120 mV with an extracellular calcium concentration of 10\(^{-4}\) M. The discrepancy between the observed suppression potential (+75 mV) and the estimated calcium equilibrium potential (+90 to +120 mV) is expected, for suppression of the ciliary response should require only that influx of Ca\(^{2+}\) in the cilium be kept below a critical level rather than be completely prevented.

The “off” response logically follows a suppressing positive pulse if it is assumed that the calcium permeability remains fully or partially turned on while the membrane potential is at or beyond the suppression level, and
turns off after the end of the current pulse with a time-course that lags somewhat behind the repolarization of the membrane. During repolarization the electrochemical potential acting on Ca$^{2+}$ rapidly increases, while permeability remains temporarily high. As a result a transient net inward calcium current flows during repolarization. This causes the intracellular calcium concentration to undergo a transient increase after the end of a suppressing potential pulse, and this produces a brief period of reversed ciliary beating, the "off" response. The suppression phenomenon and the "off" response of cilia was originally observed in Opalina (Naitoh, 1958), but at that time could not be interpreted. More recently they were documented in the hypotrich Euplotes (Epstein, 1972). Similar behavior is exhibited by the Ca-dependent release of the neural transmitter substance from the presynaptic nerve terminal (Katz and Miledi, 1967). In this case the release of the transmitter is suppressed during a strong internally positive pulse and occurs as an "off" response at the end of the pulse.

The first signs of ciliary reversal were observed with calcium responses as small as 1 or 2 mV above the electrotonic component of depolarization (Fig. 5). Data from chemically skinned paramecia (Naitoh and Kaneko, 1972) suggest that ciliary reversal in the living cell requires an increase in the concentration of intraciliary free calcium to 10$^{-6}$ M or above. Can the calcium influx calculated for such small responses (i.e. 1 mV) raise the internal concentration enough (i.e. > 10$^{-6}$ M) to produce reversed ciliary beating? A lower limit for the increment in calcium concentrations within the cilium was calculated assuming that calcium enters through the entire cell membrane including the ciliary surface membrane. The surface-volume ratio of the cilia would favor significant increments in concentration within the cilia (Eckert, 1972). The cilia of Paramecium are about 10$^{-2}$ cm long and 2 X 10$^{-3}$ cm in diameter. The quantity of divalent cations which would carry an amount of charge across the surface area of one cilium ($\sim$6 X 10$^{-8}$ cm$^2$) sufficient to produce a 1 mV depolarization of the membrane (assuming a membrane capacitance of 10$^{-6}$ F/cm$^2$) is 3 X 10$^{-22}$ mol. Since the volume of a cilium is about 3 X 10$^{-16}$ liter, this quantity will produce an increase in calcium concentration within the cilium of 10$^{-6}$ mol/liter. For calcium responses of amplitudes greater than 1 mV the increment in intraciliary calcium concentration will accordingly be higher. The calcium response of the membrane is adequate in theory, then, to supply enough Ca$^{2+}$ to the interior of the cilium to activate reversed beating.

1 This approximation neglects diffusion out into the cytoplasm through the narrow passages at the base of the cilium, and neglects binding of Ca$^{2+}$ to intraciliary proteins. Consideration of those effects would lead to a lowering of the calculated value for free Ca$^{2+}$. However, our calculation also neglects short circuiting of Ca$^{2+}$ influx by compensatory K$^+$ efflux, which undoubtedly occurs; correction for this effect would increase the value calculated for the influx of Ca$^{2+}$, and tend to offset diffusional loss and binding.
Dr. Machemer is on leave of absence from the Zoological Institute, University of Tübingen, West Germany.

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