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Damped Oscillations in Photosensory Transduction of *Halobacterium salinarium* Induced by Repellent Light Stimuli

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Halobacteria usually respond to repellent light stimuli by reversing their swimming direction. However, cells seem to be in a refractory state when stimulated immediately after performance of a reversal. I found that in this case, a special type of response is exhibited rather than spontaneous behavior. A strong stimulus induced a rhythmic pattern of successive reversals. On stimulation immediately after a reversal of swimming direction, the first of these reversals was skipped without influence on the rhythm. The results suggest that the stimulus evokes an oscillating signal which alters reversal probability but which is itself independent of the state of the motor apparatus. The oscillation has a period length of about 5 s and is damped out within a few cycles. It does not depend on the special sensory photosystem through which the stimulus is applied. The consequences of these findings for the model description of swimming behavior control in halobacteria are discussed.

Under constant conditions, *Halobacterium salinarium* (formerly *Halobacterium halobium*) reverses its swimming direction every 3 to 50 s by switching the rotational sense of its flagellar motors from clockwise (CW) to counterclockwise (CCW) or vice versa. The distribution of the interval length is highly asymmetric, with the maximum at short times of about 10 s (for a comparative compilation of different results, see reference 14; original data are presented in references 5, 7, 9, 10, and 15). Light stimuli alter the length of a swimming interval. The effect of stimulation depends on the wavelength and on the sign of the light intensity change, which is sensed by the retinal proteins sensory rhodopsins I and II (9, 20, 21, 23, 24). Stimuli which prolong a swimming interval are called attractants, and stimuli which cause a shortening of a swimming interval are called repellents. After strong repellent stimuli, all bacteria reverse their swimming direction within a few seconds. However, this response fails to occur if the stimulus is applied during the first half-second after a reversal has taken place, during the so-called refractory period. Cells regain responsiveness within the following 2 s (10, 15).

It has been postulated that the swimming behavior of *H. salinarium* is controlled by an intracellular deterministic oscillator, which triggers a switching event after completion of each cycle. It was assumed to alter sensitivity to attractant light stimuli during the cycle (15, 16). Such an oscillator would be one of the very few examples of the occurrence of a biological clock in procaryotes, besides the circadian rhythms in certain cyanobacteria (2, 11, 18). But the original results in favor of the oscillator hypothesis were recently rejected as being based on inadequate methods, and constancy of the sensitivity to attractant light stimuli during a swimming interval could be demonstrated (5). There is no evidence for oscillations in the unstimulated state.

In an alternative description of the system, the transition between the CW and CCW rotating states of the flagellar motor is regarded as a stochastic process. It is assumed that after each reversal, the motor-switching apparatus runs through a few distinct states and eventually gives rise to the next change of flagellar rotation (9, 10). These few-state models yield a good description of spontaneous motor switching. However, they need extension to be applicable to stimulus responses as well.

Starting from a reinvestigation of the refractory behavior after repellent light stimuli, this study intends to establish a unified view of the control of swimming behavior of *H. salinarium*. The length of swimming intervals of single cells, stimulated by step-like light intensity changes, was measured. I could demonstrate not self-sustained but damped oscillations in the signal transduction pathway. This damped oscillation of the signal may be introduced into the few-state models, rendering them applicable to all the different findings.

MATERIALS AND METHODS

Experiments were done with the mutant strain Flx3 of *H. salinarium* (19, 22), which contains both sensory rhodopsins but lacks the light-dependent ion pumps bacteriorhodopsin and halorhodopsin. Cells were grown for 3 days in peptone medium and diluted for the experiments with five parts of basal salt solution, buffered with 25 mM MOPS (3[*N*-morpholino]propanesulfonic acid) at pH 7.8 (5). Measurements were done by observing single cells under a video microscope through which step-like light intensity changes were applied. The wavelength of stimulating light was chosen to correspond to the respective maximum of phototactic sensitivity. Observation or background light was infrared light, $\lambda > 800$ nm, or white light at 250 $\mu W \cdot mm^{-2}$. Technical details are given elsewhere (5). The temperature was held at 23°C. With each new sample, an adaptation time of 2 min was allowed before the beginning of the measurements. Furthermore, each measured bacterium was allowed to adapt for at least two swimming intervals to prestimulus conditions after resetting of the stimulating light. Stimuli were delivered at variable delay with respect to an observed spontaneous reversal. The delay for each measurement was chosen by a computer at random from a list of given delays, spaced by 25 ms. A measurement was started when a spontaneous reversal of the observed cell was detected by the experimenter. The delay chosen by the computer was not known at this time. The experimenter's reaction time was assumed to be 0.2 s (15) and is included in the delay.

RESULTS

Repellent stimuli applied later than 2 s after a spontaneous reversal evoked a response which was independent of the delay of the stimulus (Fig. 1A), in accordance with previous results (10, 15). Almost all cells reversed their swimming direction 2 to

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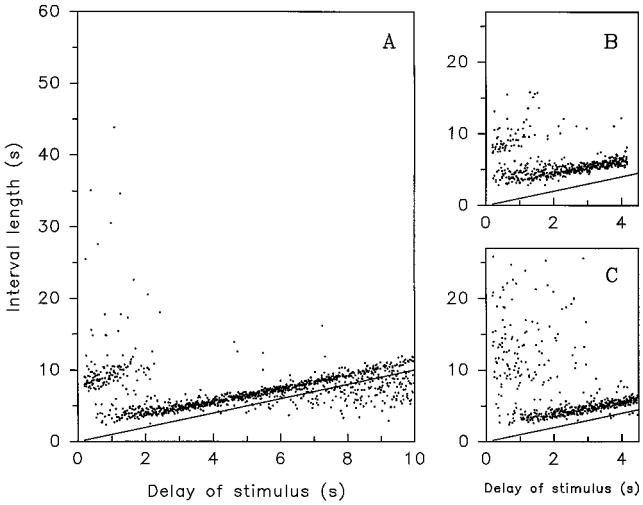

FIG. 1. Response to repellent stimuli in dependence of the time of stimulation during an interval. Each dot represents a single event. Each panel shows the results from three series of experiments. (A) Stimulation of the UV-sensitive photosystem (intermediate S_{373} of the photocycle of sensory rhodopsin I). The stimulus was a step-like increase of light at a wavelength of 370 nm by $1.2 \cdot 10^{13}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}; white background light was used. (B) Stimulation of the long-wavelength-sensitive photosystem (ground state sR_{587} of sensory rhodopsin I). The stimulus was a step-like decrease of light at a wavelength of 565 nm from $1.8 \cdot 10^{14}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1} to 0; infrared observation light was used. (C) Stimulation of the blue-light system (sensory rhodopsin II). The stimulus was a step-like increase of light at a wavelength of 480 nm from 0 to $6 \cdot 10^{13}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}; infrared observation light was used. Two-day-old cultures, in which the signal generated by sensory rhodopsin II is amplified more strongly than in older cultures (13), were used.

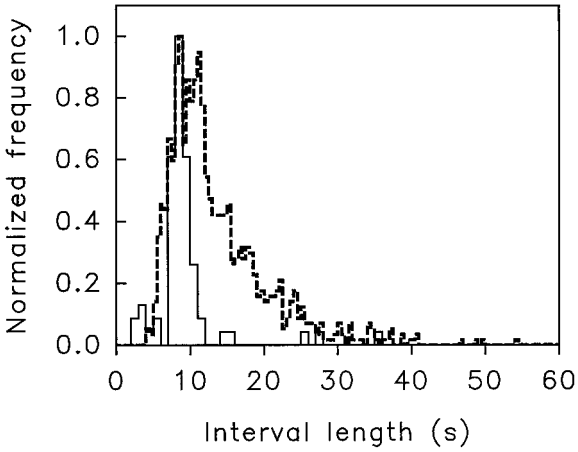

FIG. 2. Distribution of the interval length at stimulation during the refractory period, compared with spontaneous behavior. The solid line shows all events from Fig. 1A for delays smaller than 0.8 s. The dashed line shows lengths of intervals in the absence of stimulation, with data from reference 5. Histograms were normalized with respect to the maximum.

3 s after stimulation. These events are represented by the narrowly distributed population of dots above the straight line in Fig. 1A, which indicates the time of stimulation with respect to the ordinate. Dots below or just above the line represent spontaneous reversals (5).

When the delay of the stimulus was less than 2 s, an irregularity of the response was observed: with decreasing delay, the response population becomes less well bounded and contains fewer and fewer events. Instead, a second population of events occurs, centered about 8 s after stimulation. Both populations are well separated by an area of low dot density. Similar results were obtained upon stimulation through the other known sensory photosystems (Fig. 1B and C), as well as with the fully pigmented strain R1 of *H. salinarium* (data not shown). For the sake of brevity, I call the two populations of events direct and indirect responses, respectively.

It was previously concluded from averaged data that cells do not respond at all to stimulation during the refractory period (10, 15). However, the quite narrow distribution of the indirect response does not fit at all the distribution of spontaneous intervals (Fig. 2). Moreover, it runs parallel with the time of stimulation rather than with the abscissa in Fig. 1. These features qualify this population of events to represent in fact a stimulus response, though of a type different from the direct response to stimulation later during a swimming interval. The transition from the indirect to the direct response, described previously as a gradual shift of the average interval length (10, 15), turns out to be a shift of the frequencies of both distinct types of response.

The analysis of series of successive reversals yields an explanation for the irregular behavior after stimulation early in a swimming interval. A repellent stimulus was applied during the period in which direct and indirect responses overlap. The times of the first three reversals following that stimulation were measured. To classify the bacteria with respect to directly and indirectly responding cells, in a first step only the first reversal of each bacterium was analyzed. As expected from the data shown in Fig. 1, a bimodal distribution of the reversal time was obtained, with the peaks of direct and indirect responses spaced by about 5 s (Fig. 3A). In Fig. 3B, the whole series of reversals of the directly responding cells is shown. (Note that, in contrast to the data in Fig. 3A, in Fig. 3B and C the times of more than one reversal per cell are shown in relation to the time of stimulation.) Three separate peaks of high reversal frequency, which become smaller and less steep with increasing time, occur in Fig. 3B. This distribution is reproduced quite well by the distribution of the first two reversal times of the indirectly responding cells (Fig. 3C), except for the first peak, which is missing. The first and second reversals of indirectly responding cells are therefore equivalent to the second and third reversals of directly responding cells. The direct response is skipped by indirectly responding cells, without any consequences for further behavior.

Sometimes the periodic change of reversal frequency was already damped out after the second peak, and sometimes a weak fourth peak could be detected. The lifetime of this oscillation is therefore 15 to 20 s. In all experiments, the peaks were spaced by 5 to 7 s, irrespective of the photosystem through which the stimulus was applied.

The damped oscillation was induced equally well and with the same period length with all delays of the stimulus within the tested range. The second peak of the oscillation is indicated either by the indirect response (stimulation during the refractory period) or, if the cell exhibits a direct response, by its second reversal. It occurs at a constant time after stimulation, irrespective of the delay of the stimulus (Fig. 4A). The measured range of delays could be expanded to negative delays as well, by a method which was used previously to study the attractant response (5). For this, two successive interval lengths, t_1 and t_2, were measured (Fig. 4B). The stimulus was delivered at t_d, 7.5 to 11 s after the beginning of the first interval. At this time, the probability of a spontaneous reversal is maximal. So, the second reversal fell sometimes shortly before and sometimes shortly after the stimulus. The effective delay of the stimulus with respect to the second interval is $t_d -$

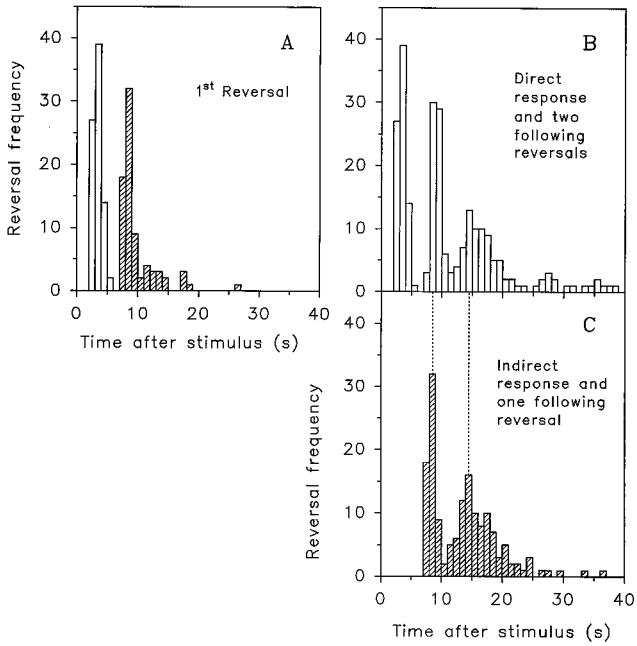

FIG. 3. Periodic change of reversal frequency after repellent stimulus. The stimulus was applied 0.7 to 1.0 s after the time of a spontaneous reversal (time zero). The stimulus was a step-like decrease of light at a wavelength of 565 nm from $1.8 \cdot 10^{14}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1} to 0; infrared observation light was used. The responses of 160 cells were measured. (A) Time of the first reversal with respect to the time of stimulation. Bacteria reversing within 5.5 s (first peak, plain bars) were classified as directly responding cells, and bacteria reversing after more than 7 s (second peak, hatched bars) were classified as indirectly responding cells. (B) Times of the first three reversals of the directly responding cells. (C) Times of the first and second reversals of the indirectly responding cells. The positions of the maxima of the data shown in panel B are indicated.

t_1 (abscissa in Fig. 4B). The length of the second interval, t_2, is given on the ordinate in Fig. 4B. The population which corresponds to the second peak of the oscillation extends continuously over the time of the reversal to negative delays. This means that even at the very time of the performance of a reversal, the inducibility of the oscillation is not altered and is thus independent of the state of the motor apparatus.

If the second reversal had not already occurred spontaneously, it was induced as a direct response by the stimulus and performed about 2 s after stimulation, as expected. In this case, t_2 is the length of the interval following this response. These events form the dense spot between delays of -2 and -1 s. This spot is continuously linked to the indirect response upon stimulation during the refractory period (delays of 0 to $+1$ s). This is an extended proof for the equivalence of the indirect response and the next reversal after a direct response.

DISCUSSION

The omission of the direct response to a repellent stimulus delivered during the refractory period may be explained by an inactivity of the motor-switching mechanism during this time. Such an inactive state, lasting for some seconds after the performance of a reversal, was previously deduced from the distribution of the length of spontaneous intervals (9, 10). In principle, such a mechanism which guarantees that a minimal period of time remains between two switching events would at the same time give an explanation for equally spaced events.

However, since the omission of the first event does not influence the position of the following ones, the rhythmic pattern of reversals found after a strong repellent stimulus cannot be due solely to such a mechanism. This effect requires that rhythmicity be generated at an earlier step of the signal trans-

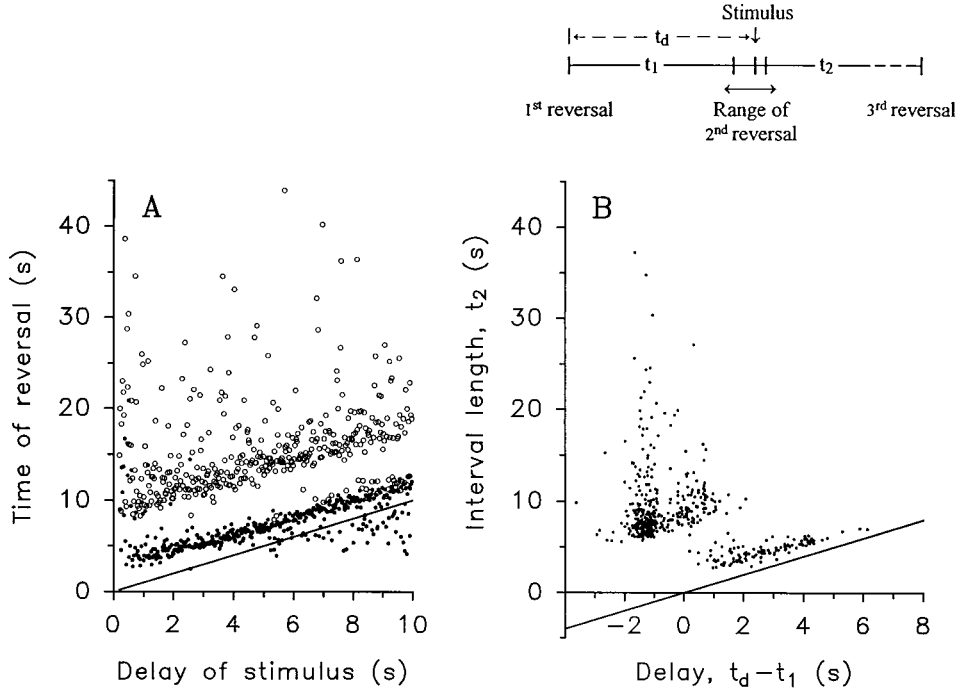

FIG. 4. Dependence of the time of the indirect response on the delay of the stimulus. (A) Times of the first (●) and second (○) reversals of each cell. The stimulus was a step-like decrease of light at a wavelength of 565 nm from $1.8 \cdot 10^{14}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1} to 0; infrared observation light was used. (B) Response to stimuli applied in close temporal proximity to a spontaneous reversal. In this experiment, the delay was calculated from experimental parameters and measured times as described in the text. The stimulus was a step-like increase of light at a wavelength of 370 nm by $1.2 \cdot 10^{13}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}; white background light was used. A total of 500 events were recorded. Similar results were obtained with step-down stimuli at 565 nm with infrared observation light.

duction chain. It has to be assumed that the signal generated by the cell after a strong repellent stimulus shows a damped oscillation. A cell which has skipped a reversal because of a block of the motor switch reverses its swimming direction not immediately after it regains switching ability but only when the signal is again strong enough. Because of the refractory state of the switch, the cell changes its swimming direction only once during a peak of the signal. The feedback loop which allows adaptation to altered environmental conditions after stimulation may well be the origin of the damped oscillation.

It can be inferred that the intracellular signal which induces a switching of the motor from CCW to CW is the same as that for the opposite process. Otherwise, after skipping one reversal induction, the cell would perform only the third and not the second reversal induced by this signal. This is fundamentally different from the situation found with *Escherichia coli*, where the switching signal (phosphorylated CheY) always induces a specific rotational sense, namely, CW rotation (1, 3, 25). But in terms of behavior, the difference turns out to be an equivalence: in the peritrichous *E. coli*, CW rotation causes tumbling of the cell, resulting in a change of swimming direction. Smooth swimming is performed with CCW rotating flagella only (6). In contrast, the polarly flagellated *H. salinarium* swims almost equally well with CW and CCW rotating flagella, but in opposite directions, parallel to ~~a direction opposite to that of~~ its cell axis (4, 8). A change of swimming direction is brought about just by the change of the rotational sense. In both organisms, the respective switching signal causes, by an appropriate mechanism, a reorientation of the cell.

The results presented here give the experimental basis for an expansion of the stochastic few-state models, which originally account only for the control of spontaneous reversals. The few-state models are based on the finding that, except during an initial phase after a reversal, the switching probability remains constant (9, 10). The behavior after attractant stimulation is correctly described, if it is assumed that an exponentially decaying signal transiently reduces the switching probability (12). By introducing into the few-state models a damped oscillation of the signal after a repellent stimulus which modulates switching probability in a periodic manner, it should now be possible to extend the models to describe behavior after repellent stimuli, including refractory behavior.

Two results currently remain as evidence for sustained oscillations in *H. salinarium*: the uniform lengths of swimming intervals occurring in suitable temporal light gradients (16) and multimodal distributions of the swimming interval length, which were reported to occur with cells treated with 3′-isobutyl-1-methylxanthine (IBMX) (17). By making use of the damped oscillation of the signal, both results can be interpreted in a straightforward manner. In a temporal light gradient, the repellent stimulus is maintained over several swimming intervals. It therefore may maintain the induced oscillation and thus cause a regular sequence of reversals. This proposal is supported by the fact that the length of swimming intervals in the gradient with the strongest synchronizing effect is 6 to 7 s, i.e., in the range of the period length of the induced oscillation. If, on the other hand, the treatment with IBMX decreased the damping in signal transduction, oscillations could build up from fluctuations.

Thus, all results published so far can be integrated within the view of the system presented here. There is no need to deduce from any of the findings the presence of an autonomous oscillator working permanently in the cells, which, in contrast, would not easily explain the rest of the results.

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