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Sensitivity of *Halobacterium salinarium* to attractant light stimuli does not change periodically

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Abstract *Halobacterium salinarium* swims alternately in both directions of its cell axis. The average time between two reversals of the swimming direction is modulated by light stimuli. It is a matter of dispute whether the sensitivity to attractant stimuli depends on the time of stimulation during an interval. This question is crucial for model descriptions of the system. I have confirmed constancy of responsiveness with cells adapted to constant conditions and have reconstructed contradicting results. These are shown to be based on inadequate experimental and evaluative methods. The assumption of self-sustained oscillations which modulate sensitivity can not be justified from the attractant response.

Key words: Phototaxis; Sensory rhodopsin; Signal transduction; Archaea; *Halobacterium salinarium*

1. Introduction

The attractant response of *Halobacterium salinarium* (formerly called *H. halobium*) to a step-like increase of long-wavelength light was first described 15 years ago [1]. In the unstimulated state, the polarly flagellated cells reverse their swimming direction about every 5 to 50 seconds. After attractant stimulation, the next reversal is postponed, whereas repellent stimuli induce a shortening of the current swimming interval. The type of the response depends on the wavelength and on the sign of the change in light intensity, sensed by retinal proteins. Photosystem sR_{587} is the ground state of sensory rhodopsin I (sR-I), photosystem S_{373} an intermediate in the photochemical reaction cycle of this pigment [2]. Sensory rhodopsin II (sR-II) constitutes a third photosystem [3–5].

It is a matter of dispute, whether the sensitivity to light stimuli varies during a swimming interval or not. In consequence, completely different models are used to describe the underlying mechanism of reversal control and signal transduction, based on its supposed periodical changing [6,7] and constant [8] sensitivity, respectively. Schimz and Hildebrand proposed an intracellular deterministic oscillator which controls the reversals of swimming direction and periodically modulates responsiveness to attractant stimuli [6,7,9]. In contrast, Marwan and Oesterhelt, as well as McCain et al., regard the occurrence of reversals as a poissonian process [5,8]. An attempt to find autonomous periodicity within consecutive swimming intervals by autocorrelation analysis turned out negative [10].

To resolve the dispute it has to be shown, by which modifications in experimental details and evaluation procedures the divergent findings are obtained. With this aim in view, a method of data acquisition was developed, which allows direct visualisation of swimming interval lengths vs. delay of stimulation after the last reversal of swimming direction. The direct judgement of raw data may unravel structural features within the data sets which are masked after data reduction by averaging.

2. Materials and methods

The bacterial strains R1, containing all retinal proteins known in *H. salinarium*, and Flx3, lacking bacteriorhodopsin as well as halorhodopsin [11], were used. Cells were selected for motility and chemotactic competence [1], grown for 3 days under standard conditions [12], and diluted 10-fold with basic salt solution (NaCl 250 g/l, $MgSO_4 \cdot 7H_2O$ 20 g/l, *tri*-Na-citrate $\cdot 2H_2O$ 3 g/l, and KCl 2 g/l), buffered with 25 mM MOPS, pH 7.8.

Measurements were done as single cell observations in a swimming-chamber of 0.2 mm depth, using a video microscope. Infra-red light of $\lambda > 800$ nm was used for observation (cut-off filter RG 830, Schott & Gen., Mainz). Only in the experiments with photosystem S_{373}, white light of 250 $\mu W \cdot mm^{-2}$ intensity was used in order to produce this intermediate of the sR-I photocycle. The microscope was equipped with phase contrast optics and an incident light illuminator in connection with a second light source for stimulation (200 W Hg-lamp, monochromator Zeiss M4Q III) [12]. Step-like light stimuli were applied by opening or closing an electronic shutter in the path of the stimulating light. The light path was completely screened in order to protect the bacteria from all but the experimentally applied light. Background and stimulating light were measured in the plane of observation, within the field visible on the video monitor [13]. The temperature of the preparation was hold at 23°C by means of a custom made peltier-regulated microscopic stage.

For stimulus application and data recording a microcomputer was used, to which observed reversals of a cell (beginning and end of a swimming interval) were signalled by pressing a button. The experimenter's reaction time was assumed to be 200 ms [7]. The computer delivered the stimuli by controlling the electronic shutter. The respective delay between an observed reversal and the stimulus was chosen by the program at random from a list of given delays, spaced by 25 ms. This ensures that a possible drift in responsiveness may not systematically influence the results. After resetting of stimulating light, an adaptation time of at least two swimming intervals was left before beginning the next measurement. Each series of measurements was carried out 3 times, the combined results of these series (1176 data points) are shown in a single diagram. Methodological details of measurements of different type are given in the legends to the respective figures.

3. Results and discussion

3.1. The attractant response

The attractant response was found to be similar in both investigated strains of *H. salinarium*, and for all three photosystems (Fig. 1). No qualitative influence of bacteriorhodopsin, which acts as a sensory receptor under certain conditions [15–17], was observed, though it is stimulated together with sR_{587} by light of 565 nm wavelength.

In each diagram, two populations of dots are well separated by an area of very low dot density. This gap between the two

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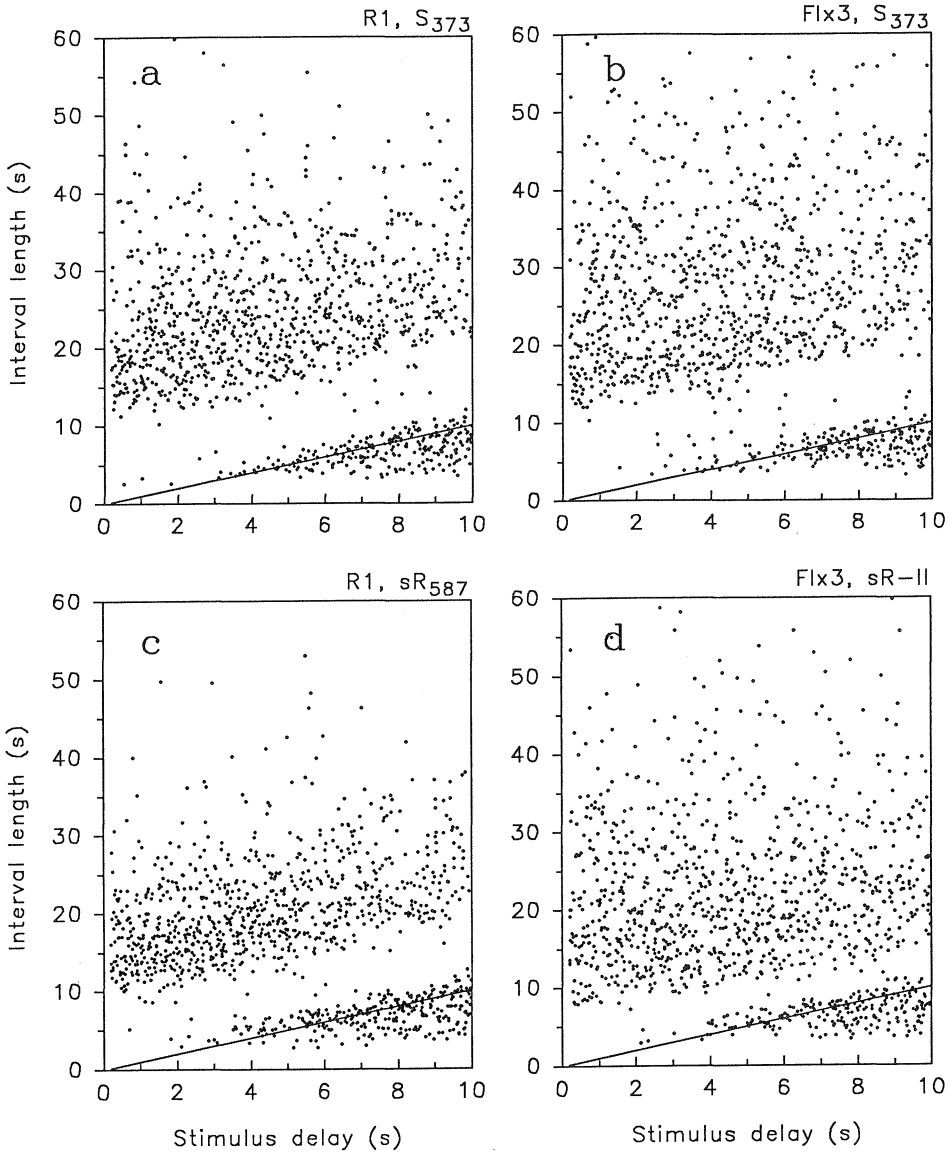

Fig. 1. Effect of attractant stimuli in dependence of the time of stimulation after the last spontaneous reversal, designated time zero. Randomized order of delays during each experiment (see section 2). Each dot represents a single measurement. The straight line indicates the time of stimulation with respect to the ordinate. (a) Strain R1, photosystem S_{373}, stimulus: step-down 370 nm, fluence rate $6 \cdot 10^{12}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}, buffer was omitted from the basic salt solution for keeping comparability with previous results [7,8]; (b) strain Flx3, photosystem S_{373}, stimulus: step-down 370 nm, fluence rate $6 \cdot 10^{12}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}; (c) strain R1, photosystem sR_{587}, stimulus: step-up 565 nm, fluence rate $6 \cdot 10^{13}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}; (d) strain Flx3, photosystem sR-II, stimulus: step-down 480 nm, fluence rate $3 \cdot 10^{13}$ photons $\cdot$ mm$^{-2} \cdot$ s^{-1}, 2-day-old cultures were used in order to ensure high sensitivity of this photosystem [14]. The response of strain Flx3 mediated by photosystem sR_{587} is shown in Fig. 2.

populations represents the transient reversal suppression by an attractant light stimulus [8], whereas the upper population of dots shows the correlated postponed reversals. The lower population is divided into two parts by the straight line which indicates the time of stimulation. Dots below this line correspond to reversals occurring spontaneously before the stimulus was delivered. Since the population is homogeneous, the dots in the area up to a few seconds after stimulation represent spontaneous events as well. This means that the stimulus exhibits its full effect in suppressing reversals only about 3 s after stimulation, which is indicative for a signal processing time. The same effect can be seen in previous data [8] as well.

Table 1
Slope of the regression of the upper population of reversals in the diagrams of the attractant response

Strain, photosystem	Data from Fig.	Attractant response, slope of regression
R1, S_{373}	1a	0.91
Flx3, S_{373}	1b	0.92
R1, sR_{587}	1c	1.04
Flx3, sR-II	1d	0.55
Flx3, sR_{587}	2	1.23

All events occurring later than 6 s, for experiment 1d later than 4 s after stimulation, are considered.

Onset and duration of the reversal suppression with respect to the time of stimulation are independent of the delay of the stimulus. The same is true of the distribution of postponed

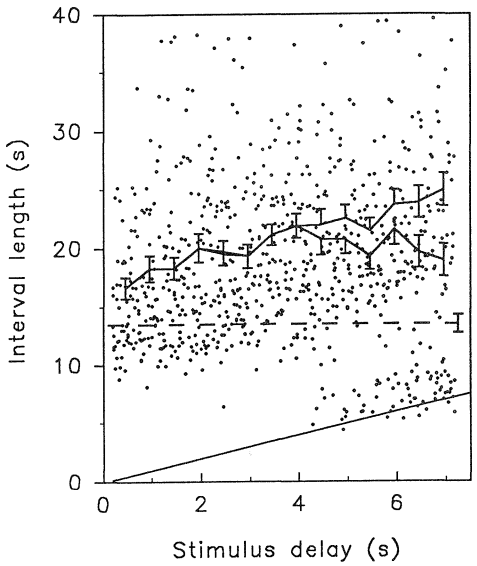

Fig. 2. Apparent attractant response under inclusion of different populations of events into averaging. Experiment driven as in Fig. 1. Single events, and average±s.e.m. for sectors of 0.5 s width each (max. 60 events) are indicated. Considered in averaging is: only the upper population of reversals (upper curve); each reversal occurring after delivery of the stimulus (lower curve). The straight line has the same meaning as in Fig 1. For comparison, the average ± S.E.M. of the length of 60 intervals in the absence of stimulation is given (dashed line). Strain Flx3, photosystem sR_{587}, stimulus: step-up 565 nm, fluence rate $3 \cdot 10^{13}$ photons $\cdot mm^{-2} \cdot s^{-1}$.

reversals. The slope of the average curve for the upper population is close to one (Table 1), indicating constant sensitivity during the whole swimming interval. The response mediated by sR-II was studied with submaximal stimuli only and shows a reduced slope. The same effect occurs with the other photosystems upon stimulation with lower intensity.

3.2. Reconstruction of earlier results

If the splitting into two populations of data points is not detected, at least those spontaneous reversals which occur after delivery of the stimulus will be included in an evaluation based on averaging. This results in a curve showing an apparent decrease of sensitivity, which starts at the onset of the population of spontaneous reversals, even if the reversals occurring before stimulation remain excluded (Fig. 2, lower curve). The correct analysis of the data shows constant sensitivity over the whole range examined (Fig. 2, upper curve).

When the adaptation time between resetting the stimulus and beginning of the next measurement was omitted, I found an initial slope of the attractant response of 1.6 (Fig. 3, lower curve). This increased slope is due to an initial reduction of responsiveness in comparison to the control experiment (Fig. 3, upper curve), decaying within about 3 s. Resetting an attractant stimulus is a repellent stimulus which therefore brings about the transient reduction of responsiveness. In contrast to other experimenters [1], Schimz and Hildebrand did not find any influence of light stimuli on intervals which follow the primary response [7]. Therefore, in many of their experiments no adaptation time was left (A. Schimz, personal communication). Their finding of an initial increase of sensitivity with a slope of 1.64, close to the value reported here, may be ascribed to this method. The combined effects of both errors, inclusion of spontaneous reversals in evaluation and insufficient adaptation, allow the reconstruction of the sawtooth-shaped change in responsiveness reported by Schimz and Hildebrand [7].

3.3. Sensitivity in temporal proximity to a reversal

The observed constancy of responsiveness confirms the results of McCain et al. for delays greater then 1 s [8]. However, these authors concluded from experiments with light pulses, which evoked a submaximal response, that sensitivity to attractant light is zero at the time of a reversal.

In the measurements reported up to now the stimulus could only be delivered after detection of a reversal but not before or at the time of the beginning of a swimming interval. The following strategy allowed an extension of the range of delays: The stimulus was delivered at the time at which a reversal was expected, i.e. 7.5–11 s after a preceding reversal has been observed. Therefore, stimulation took place sometimes shortly before, sometimes shortly after a reversal (Fig. 4). No deviation from constancy of responsiveness was found, neither at the beginning nor at the end of a swimming interval.

3.4. Interval lengths in the unstimulated state

A further argument to regard the swimming behaviour of *H. salinarium* as being under the control of an oscillator was the distribution of the lengths of spontaneous swimming intervals (Fig. 5), which was described in terms of a normal distribution of log interval lengths [6]. Using large sets of data, I found the distribution of log interval lengths being asymmetric (inset on Fig. 5). The χ^2-test shows a highly significant deviation from log normal distribution. The error probability when discarding the hypothesis of agreement of both distributions is $P < 0.0005$ in this case. 9 out of 14 series with 400 to 1000 events each, measured on different or on a single cell, gave statistically significant deviations from the log normal distribution (data

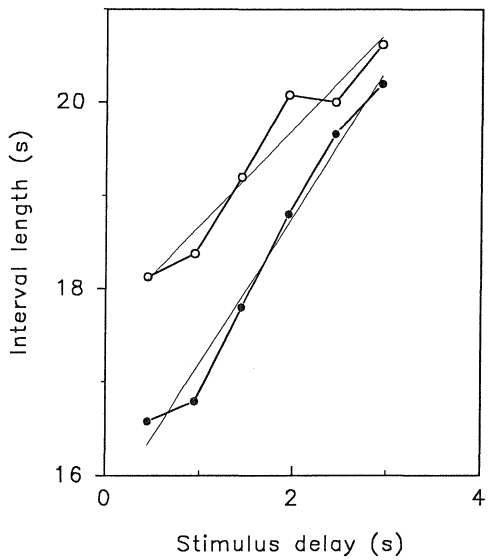

Fig. 3. Influence of a preceding repellent stimulus on the attractant response. Filled circles: The single measurements were started at the time of a reversal which was induced by a repellent stimulus, given as resetting of the attractant light. Open circles: response of dark adapted cells. Strain Flx3, photosystem sR_{587}; attractant stimulus: step-up 565 nm, fluence rate $3.7 \cdot 10^{13}$ photons $\cdot mm^{-2} \cdot s^{-1}$. Basic salt solution without buffer. The experiment was driven like in Fig. 1, but mean values of 60 measurements each are shown (bin width 0.5 s). For comparison only, regressions (straight lines) are indicated.

not shown). In contrast, the description of the right branch of the interval distribution as an exponential decay [5,8], in analogy to a model describing the analogous distribution in the swimming behaviour of *Escherichia coli* [18], fits quite well in most cases.

3.5. Conclusion

The postulate of self-sustained oscillations as the basis of the control of swimming behaviour in *H. salinarium* does not find support in the response to attractant light stimuli. All arguments which led to the specific oscillator hypothesis of Schimz and Hildebrand [7] were disproved. However, my results are not a proof for the absence of any type of oscillator from halobacteria. Model calculations carried out by Naber [19] demonstrate that both assumptions, motor-switching as the result of a poissonian process, and a stochastic oscillator, which does not directly modulate sensitivity but merely triggers reversals of swimming direction, are compatible with the results reported here.

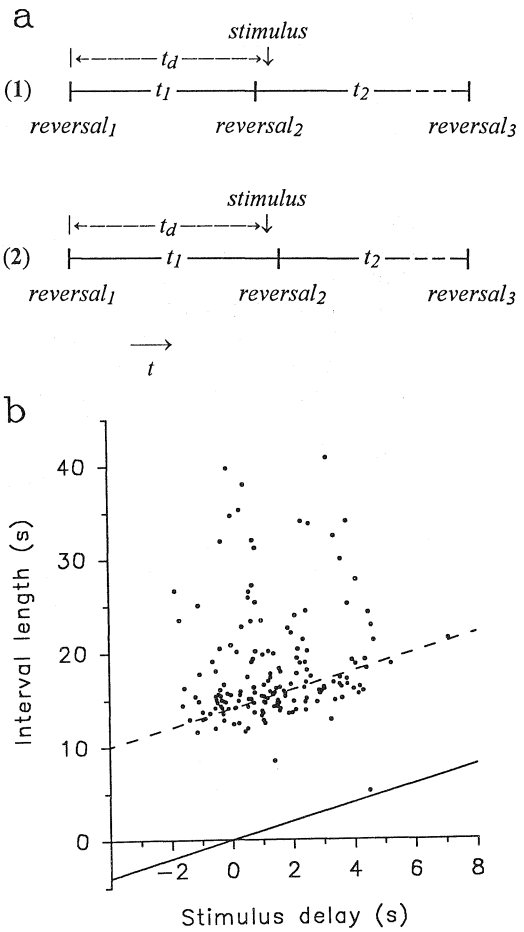

Fig. 4. Response to attractant light stimuli delivered in close temporal proximity to a spontaneous reversal. (a) Experimental programme. The length of two successive intervals, t_1 and t_2, was measured. After time t_d (7.5–11 s), the stimulus was delivered. The time of stimulation with respect to the beginning of t_2 is given as t_d minus t_1. The length of the second interval, t_2, is shown in (b). The cases of interest are: (1) reversal$_2$ occurs before delivery of the stimulus, therefore t_2 is a 'usual' attractant-stimulated interval; (2) reversal$_2$ occurs after delivery of the stimulus, during the time when only part of the population shows reversal suppression. t_2 is an attractant interval with the stimulus applied with '*negative* delay'. (If the response occurs already in t_1, reversal$_2$ is postponed largely by the stimulus. The delay with respect to t_2 is largely negative and does not fall within the shown range.) (b) Effect of attractant stimuli as given by the length of interval t_2. Solid line: time of stimulation with respect to the ordinate. Dashed line: parallel to this line through the response population. Case (1) constitutes all events shown with positive delay on the abscissa, case (2) with 'negative delay'. Strain Flx3, photosystem sR_{587}, stimulus: step-up 565 nm, fluence rate $1.2 \cdot 10^{14}$ photons $\cdot mm^{-2} \cdot s^{-1}$.

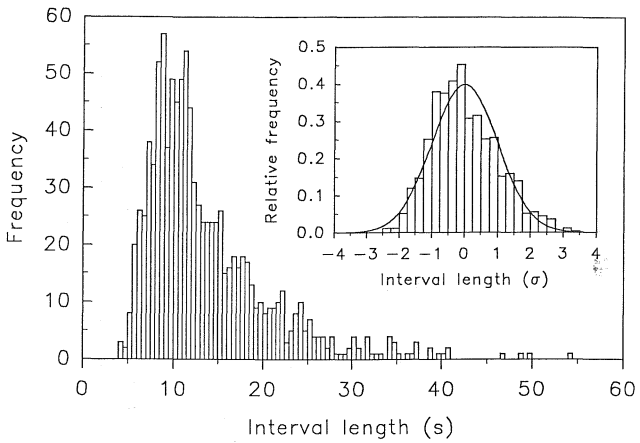

Fig. 5. Distribution of the length of spontaneous intervals. 1000 intervals, measured with different cells. Strain Flx3, infra-red observation light. Inset: Distribution of the log data, normalized. The bell-shaped curve gives the normal distribution.

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