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Oscillating signals in the sensory pathway of halobacteria induced by periodic light stimuli

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Abstract The swimming behaviour of *Halobacterium salinarium* can be modulated by light. Changes of the light intensity that induce reversals of the swimming direction are called repellent stimuli, those that suppress reversals, which otherwise would occur spontaneously from time to time, are called attractant stimuli. Bacteria were stimulated by periodic pulse-like stimuli, and the frequency of induced reversals was recorded. Stimulation with a period length between 16 and 6.5 s let the cells reverse periodically with the frequency of the external force. After the stimulation had been stopped, the cells continued to reverse periodically for 3 to 9 periods which, however, switched to a value of about 6 to 8 s, independent of the frequency of preceding stimulation. This endogeneous oscillation was most distinct when the stimulation period either equalled the endogeneous period or was twice or half of its length. During the endogeneous oscillation, the responsiveness to an attractant stimulus showed a pronounced phase-dependence. These results point to the oscillation of a signal in the sensory pathway which, different from our former assumption, seems to be not self-sustained but has to be set going by external stimulation.

Key words Oscillation · Periodic stimulation · Phase-response behaviour · Phototaxis · *Halobacterium* · Archaea

Introduction

Halobacterium salinarium swims by rotating polarly inserted flagella. Every few seconds the sense of flagellar rotation changes and the cell reverses its swimming direction (Alam and Oesterhelt 1984). By means of retinal proteins (rhodopsins) the organisms can detect light stimuli and thereby orient themselves in their environment (Schimz et al. 1983; Spudich and Bogomolni 1984). A light-increase in the yellow-green or a light-decrease in the blue/UV, a so-called attractant stimulus, suppresses reversals for some ten seconds and thereby prolongs the swimming intervals. An intensity change of the opposite sign, i.e. a light-decrease in the yellow-green or light-increase in the blue/UV, a so-called repellent stimulus, induces a reversal and thus shortens the swimming intervals (Hildebrand and Dencher 1975; Spudich and Stoeckenius 1979; Hildebrand and Schimz 1983 a). In free-swimming cells, clockwise (CW) and counterclockwise (CCW) rotation of the flagellar motor are equivalent at least with regard to the average length of the swimming intervals and the responsiveness to light stimuli (Hildebrand and Schimz 1985).

The frequency distribution of the intervals between spontaneous reversals is asymmetric (Hildebrand and Schimz 1985). Its exponential decrease gave rise to models which describe the spontaneous switching of the motor as a stochastic process, i.e. the essential step between the CW and CCW states was assumed to occur with a constant probability per unit time (Marwan and Oesterhelt 1987; McCain et al. 1987). At the same time, several observations pointed to an oscillatory activity of the cell. These were in particular: a phase-response relationship in the case of attractant stimulation (Schimz and Hildebrand 1985); a non-random behavioural pattern in temporal light gradients (Schimz and Hildebrand 1989), phase-locking, period-doublings, and presumably chaotic motion during periodic stimulation and upon changes of a supposed control parameter to certain constant levels (Schimz and Hildebrand 1992). We therefore assumed that spontaneous transitions between the two states of the motor were also controlled by oscillatory activity of the cell. Recent experiments, however, did not confirm the phase-dependence of the response when the cells were sufficiently adapted to constant light (Krohs 1994). This result, together with the observation of a transient periodicity in the motor behaviour after repellent stimulation (Schimz and Hildebrand 1992; Krohs 1995), suggests a stimulus-induced damped oscillation as postulated by Krohs (1995) instead of a self-

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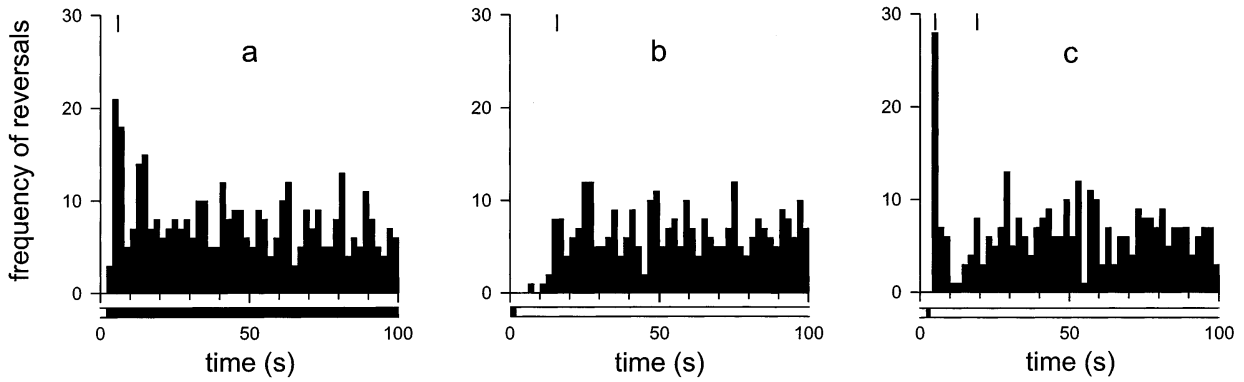

Fig. 1 a–c Time-series of reversals of the swimming direction of *H. salinarium* after single stimuli. Stimuli were given 2 s after a spontaneous reversal (t = 0), and successive reversals were recorded from the same bacterium. The number of reversals in 2-s bins is plotted vs. time. Light and dark sections are marked on the *abscissa. Vertical bars* indicate the average time at which the first reversal after a stimulus occurred. N = 50 cells in each series. **a** Repellent stimulus; step-down at 565 nm. **b** Attractant stimulus; step-up at 565 nm. **c** 1.5 s pulse; step-down followed by step-up, 565 nm

sustained one. In this paper we demonstrate a transient intrinsic oscillation induced by brief periodic stimulation and its dependence on the stimulation regime.

Materials and methods

Halobacterium salinarium (formerly *H. halobium*), strain Flx3 (Spudich and Spudich 1982), was grown for 3 – 4 days in standard medium as described previously (Hildebrand and Schimz 1983 b). The culture was diluted 10-fold with peptone-free medium, and the cell suspension was observed in a glass chamber of about 0.2 mm depth through a phase-contrast microscope connected to an IR-sensitive video system. A single bacterium in the sample was selected, and successive intervals between reversals of the swimming direction were measured with an electronic stopwatch connected to a printer. Light for periodic stimuli came from a DC-driven 150 W Xe-lamp (Osram XBO 150) equipped with a band pass-filter, $\lambda_{max} = 565$ nm, half width 13 nm. The light intensity in the plane of the sample was 2.5×10^{14} photons$\times$mm^{-2} s^{-1}. Stimulating light was directed through an incident-light illuminator and the objective onto the sample. Periodic dark pulses of 1.5 s duration were produced by closing and opening an electronic shutter; the frequency was set by a pulse-generator. The stimulation regime was started after a spontaneous reversal had been observed. For technical reasons, the program did not directly start with a pulse buth with a delay that equals the gap between pulses. Reversals were recorded while 9 dark pulses were given and the train ended with a light-off step. Subsequently, 10 further reversals were recorded with the same cell. Usually, about 10 such runs were carried out with one preparation. Single repellent or attractant stimuli were given 2 s after a spontaneous reversal. Prior to each series, the cells were allowed to adapt to light of 565 nm wavelength for 3 to 5 min. To measure phase-response curves, test stimuli were obtained from a DC-driven 200 W Hg-lamp (Osram HBO 200) connected to a Zeiss monochromator M4QIII. The intensity at 565 nm (half width 30 nm) was 9×10^{13} photons$\times$mm^{-2} s^{-1}. Observation light came from a 150 W Xe-lamp with an inserted IR filter, $\lambda > 830$ nm (Schott & Gen., RG 830). The temperature of the sample was kept at 25 °C by means of a peltier-controlled microscopic stage. Additional details will be described in the figure legends.

Results and discussion

Periodic light stimuli cannot be given but by switching the light on and off. In a pulse-like stimulus, however, the on-flank and the off-flank are seen by the organism as a sequence of two opposite stimuli. A single step-down stimulus at 565 nm induces a reversal within 3 to 5 s (Fig. 1 a). The second peak, which occurred 11 to 13 s after the stimulus will be discussed later. A single step-up stimulus at 565 nm acts as an attractant stimulus and suppresses reversals for some time (Spudich and Stoeckenius 1979; Hildebrand and Schimz 1983 a). The first events occurred 13 to 15 s after the stimulus (Fig. 1 b). A sequence of both, namely a dark-pulse, elicited both types of response, one after another: a reversal-induction 3 to 4 s after the off-flank of the pulse followed by a suppression of reversals that lasted up to about 14 s after the on-flank (Fig. 1 c).

In the following experiments, the cells were stimulated by periodic dark pulses at selected frequencies, ending with a repellent stimulus. Examples of the obtained time series are shown in Fig. 2. In parallel, the frequency of interval lengths in subsequent time windows (Fig. 3) is given as a more sensitive device to look for the temporal development of behaviour. During periodic stimulation, reversals occurred periodically with the same frequency as the external force, provided that the period of stimulation was within the range 16 to 6.5 s. The delay between the off-flank of the pulse and the induced reversal was about 3.5 s throughout. The distribution of interval lengths during periodic stimulation showed steep and narrow maxima

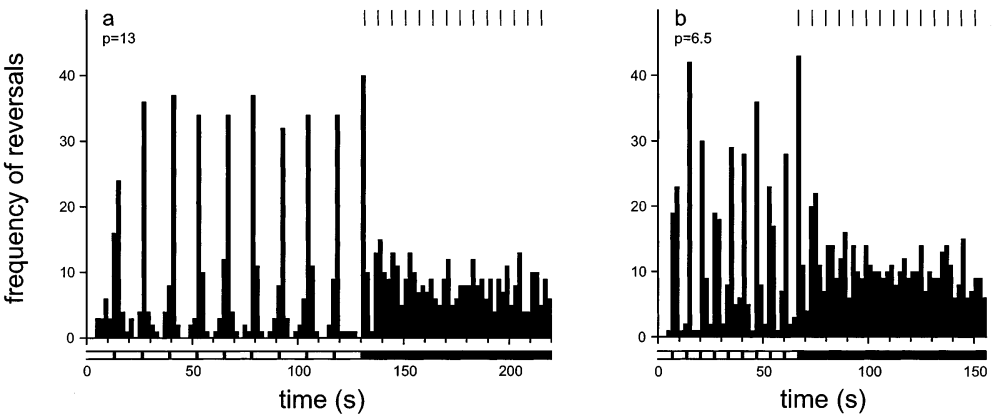

Fig. 2 a, b Time-series of reversals during and after periodic stimulation. Successive reversals during stimulation and thereafter were recorded from the same bacterium. T = 0 refers to a spontaneous reversal. The *vertical bars* indicate a period of 6.5 s beginning with the last stimulus-induced reversal. *P* denotes the period of stimulation in seconds. The number of bacteria in each series was N = 50

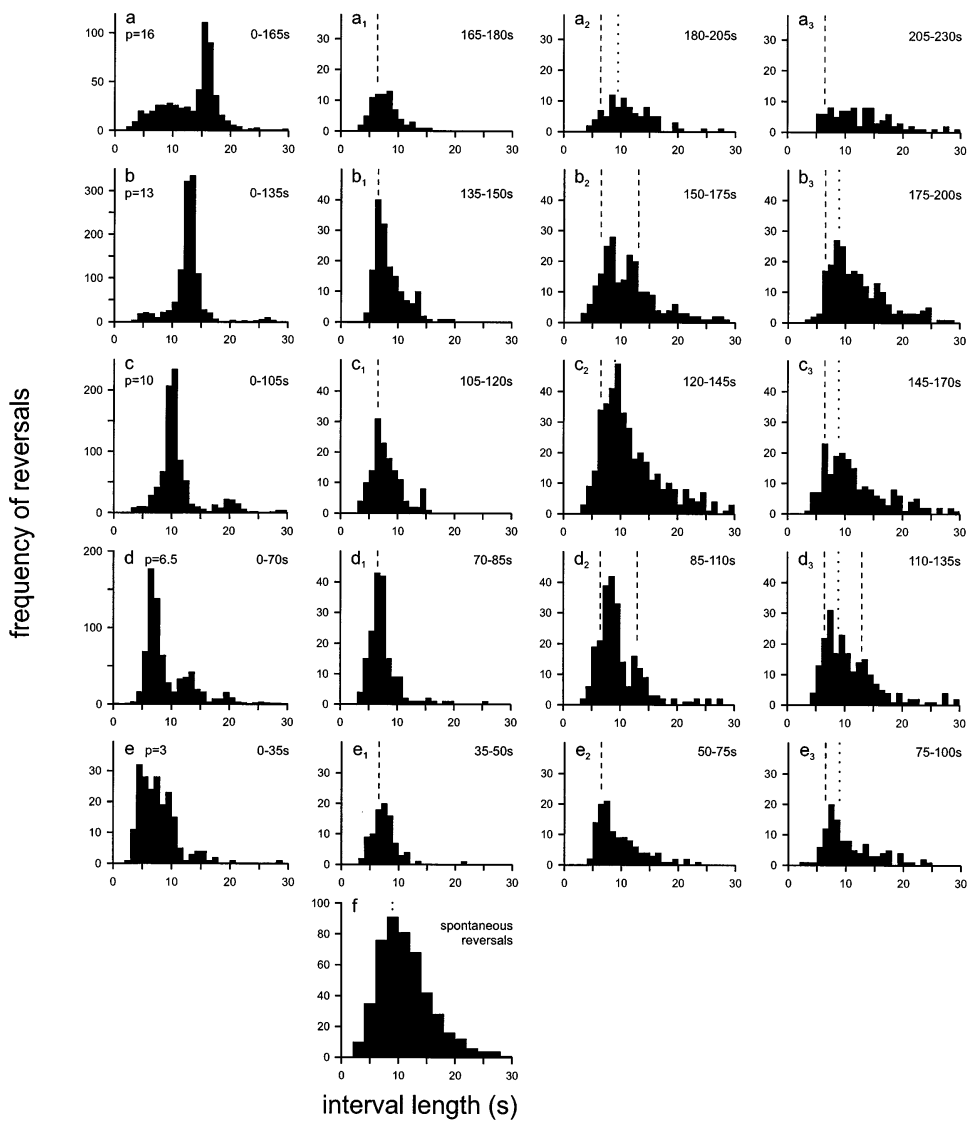

Fig. 3 a–f Frequency distribution of swimming intervals during periodic stimulation (a–e) and during subsequent time sections thereafter (a_1–e_3). The number of bacteria were N = 50 in series a and e, N = 100 to 110 in b, c, and d; N = 1, 473 intervals in f. The respective time-windows are given on each diagram in seconds; t = 0 refers to the last spontaneous reversal before stimulation as in Fig. 2. *P* indicates the period of stimulation in seconds. The *vertical dashed lines* mark an interval length of 6.5 s or the twofold; the *dotted lines* indicate the mean interval length between spontaneous reversals of about 9 s obtained from f

(Figs. 3 a–e) which met the respective periods of stimulation and differed considerably in shape from the broad and asymmetric distribution of intervals in the spontaneous state (c.f. Fig. 3 f). With stimulation periods of 13 to 6.5 s, period doublings showed up besides the main peaks (Figs. 3 b–d). Period 3 produced a broad and unstructured distribution (Fig. 3 e). Previous experiments with much longer sequences of 200–600 periodic stimuli suggested that this frequency range of stimulation may lead to chaotic motion (Schimz and Hildebrand 1992).

After periodic stimulation was stopped, reversals continued to occur periodically and were synchronized for

some time, but now with a new interval length of 6 to 8 s, which was independent of the frequency of preceding stimulation and significantly shorter than in the unstimulated state. The interval distributions during the first 15 s after the stimulation protocol showed more or less sharp maxima in all series (Fig. 3 $a_1 - e_1$). The extent and duration of this endogeneous periodicity was not exactly the same in all series. In the subsequent window from 15 to 40 s after stimulation, the 6 to 8 s interval clearly persisted when the stimulation period had been 13 s (Fig. 3 b_2), 6.5 s (Fig. 3 d_2), and 3 s (Fig. 3 e_2). In addition, roughly the twofold interval length showed up at least in two series (Fig. 3 b_2, d_2, d_3). Subsequent to stimulation with period lengths of 6.5 and 3 s, the endogeneous period of 6 to 8 s even persisted up to 65 s after stimulation (Fig. 3 d_3, e_3), whereas in the case of 16 s, 13 s, and 10 s the interval length approached the spontaneous value of 8 to 10 s by that time (Fig. 3 a_2, b_3, c_2). In two cases both the endogeneous period and the mean interval in the spontaneous state showed up side by side (Fig. 3 c_3, d_3).

Time-series like those presented in Fig. 2 confirm the different effectiveness of the period of stimulation: Synchronization of reversals could be detected for at least 3 cycles after stimulation; it lasted even longer with particular stimulation periods of 13, 6.5 (Figs. 2 a, b), and 3 s (not shown). Another parameter to influence the persistence of the initiated endogeneous oscillation was found to be the duration of the periodic stimulation. When the stimulation at period 13 was cut down to 5 cycles, the subsequent oscillation was reduced to 3 cycles (data not shown).

We know that already a single repellent stimulus induces a damped oscillation that persists for one or two cycles after the response (Krohs 1995). Accordingly, Fig. 1 a shows a first peak of reversal frequency as a response to the stimulus and a second peak about 8 s later, but no marked further periodicity. While a single repellent stimulus evokes only a weak oscillating signal, periodic stimulation obviously leads to an amplified signal that persists for a longer time depending on the stimulation period. The most effective way to set going the endogeneous oscillation, is the use of a stimulation period that either equals the endogeneous period, or is of half or twice its length.

It is to be expected that there will be a difference whether the train of periodic stimuli ends with a repellent or an attractant flank. Under the latter condition, the attractant flank suppressed the endogeneous reversals for at least 12 to 16 s in the same way as a single attractant suppressed spontaneous reversals (Fig. 1 b). Thereafter, reversals occurred in a synchronized manner for about 6 cycles of 6.5 to 10 s lengths (data not shown). This observation suggests that the oscillating signal induced by the repellent stimulus is not cancelled by the attractant signal and that repellent and attractant signals are qualitatively different.

Finally, we readdressed the question of whether the sensitivity to an attractant stimulus shows a phase-dependency, i.e. whether on the basis of an oscillating signal the sensitivity to stimuli also oscillates. When an attractant test stimulus was applied during the first swimming interval following periodic stimulation (Fig. 4) or during the second one (data not shown), the response showed a marked dependence on the moment of stimulation. As in former experiments that were done under slightly different conditions (Schimz and Hildebrand 1985; Hildebrand and Schimz 1990), the response reached a maximum when the stimulus was delayed about 2 s with respect to the last induced reversal (t = 0). When the same experiment was performed during the tenth interval after periodic stimulation, i.e. when the endogeneous oscillation had faded away, no significant phase-dependence of the response was left (data not shown). This is in agreement with the results obtained by Krohs (1994), when he used cells which had been sufficiently adapted to constant light. Data points below the dashed line in Fig. 4 result from those cells, which, due to the initiated endogeneous oscillation, reverse with a period of 6 to 8 s before they see the attractant stimulus. During the first cycle, 75–80% of the cells behaved in that way. The remaining cells, however, skipped this reversal, and only those could be used for measurements at longer delays. The phase-response curve shows a second maximum between 7 and 8 s. This indicates that also in the case when reversals are skipped the endogeneous oscillation goes on, although the amplitude may be too small to trigger a motor event.

A main result of this work is that the oscillation that persists after the periodic stimulation was terminated, shows a period that is invariant and almost constant what-

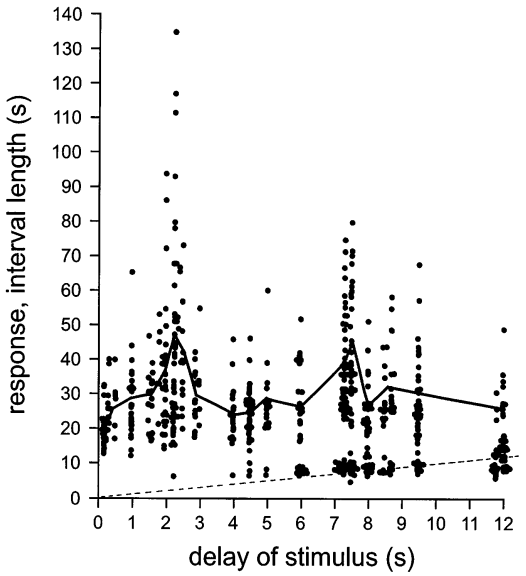

Fig. 4 Phase-response relation of the attractant response during the first swimming interval after periodic stimulation. Bacteria were stimulated as described in Fig. 2 a and a single attractant stimulus (step-up at 565 nm) was given with various delays after the last induced reversal (t = 0). The interval length between that reversal and the next one was measured as the response. *Data points* represent single measurements from different cells. The *dashed line* marks the moment of stimulation with regard to both axes and separates reversals which occurred before and after the stimulus. Points up to 3 s above this line are regarded as spontaneous reversals (Krohs 1994) and were omitted from the calculation of the average values, that are connected by *solid lines*

ever the period of entrainment may have been. The question arises of whether stimulation synchronizes a pre-existing free-running but noisy oscillation, that controls spontaneous reversals, or whether it initiates the oscillation. In our experiments, the trains of periodic stimuli start at various times after a spontaneous reversal, i.e. at different "phase-angles". Nevertheless, neither a phase-shift in the entrained oscillation nor changes of the period of endogeneous oscillation could be observed. This would be rather in agreement with the hypothesis that stimulation triggers an oscillating response at any given time.

The initiation of damped endogeneous oscillation by stimulation has parallels in higher developed organisms. For example, 40–60 Hz oscillations in the mammalian brain are initiated by visual stimuli and persist for a short time. They are assumed to be essential for information processing (Gray et al. 1989; Podvigin et al. 1992). The features which we describe here in a prokaryote are also concerned with processing of photosensory information.

According to the results which we presented here, we have to assume that different from our former assumption of a self-sustained oscillation in *Halobacterium* (Schimz and Hildebrand 1985, 1992) oscillation is initiated by stimulation as proposed by Krohs (1995). At present we do not know what parameters determine the frequency of the endogeneous oscillation. A possible source for its generation in the sensory pathway may be the feedback loop that connects the excitatory signal to the methylation of transducer proteins and thereby causes adaptation (Yao and Spudich 1992; Marwan et al. 1995). In previous experiments we observed that inhibition of methylation increases the lifetime of sensory signals (Hildebrand and Schimz 1990) and probably shifts the range at which the cells become entrained (Schimz and Hildebrand 1989, and unpublished results).

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