Competition-Integration of Blue and Orange Stimuli in *Halobacterium salinarum* Cannot Occur Solely in SRI Photoreceptor

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ABSTRACT Experiments on the integration of blue and orange stimuli in *Halobacterium salinarum* were performed by using different combinations of blue and orange steps. The results show that the prevalence of the blue stimulus over the orange one depends on both the blue and the orange light intensities. A quantitative analysis of the current hypotheses on the phototransduction of orange and UV-blue light stimuli is presented, showing that the balancing between the two antagonistic stimuli should depend only on the intensity of the blue stimulus and not on that of the orange one, provided that the combination of the two stimuli occurs linearly at the photoreceptor stage. We conclude that blue and orange stimuli elicit distinct intracellular signals whose integration occurs downstream of the photoreceptor.

INTRODUCTION

H. salinarum shows step-up photophobic motor responses to UV-blue and blue-green lights whereas orange light acts as an attractant stimulus. It is widely accepted that a background orange light is required for responses to UV-blue stimuli and it is also commonly observed that, at the onset of "white" light, the repellent stimulus prevails over the attractant one. The cell membrane of *H. salinarum* contains two sensory rhodopsins, SRI and SRII. Each of these pigments undergoes a photocycle upon light absorption. The photocycle of SRII is a single loop, while SRI behaves as a photochromic pigment, showing a one-photon and a two-photon cycle (Spudich and Bogomolni, 1988); its long-lived intermediate, S_{373}, formed from earlier intermediates lasting a few hundreds μs, can absorb UV-blue light to yield S^b_{510}. It has been proved that S_{373} is the signaling state for the responses to orange light (Yan and Spudich, 1991). Responses to UV-blue light stimuli have also been ascribed to SRI. The evidence for this is qualitative and comes from the reported fact that responses to UV-blue light only occur against an orange background, making possible the formation of S_{373} and the absorption of UV-blue light by SRI. Stronger evidence is given by the fact that the onset of an orange light on an UV-blue background elicits a step-up photophobic response, in perfect agreement with SRI photocycle (Spudich and Bogomolni, 1984). A tentative explanation of UV-blue responses by the decrease of S_{373} due to UV-blue light absorption was considered (Marwan et al., 1995). However, as pointed out by Hoff et al. (1997), photophobic responses are triggered by the simultaneous onset of orange and UV-blue light, a stimulation which induces an increase of both S_{510} and S_{373}, thus indicating S^b_{510} as the putative signaling state for UV-blue stimuli.

The information available on the transduction chain in *H. salinarum* is not limited to pigment photocycles (for a recent review, see Hoff et al., 1997). It has been shown that both pigments are closely associated to specific transducers, HtrI and HtrII, respectively. The Htr proteins both have a signaling domain and two methylatable domains. SRI and SRII pigments are each closely associated with specific methyl-accepting transducers, HtrI and HtrII, respectively (Yao and Spudich, 1992; Zhang et al., 1996). The Htr proteins are thought to control the CheA phosphotransferase activity on CheY (Rudolph et al., 1995), which in its phosphorylated form acts as a diffusible switching signal on the flagellar motor. Basically this excitation model implies that a single kind of signal is generated by light stimulation, the action of different stimuli being mediated by the activity level of CheA. Therefore, in this view, responses to UV-blue and orange light are closely associated, sharing a common root in the signaling mechanism. The idea that the activated receptors enhance or depress the autophosphorylating CheA was originally proposed for *H. salinarum* from eubacterial chemotaxis, and has been recently reformulated in a very clear and precise way, that allows to treat it mathematically. According to Spudich and Lanyi (1996), the basis for signaling is a unitary mechanism, that should account both for signaling in sensory rhodopsins and for ion pumping in bacteriorhodopsin and halorhodopsin. In sensory rhodopsins, two conformations of the SRI-HtrI photosensor are envisaged. The bias, specific of the spectroscopic state, in the shuttling between A (attractant) and R (repellent) conformation, shifts the signaling against or in favor of the reversal events (Spudich and Lanyi, 1996; Hoff et al. 1997; Jung and Spudich, 1998). Conformation R activates the CheA kinase, whereas conformation A inhibits its activity; this clearly implies that blue and orange stimuli combine with each other in a simple additive way at the level of the photoreceptor, unless photoreceptors are organized in specialized structures.

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In this paper we report results on the integration of blue and orange stimuli and discuss them in terms of the unitary mechanism based on the assumption that the signaling is basically due to the phosphorylation of a single species (CheA). The consequences of this assumption will be compared with the data.

MATERIALS AND METHODS

Experiments were carried out on the Flx15 mutant strain of *H. salinarum* (BR^-, HR^-, SRI^+, $SRII^+$). Cells were grown under standard conditions (Spudich and Spudich, 1982). Two quartz-iodine lamps, focused on the sample, were used to stimulate the sample; the combination of stimulation and observation lights was obtained by two beam splitters. The sample under the microscope was observed in dark field with infrared illumination using a 780-nm long-pass filter (RG 780 Schott, Germany).

In order to select orange, green or blue light, interference filters (Balzer K60, 600 ± 25 nm; Balzer K40, 400 ± 25 nm; Balzer K50, 500 ± 25 nm) were used. The respective maximum light intensities were: I_{max} = 49 mW cm^{-2}, equivalent to about $1.5 \cdot 10^{17}$ photons cm^{-2} s^{-1}, for orange light; I_{max} = 5.2 mW cm^{-2}, equivalent to about $1.0 \cdot 10^{16}$ photons cm^{-2} s^{-1} for blue light; I_{max} = 26.8 mW cm^{-2}, equivalent to about $6.7 \cdot 10^{16}$ photons cm^{-2} s^{-1}, for green light.

Stimulus delivery, data acquisition and analysis were performed by a program run by a 486 PC equipped with a frame-grabber card (Matrox Pip 1024). The program acquires video images of the sample, calculates and stores the cell coordinates (within about 0.35 s, depending on the number of objects in the field), traces the cell trajectories and counts the reversals. Several (usually 10) files are stored to increase the number of observed cells; cumulative data are displayed as a histogram of the reversal frequency vs. time. Details on the apparatus for light stimulation and for data analysis are reported elsewhere (Lucia et al., 1996). A photoresponse index (*r*) is evaluated as follows:

$$r = \frac{P_r - P_c}{100 - P_c}$$

where P_r is the percentage of reversals in the response peak and P_c is the percentage of reversals in a histogram region far away from the response peak.

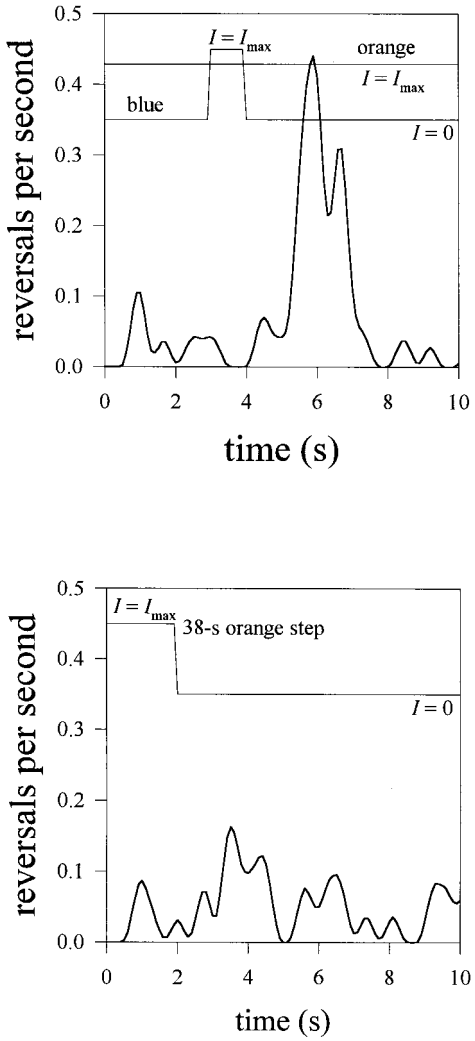

FIGURE 1 Photoresponses of a 1-day old sample: in the top panel, a good response (r = 0.63) to a blue pulse against an orange background is reported; in the bottom panel, a poor response (r = 0.22) to the offset of a 38-s orange step can be observed. The response to a green pulse showed r = 0.42. Light intensities were I_{max} in each case. Average cell number was 60 in both experiments.

RESULTS

Sensitivity to different photostimuli varies with growth phase

The sensitivity to stimuli of different wavelength varies considerably during cell growth (Hildebrand and Schimz, 1983; Otomo et al., 1989, Tomioka et al., 1986). Usually we observed, as already reported, that the sensitivity to blue-green stimuli appears before than that to orange and UV-blue stimuli. We have not carried out a systematic study on the onset of the sensitivity to different colors, but occasionally we get results (Figs. 1 and 2) which seem to be relevant to the mechanism of color sensitivity. We observed that in the early growth phase it is possible to get responses to blue pulses while the sensitivity to orange step-down is very low (Fig. 1). In the case of Fig. 1, we also tested the response to green pulses: it was present, but consistently lower than that to blue pulses. On the other hand, on a different, older culture sample, we observed responses to orange stimuli, but not to blue stimuli over an orange background (Fig. 2).

The competition between blue and orange stimuli

We studied the competition and integration of blue and orange stimuli by using different combinations of blue and orange steps. The strain used (Flx15) can synthesize both SRI and SRII, so that cells can respond both to UV-blue and to blue-green stimuli. More important, the responses due to the light filtered by a Balzer K40 filter could in principle be due either to SRI or SRII, because the absorption of SRII is still high around 400 nm. The sample of Figs. 3-6 presented in fact responses to both green (Balzer K50) and blue

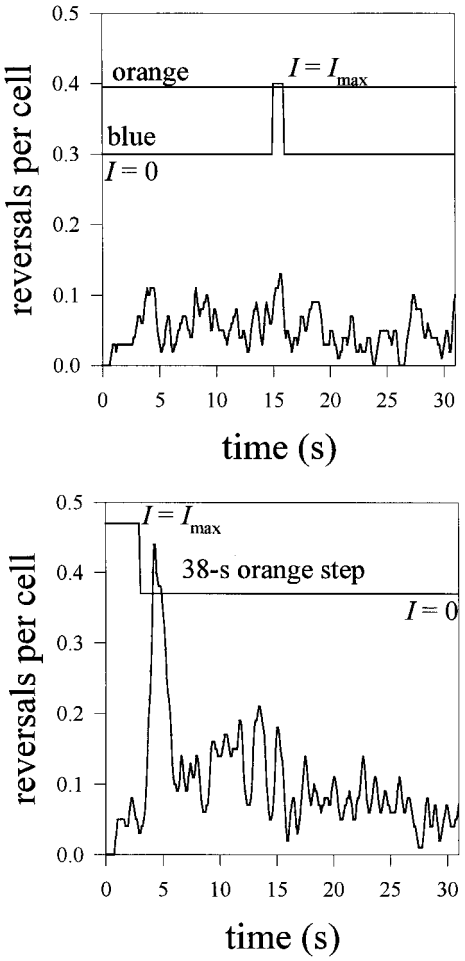

FIGURE 2 Photoresponses of a 4-day old sample: in the top panel, the response to a blue pulse against an orange background is absent; in the bottom panel, a strong response ($r = 0.77$) to the offset of a 38-s orange step can be observed. Light intensities were I_{max} in each case. Average cell number was 130 in both experiments.

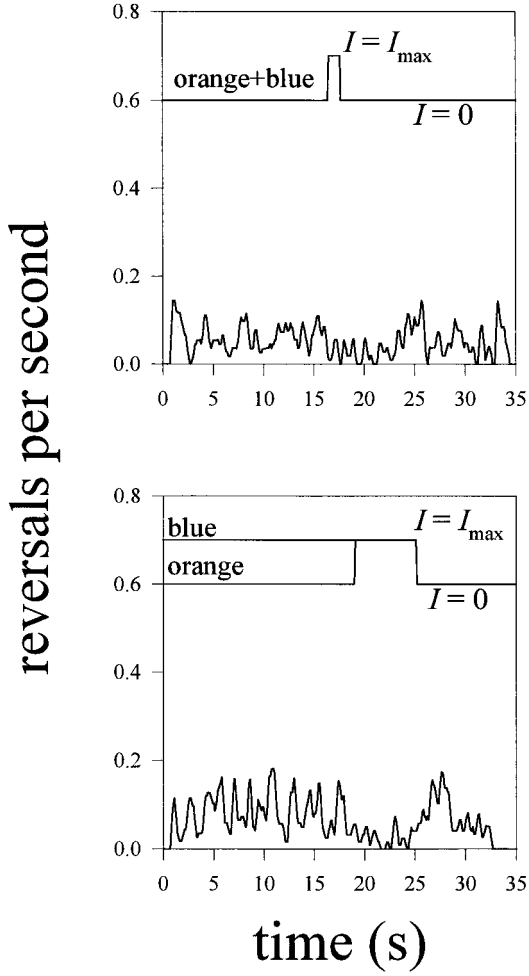

FIGURE 3 Anomalous responses of a 3-day old sample to stimuli of maximal intensity. In the top panel the absence of the typical photophobic response to a pulse of orange and blue light (concomitantly delivered by two different light sources) is apparent. The bottom panel shows the depression of the reversals at the step-up of an orange light against a blue background, at odd with the classic experiment reported by Spudich and Bogomolni (1984). Average number of cells was 120 in both experiments.

(Balzer K40) stimuli. However, the responses of Figs. 3 and 4 are unlikely to be due to SRII, because they did not occur without an orange background, a criterion usually accepted to distinguish between SRI and SRII. The sample of Figs. 5 and 6 presented responses to blue stimuli alone, also in absence of orange background; however, responses to green pulses were lower than those to blue pulses and the response at the onset of an orange step on a blue background (Fig. 6, bottom panel) is typical of SRI and in fact considered a convincing proof of the SRI-mediated blue responses (Spudich and Bogomolni, 1984). As a conclusion, we ascribe the responses reported in Figs. 3–6 to SRI.

The experiments reported in Fig. 3 surprisingly show that this sample did not present any photophobic response when an orange+blue pulse of one second and of maximal intensity was delivered or when an orange step-up was applied over a UV-blue background (as it occurred in Spudich and Bogomolni, 1984). Indeed, the orange step-up over a blue-light background clearly induced a depression of reversals. However, a blue pulse elicited a photophobic response when applied at least with a 10-s delay with respect to the orange step-up while its effectiveness was null with a 3-s delay (Fig. 4). In Fig. 4 it is also shown that, by reducing the intensity of the orange step-up, the response to the blue pulse delivered within a short delay could partly be restored. A behavior similar to that depicted in Fig. 4 also occurred with green stimuli (data not shown).

Data obtained from a different culture are reported in Figs. 5 and 6. In this case, a white pulse or a maximal blue+orange pulse elicited a photophobic response, and a photophobic response was also obtained at the onset of the orange light on a blue background (data not shown). How-

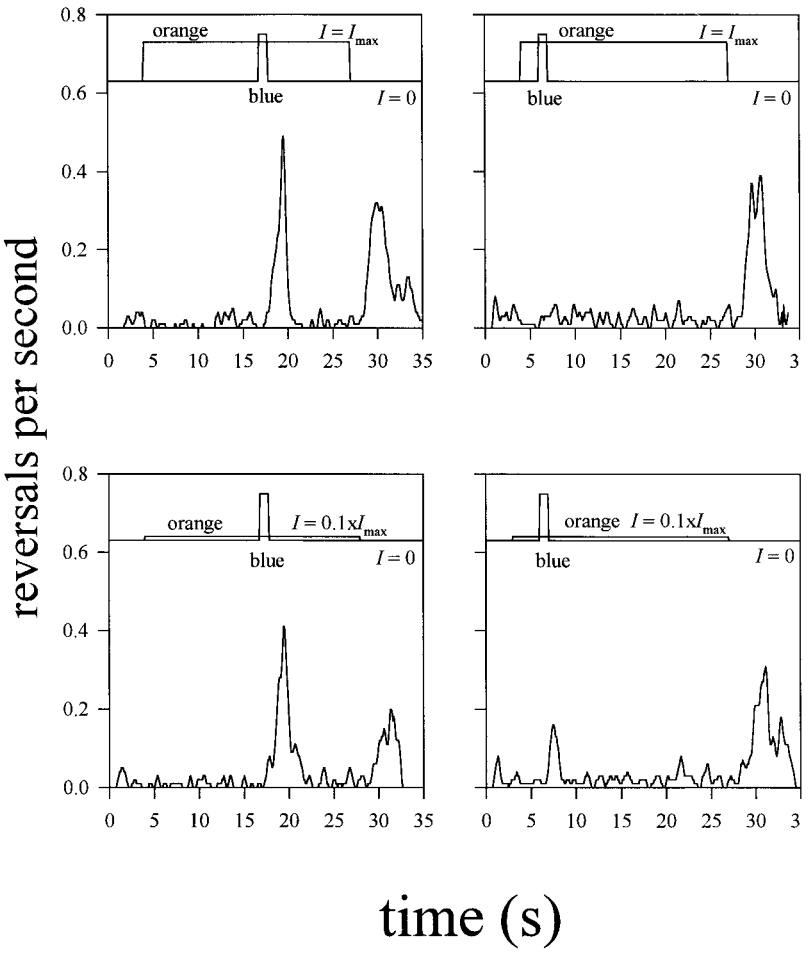

FIGURE 4 Same sample as in Fig. 3. (*Left*) Response to a late blue pulse, delivered at least 10 s after the orange step-up. Orange light intensity was maximal in the top panels and equal to $0.1 \times I_{max}$ in the bottom panels. (*Right*) No response to an early blue pulse (3-s delay) at maximal intensity of the orange background (top panel), partially restored at $I = 0.1 \times I_{max}$ (*bottom panel*). Average number of cells, 160.

ever, when the blue light intensity was reduced to 50%, the same sample displayed the "anomalous" behavior described in Figs. 3 and 4. With $I_{blue} = 0.5 \cdot I_{max}$ the orange stimulus dominated over the blue one (Fig. 5). Moreover, by reducing the intensity of the orange light it was still possible to restore the typical photophobic responses to the blue pulse at short delays from the orange step-up and to the orange step-up applied over a blue background (Fig. 6).

Expectations from the shuttling model of CheA activation

Let us analyze the model based on the photocycle of SRI and on the assumption that blue and orange stimuli have a common output downstream of the receptor, namely both control the phosphorylation level of CheA. According to the most recent formulation of this model, we will analyze its consequences speaking in terms of the shuttling between two conformations of the photosensor.

Firstly, we set in mathematical form the A-R hypothesis (A indicating the attractant and R the repellent conformation). Let α_{587}, α_{373}, and α_{510} are the probabilities of conformation R under non-adapted conditions for the ground state and for the intermediates S_{373} and S^b_{510}, respectively. Obviously, it is necessary that $\alpha_{373} < \alpha_{587} < \alpha_{510}$, because S_{373} shifts the equilibrium toward the A conformation, while S^b_{510} acts in favor of the R conformation.

The probability of finding a molecule in conformation R at the simultaneous onset of orange and blue stimuli is then given by the law of compound probabilities, getting:

$$\alpha = (S_{587}\alpha_{587} + S_{373}\alpha_{373} + S^b_{510}\alpha_{510}) \quad (1)$$

where S_{587}, S_{373}, and S^b_{510} are the fractions of the pigment in the corresponding spectroscopic states.

This expression allows calculating how many molecules of S_{373} will balance the signal coming from a single S^b_{510} molecule. When this occurs, conformation R will form with the same probability as for the ground state ($\alpha = \alpha_{587}$) and we get:

$$\alpha_{587} = (1 - S_{373} - S^b_{510})\alpha_{587} + S_{373}\alpha_{373} + S^b_{510}\alpha_{510} \quad (2)$$

whence

$$S^b_{510}/S_{373} = (\alpha_{587} - \alpha_{373})/(\alpha_{510} - \alpha_{587}) \quad (3)$$

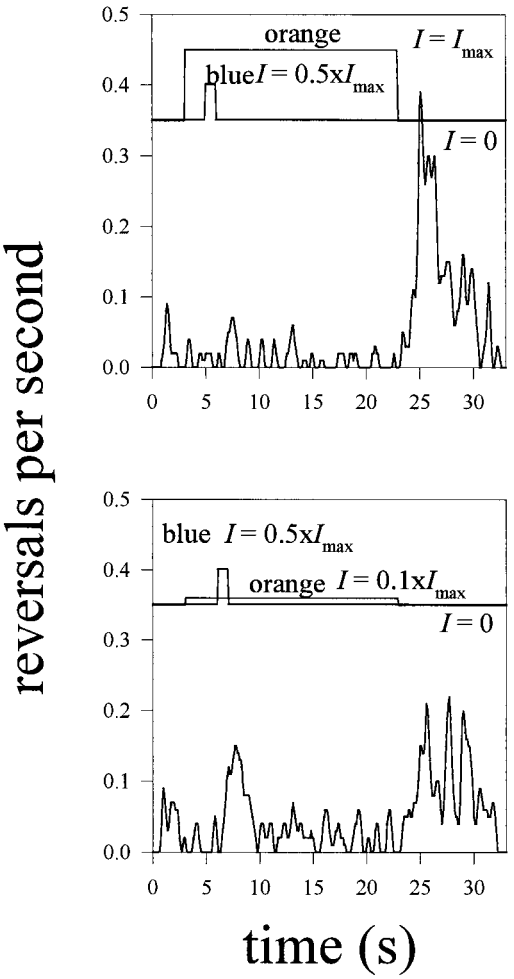

FIGURE 5 Anomalous behavior of a 2-day old sample, obtained by lowering the blue intensity at $0.5 \times I_{max}$. The top panel shows the absence of the response to a blue pulse at short delay from the orange step-up, while the bottom panel illustrates the restoring of the response when the blue pulse is delivered against an orange background of reduced intensity ($I = 0.1 \times I_{max}$). Average number of cells, 90.

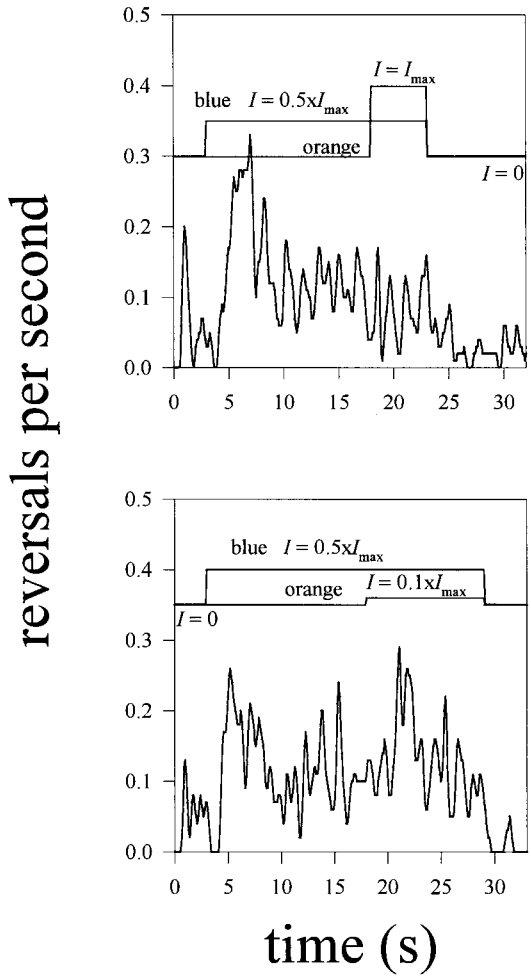

FIGURE 6 Responses of the same sample of Fig. 5 to an orange step-up against a blue background (intensity = 50% of I_{max}). In the top panel, where no response to the orange step-up is observed, the orange light intensity was I_{max}; in the bottom panel, where a photophobic response to the orange step-up is observed, the orange light intensity was 10% of I_{max}. Note that the first peak of reversals in both panels is due to the blue light onset, in the absence of an orange background. The average cell number was 90 in both cases.

Let us define the balance parameter B as:

$$B = (\alpha_{587} - \alpha_{373})/(\alpha_{510} - \alpha_{587}) \tag{4}$$

B depends only on α_{587}, α_{373}, and α_{510}, i.e., on the molecular characteristics of SRI and its intermediates, while it does not depend on the light stimuli. We can state that the bias will be in favor of the reversal of motion according to the expression

$$\frac{S^b_{510}}{S_{373}} > B \tag{5}$$

When $S^b_{510}/S_{373} = B$, the antagonistic orange and UV-blue stimuli will balance each other, for $S^b_{510}/S_{373} > B$ the UV-blue stimulus will "win" over the orange one, and vice versa for $S^b_{510}/S_{373} < B$. For example, if $\alpha_{587} = 0.5$, $\alpha_{373} = 0.49$, and $\alpha_{510} = 1$, B is equal to 1/50, and 50 molecules of S_{373} will balance the effect of a single S^b_{510} molecule; a photophobic response will occur at the onset of a UV-blue+orange light when the effect of light stimulation is such that $S^b_{510}/S_{373} > 1/50$.

Now let us consider the photocycle equation describing the formation and decay of S^b_{510} to get the ratio S^b_{510}/S_{373}. We have:

$$\frac{dS^b_{510}}{dt} = \sigma_{blue} S_{373} I_{blue} - \frac{S^b_{510}}{\tau_{510}} \tag{6}$$

In the expression above σ_{blue} is the cross-section for UV-blue light, I_{blue} is the intensity of blue light (in photons/cm^2 s), and τ_{510} is the lifetime of S^b_{510}.

From Eq. 6 at steady state (dS^b_{510}/dt) we get

$$\frac{S^b_{510}}{S_{373}} = \sigma_{blue} I_{blue} \tau_{510} \quad (7)$$

Eq. 7 shows that the ratio S^b_{510}/S_{373} does not depend on the orange intensity, but only on the intensity of the UV-blue stimulus. Then, as a consequence of Eq. 5, if an UV-blue stimulus "wins" over an orange one this will occur at any level of the orange light. There is an obvious limitation: the intensity of the orange light should produce a sufficient amount of S_{373}. We can state that, if an UV-blue stimulus predominates over an orange one of intensity I_T, the same will continue to occur at any level of the orange light higher than I_T.

It is worthwhile to note that the conclusion reported above in italics holds not only for the shuttling between two conformation but also for any mechanism in which the composition of the signal stemming from S_{373} and from S^b_{510} is assumed to be linear.

CONCLUSION

Let us consider first the results reported in Figs. 1 and 2. Two kinds of relevant behavior were observed in these samples: they either responded to orange stimuli, but not to blue stimuli over an orange background, or they responded to blue stimuli, but not to orange stimuli. This is quite puzzling if blue and orange responses are thought to be mediated by the same pigment (SRI) and through the same basic mechanism (the shuttling between two conformations of the photosensor in different spectroscopic states), although the idea could be conceived that in the sample of Fig. 1 some metabolites block the signaling in S_{373} but do not affect it in S^b_{510}, or vice versa in the case of Fig. 2. The dependence of the responses on growth phase clearly deserves further investigation, but this is beyond the scope of the present work.

Strong evidence against the above-mentioned model comes from the experiments described in Figs. 3 to 6. Here the occurrence of the typical photophobic responses (to a blue pulse in the presence of an orange background and to an orange step applied on a pre-existing blue background) is tested with several combinations of orange and blue light intensities. These results show that the competition between orange and blue stimuli is not always in favor of the blue stimulus, even at maximal light intensity (Figs. 3 and 4). When, as it usually occurs, the blue stimulus predominates at maximal intensities, it is possible, by lowering the blue intensity (to half of its maximal value in Figs. 5 and 6), to get a situation in which the orange stimulus predominates, a quite obvious result. The important point is however that, under this condition (orange predominating), lowering the orange stimulus restores the predominance of the blue stimulus. The last sentence may appear trivial: this is just what occurs in the competition between green and orange stimuli. But it must be borne in mind that blue-green and orange stimuli impinge on different pigments, while the response to UV-blue light is supposed to depend on SRI and more precisely on the amount of the intermediate S_{373} formed under the action of orange light. In "Expectations from the shuttling model of CheA activation" a quantitative discussion of this point is reported, showing that the predominance of orange over UV-blue stimuli should not depend on the orange light intensity but only on that of the UV-blue light. Therefore, in the frame of the model, lowering the orange intensity cannot induce the predominance of blue stimuli. This mismatch between the prediction of the model and the results of the experiments clearly shows that the control of CheA activity in the photochromic photoreceptor SRI (through the shuttling between two conformations of the photosensor SRI-HtrI) cannot be the unique basis for signaling. To make this point more clear: a nonlinear integration of blue and orange stimuli can occur if and only if, together with the modulation of CheA activity, something else occurs and a signal X is sent from the receptor, presumably through the cytoplasm, to the motor switch.

Unless photoreceptor clustering occurs, the integration of different stimuli at the photoreceptor stage is necessarily linear, because no interaction is expected between individual photoreceptors. The above statement has nothing to do with the non-linearity of the relationship between the concentration of the signaling states and the stimulus light intensity (this non-linearity is obvious and implicit in the photocycle equations). Analogously, considering the case of eubacteria, the non-linearity between the bias in favor or against run and tumbles and the concentration of attractants or repellent stimuli is not connected with the above argument. The non-linearity that we see as a necessary consequence of our results concerns the composition of the internal signals (the plural is important) generated by blue and orange stimuli. The need for a nonlinear composition brings us to shift the integration of the two pathways from the photoreceptor to the cytoplasm.

The existence of two cytoplasmic signals could also account for the color specificity of reported delayed effects of stimuli, occurring after the delivery of a stimulus on a time scale much longer than that of the photocycle (MacCain et al., 1987; Lucia et al., 1996, 1997; Cercignani et al., 1998).

However, an alternative interpretation of the experimental results of the present paper could also be considered: either shuttling is not the unique source of signaling or the hypothesis that S^b_{510}, or any species present in amounts proportional to it, mediates the responses to UV-blue light does not hold. The issue concerning the signaling nature of S^b_{510} deserves more attention and can further be investigated by testing whether or not there is a correlation between the amount of S^b_{510} and the photoresponses to UV-blue light stimuli. In this context the recent report on a haem-contain-

ing, blue-absorbing receptor in *H. salinarum* (Hou et al., 2000) could be relevant, although its possible role as a photosensor is only speculative.

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