In vivo imaging of calcium accumulation in fly interneurons as elicited by visual motion stimulation

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ABSTRACT The computation of motion plays a central role in visual orientation. The fly has been successfully used as a model system for analyzing the mechanisms underlying motion detection. Thereby, much attention has been paid to a neuronal circuit of individually identifiable neurons in the third visual ganglion that extracts different types of retinal motion patterns and converts these patterns into specific components of visual orientation behavior. The extended dendritic trees of these large cells are the sites of convergence of numerous spatially distributed local motion-sensitive elements. As is revealed by in vivo microfluorometric imaging, these cells accumulate calcium during activation by visual motion stimulation. The spatiotemporal pattern of calcium distribution shows the following characteristics: (i) calcium accumulation is first spatially restricted to those dendritic branches that are depolarized by the retinotopic input, (ii) during ongoing motion stimulation calcium may also accumulate throughout the cell and, in particular, in regions that do not receive direct synaptic input. These experiments successfully monitor the intracellular distribution of activity-dependent ions in visual interneurons of living animals stimulated by their natural synaptic input.

Spatial integration of local motion signals is one of the key processing steps in motion vision. Studies of the visual ganglia of insects (1), the middle temporal area of monkeys (2, 3), and the medial superior temporal area (4, 5) of monkeys reveal that integration plays a decisive role in tuning motion-sensitive cells to particular types of retinal motion patterns. This processing step is assumed to be accomplished by the distributed action of large numbers of synapses subserving upstream local motion-sensitive elements. The spatiotemporal characteristics of dendritic integration have so far been analyzed only by conventional electrophysiological techniques. However, optical recording techniques are now available for monitoring the two-dimensional distribution of neuronal activity in single nerve cells. They have been successfully applied to analyze dendritic integration of synaptic input in nerve cells such as hippocampal pyramidal cells or cerebellar Purkinje cells (6–9). For methodological reasons, these studies have been done mostly in cell culture or brain slices rather than in the whole animal (6–9).

As is reported here, optical recording techniques can now also be applied in vivo to study dendritic integration of local motion information. Because of their peculiar geometry, the directionally selective motion-sensitive large-field cells in the third visual ganglion of flies (1) are particularly useful for such an analysis. Their dendritic trees are almost two dimensional and are located within a thin superficial layer of the brain. With only minimal dissection of the animal, large parts of their branching pattern can thus be visualized in the focal plane of a microscope after injecting the cells with a fluores-
The lower trace represents the membrane potential the schematic imaging spike-like station filters (bandwidth, tometrics, ultralow dark intensity Va) head (CH and (CE 512) A) until the sequence a controller board (NU 200). Images were acquired and evaluated by a software package (IPLab, Signal Analytics, Vienna, Va) on a Macintosh IIx computer (Apple).

**Experimental Procedure.** The dye was injected into the cell (10–30 min; –1 to –3 nA) until the major cellular processes could be clearly discerned from the background (Fig. 2A). After injection of the dye, 10–30 min was allowed for the dye to diffuse throughout the cell before the experiment started. However, the most distal dendritic branches on which most of the input synapses of these cells reside (16) usually could not be recognized in the fluorescence images in vivo, as is suggested by a comparison with anatomical data using conventional dyes and sectioned material (15–17). At rest, the fluorescence intensity of the main cell branches was typically 2–5 times higher than background. In some of the experiments, the electrical activity of the cell was simultaneously recorded with an intracellular electrode to ensure that it responded in its normal way (Fig. 1). During the experiments, a sequence of pictures was taken at 1.5-s intervals under epifluorescence illumination of the cell at 380 nm. Five control pictures were taken preceding stimulus presentation followed by 20 images during stimulus motion. Another 15 images were taken after cessation of pattern motion. To determine the resting calcium concentration (13), two pictures were taken with excitation filters centered at 380 (bandwidth, 10 nm) and 349 (bandwidth, 10 nm), respectively, before and after the experiment.

**Image Processing.** From the raw fluorescence images, the changes in the relative fluorescence ($\Delta F/F$) were determined in the following way. The first picture of the sequence was used as a reference. This picture was subtracted from each picture of the sequence. The resulting difference picture was then divided by the reference picture pixel by pixel. A decrease in $\Delta F/F$ corresponds to an increase in cytosolic calcium concentration (13). Because of movements of the brain that may occur in a living animal, the reference image and successive images may be misaligned, leading to artefacts in the relative fluorescence image. Care was taken to evaluate only those image sequences where these artefacts did not occur or, at least, where they were considerably smaller than the stimulus-induced calcium signals. Compare, e.g., in Fig. 2 B–D positive (dark) values to the right of the axon with the signals in the dendrite. The calculation of the change in relative fluorescence described so far does not take into account the contribution of the background to the intensity measured at each image pixel. Therefore, the background must be subtracted to determine the true relative fluorescence change. However, calculation of the $\Delta F/F$ value requires, in this case, a clear distinction of the cellular processes from the background. Otherwise, the denominator may become very small and, hence, the corresponding $\Delta F/F$ value will be extremely prone to noise. We therefore determined the background-corrected $\Delta F/F$ value only for the axon and the main dendritic branches of the cells. For similar reasons, determination of the resting calcium level also requires background subtraction and was consequently calculated only in those regions where the cell could be clearly discerned from background in the raw fluorescence intensity image. Here, the resting calcium concentration was in the range between 20 and 60 nM. From known resting calcium concentration and the background-corrected change in relative fluorescence, the absolute calcium concentration can be calculated for each image frame (ref. 18; V. Lev-Ram, H. Miyakawa, N. Lasser-Ross, and W. N. Ross, personal communication). Variations of the background fluorescence within an image has to be considered as the major source of uncertainty for the values given below. These variations can affect the measurement of the resting calcium concentration.
as well as the calculation of the change in relative fluorescence, but they are unavoidable because of the inhomogeneous background fluorescence of the in vivo preparation.

RESULTS

Motion stimulation leads to localized fluorescence changes in the dendrite. As shown in Fig. 2 B–D, an HSE cell (11, 15) was stimulated by horizontal motion of a grating pattern in either the dorsal or the ventral part of its receptive field, respectively, or in both regions simultaneously. The resulting changes in relative fluorescence are shown in Fig. 2 B–D after 9 s of motion. The fluorescence changes are restricted to those parts of the dendrite that are activated directly by the retinotopic motion input. During motion stimulation in the dorsal part of the receptive field, there is a calcium response only in the vicinity of one of the main dorsal dendritic branches (Fig. 2B). In contrast, when there is motion in the lower part of the cell’s receptive field, only areas at the terminals of two ventral dendritic branches indicate an increase in cytosolic calcium. When both patterns move simultaneously, both parts of the dendrite reveal pronounced fluorescence changes (Fig. 2D). This experiment has been done in a total of 15 neurons of various types (HS, VS, CH; for the anatomy and functional properties of these cells, see ref. 19). In all cases, a calcium increase was localized in those dendritic regions that correspond to the part of the stimulated visual field. A quantitative estimate of the Ca

\[ Ca^{2+} \] concentration cannot be given here, because this requires the background fluorescence to be subtracted from the image before the relative fluorescence changes are determined (13) (see Materials and Methods). Without background subtraction, the relative fluorescence changes might be significantly underestimated. However, those parts of the cell that show the most pronounced fluorescence changes are not the main dendrites but rather the fine distal dendritic branches that cannot be discriminated from the background in the raw fluorescence intensity image (compare Fig. 2A with Fig. 2 B–D). Nevertheless, the data shown in Fig. 2B–D can at least be compared qualitatively with each other since, within the labeled regions, neither the density of dendritic branching nor the fluorescence intensity of the background varies systematically. Despite the limitations of an in vivo preparation, the localized changes in relative fluorescence indicate a local retinotopic calcium accumulation. Hence, this experiment visualizes directly the retinotopic input organization of visual interneurons.

Calcium accumulation may not always remain restricted to the fine branches of the neurons throughout the time of stimulation. In the example shown in Fig. 3, another motion-sensitive cell of the third visual ganglion, a VS1 cell (17, 20), was injected with fura-2 and subsequently stimulated in the ventral part of its receptive field with downward motion (Fig. 3A). Fig. 3 B and C shows the relative fluorescence changes at two instants of time, 6 and 27 s after the stimulus pattern started moving. First, only the ventral branches of the cell’s dendrite are labeled (Fig. 3B), consistent with the known map of the receptive field on this region. In addition, the major ventral dendrite along with the soma and the axon show some slight labeling. After 27 s (Fig. 3C), the labeling has generally increased. Furthermore, it is no longer localized but has spread, in particular to those parts of the dendrite that do not receive synaptic input themselves from the motion stimulus used here. In the experiment shown in Fig. 3 branches of the cell are labeled that can be clearly distinguished from the background in the raw fluorescence images (Fig. 3E), and it is possible, therefore, to calculate the background-corrected fluorescence changes in these areas. This was done at five different locations, indicated by windows in Fig. 3D. Fig. 3E shows the time course of the relative fluorescence change at
four locations on the dendrite (d1–d4) and the soma (s) of the cell. Calcium accumulation starts in those dendritic branches that are activated during motion stimulation by retinotopic synaptic input (d1 and d2). At these locations, the relative calcium changes are greatest throughout stimulation (∼25%). The change in relative fluorescence of the soma also starts right at the beginning of stimulation. In contrast, the fluorescence signal in the dorsal branches of the cell’s dendrite follows with a delay of 10–15 s. The fluorescence signals reach a plateau that is maintained for the duration of the stimulus and subsequently decline to the background level within the next 10–20 s. When the resting Ca²⁺ concentration is known, the background corrected change in relative fluorescence can be transformed into absolute Ca²⁺ concentration (ref. 18; V. Lev-Ram, H. Miyakawa, N. Lasser-Ross, and W. N. Ross, personal communication). This has been done for the data shown in Fig. 3. With respect to a resting Ca²⁺ concentration of 50 nM, a 10% decrease in background-corrected ΔF/F corresponds to a Ca²⁺ concentration of 84 nM and a 30% decrease in ΔF/F corresponds to a Ca²⁺ concentration of 185 nM.

**DISCUSSION**

The data illustrate that the processing of motion information in the dendritic tree of the large-field cells in the third visual ganglion of the fly is accompanied by intracellular calcium accumulation. Calcium rapidly accumulates in those regions of the dendrite that receive direct retinotopic synaptic input (Fig. 2). Preliminary experiments suggest that local Ca²⁺ accumulation depends on the stimulus strength in a graded way. During ongoing stimulation, calcium may also accumulate throughout the cell. In several experiments, the global calcium signal was larger than the initial local transients and developed faster than in the example shown in Fig. 3. Although this has not yet been analyzed quantitatively, it seems that this global signal is triggered by stronger stimulation.

In contrast to the vast amount of data available on the properties of the large-field cells of the fly in response to visual stimulation and on their significance in visual orientation behavior (1, 12), almost nothing is known so far about the ionic basis and the pharmacological characteristics of the ion channels underlying their potential changes. The same is true for the functional role of calcium in the motion-sensitive large-field cells. However, there are two electrophysiological phenomena of these cells that might be linked to calcium regulation. (i) Calcium may underlie membrane potential changes such as the spike-like depolarizations (Fig. 1). It is still not clear where in the cell these spikelets are generated. Active membranes in dendrites and, in particular, dendritic spikes have been found recently to be quite common in nerve cells (for review, see refs. 21 and 22). (ii) Adaptational changes have been proposed in the motion-detection system of the fly (23–25). Calcium may act as a second messenger that controls these changes in the sensitivity of the cell to motion stimulation, for instance, by activating calcium-dependent potassium channels. By comparing the dynamic properties of the large-field cells and their local motion-detecting input elements (14, 26), at least part of the decline in response amplitude in the large-field cells during maintained motion stimulation (Fig. 1) may be due to such
adaptational changes. Interestingly, these changes have a time course that is reminiscent of the time course of calcium accumulation in the cell (compare Fig. 1 Lower right and Fig. 3F). Future experiments with photolabile calcium cages (27, 28) should reveal whether calcium does indeed modulate the sensitivity of the cell to motion stimulation.

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