The Subcellular Distribution of Calcium in the Visual Cells of Crayfish, Observed with the Combined Oxalate-Pyroantimonate Technique, Depends on Extracellular Calcium

Hans Jarminowski and Hennig Stieve

Institut für Biologie II (Zoologie), RWTH Aachen, Kopernikusstraße 16, D-52074 Aachen, Bundesrepublik Deutschland

Z. Naturforsch. **50 c,** 581–587 (1995); received March 9/April 12, 1995

Orconectes, Calcium, Electron Microscopy, Photoreceptor, Antimonate

1. With the combined oxalate-pyroantimonate method we determined the subcellular distribution of calcium in the photoreceptor cells of the crayfish *Orconectes limosus*.

2. The calcium antimonate deposits were identified with the electron microscope. Energy-dispersion X-ray analysis verified that the electron dense deposits contained calcium and antimony.

3. Calcium antimonate deposits were found in the submicrovillar cisternae, mitochondria, multivesicular bodies, and pigment granules, as well as in the cytoplasm.

4. The calcium deposits in the cytoplasm of the retinula cell and in the pigment granules were reduced after incubation of the retinae in a solution with a lowered calcium concentration and even more after we permeabilized the plasma membrane with the saponin β-escin.

5. We suppose that the calcium-storing pigment granules participate in the regulation of the cytosolic calcium concentration of the crayfish photoreceptor cells.

Introduction

In photoreceptor cells of invertebrates calcium plays an important role in light adaptation and excitation. Illumination causes an increase in the cytosolic calcium ion concentration in the *Limulus* ventral nerve photoreceptor cell (Brown and Blinks, 1974; Maaz and Stieve, 1980; Nagy and Stieve 1983; Ivens and Stieve, 1984; Levy and Fein, 1985; Payne *et al.*,1988). The light-induced increase in the intracellular calcium concentration follows the receptor current with some delay (Stieve and Benner, 1992; Ukhanov *et al.*, 1995). After strong illumination this increase is smaller in calcium-free external solution, but is larger in raised external calcium solution (Brown and Blinks, 1974). Electrophysiological experiments with crayfish ommatidia showed that the amplitude of the transient receptor potential does not change significantly after incubation in a solution containing a low calcium concentration. When calcium is additionaly lowered by adding 10 mmol/l EGTA, the receptor potential is reduced, but there is no difference in this reduction no matter whether the retina is previously illuminated or kept in darkness for one hour (Becker *et al.*, 1988).

With the combined oxalate-antimonate technique we were able to detect calcium in the cytoplasm as well as in other cell compartments. Calcium was first specifically precipitated by oxalate whereafter other cations were washed out. Subsequently the calcium oxalate was converted to water-insoluble calcium pyroantimonate by addition of osmium and pyroantimonate (Borgers *et al.*, 1981; Slocum and Roux, 1982).

We already showed (Jarminowski *et al.*, 1993) that photoreceptor cells of the crayfish contain three kinds of actively calcium-accumulating organelles: submicrovillar cisternae, mitochondria, and pigment granules. In this study we show the distribution of calcium within the cytoplasm and organelles in dependence of the extracellular calcium concentration. After incubation of the retinae in a solution with a lowered calcium concentration the calcium within the retinula cells, the tapetum cells and the pigment granules is reduced.

Part of these results have already been published in abstract form (Jarminowski and Stieve, 1994).

Reprint requests to Dr. H. Jarminowski.
Telefax: (0241) 88881 33.

0939–5075/95/0700–0587 $ 06.00 D

Material and Methods

Animals

Adult crayfish, *Orconectes limosus R.*, were obtained from a commercial dealer (Havelfischerei Liptow und Gabriel, Berlin, Germany) and kept under laboratory conditions at 15 °C before preparation.

Isolation of the retinae

After removal of the eye stalks at noon at room light, the optic neuropile together with the retina was pulled out of the eye stalk with forceps. Then most of the nerve tissue including the lamina ganglionaris was cut off with scissors.

Reagents

All reagents except for the oxalic acid (Merck, Darmstadt, Germany) and the potassium hexahydroxoantimonate (Riedel-de Häen, Seelze, Germany) used in this study were purchased from Sigma (Deisenhofen, Germany).

Experimental procedure

We did three different kinds of experiments with five retinae each. In one group retinae were fixed immediately after preparation without any pre-treatment. In the second group of experiments retinae were incubated for one hour in a physiological saline (10 mmol/l Tris, 203 mmol/l NaCl, 5 mmol/l KCl, 3 mmol/l $MgCl_2$, pH 7.5) without added calcium (Ca concentration was estimated to be <1 µmol/l, called "calcium-free" in the further text). In a third group the retinae were treated for 10 min with the saponin β-escin (50 µg/ml physiological saline) to permeabilize the plasma membranes before incubation with the "calcium-free" physiological saline. In the second and third group of experiments the retinae were treated at room temperature in the above described way to reduce the cytosolic calcium before fixation. All treatment was done at natural room light.

Electron microscopy

After preparation and incubation with the different salines the retinae were fixed in 3% glutaraldehyde and 90 mmol/l potassium oxalate for 2 hours on ice. Afterwards the retinae were washed for 24 hours on ice in 7.5% sucrose, containing 90 mmol/l potassium oxalate.

The oxalate-bound calcium was transformed into an electron-dense, water-insoluble precipitate of calcium pyroantimonate during postfixation. The fixative contained 1% osmium tetroxide and 2% potassium hexahydroxoantimonate in 0.01 N acetic acid. The retinae were fixed for 2 hours on ice. After washing in distilled water the retinae were dehydrated in a series of acetone and embedded in Spurrs resin.

To dissolve the pyroantimonate in 0.01 N acetic acid the solution was heated to 80 °C in a covered plastic container.

The pH of all solutions was adjusted to 7.4 with potassium hydroxide.

Ultrathin sections were made with an Nova ultramicrotome (LKB), mounted on formvar-coated copper grids, and observed after poststaining with uranyl acetate and lead citrate in a Philips EM 300 electron microscope.

X-ray microanalysis

To check whether the precipitate contains calcium, we did an energy dispersion X-ray analysis in a JEM 2000 FX II electron microscope with an EDX Tracor TN 5502. We analyzed the same sections (Fig. 2) which we had used for the routine electron microscopy.

Results

Electron microscopy

The three kinds of experiments provided different results.

1. Fixation without any pre-treatment

In the retinae fixed immediately after preparation without further pre-treatment precipitates of calcium antimonate are distributed all over the photoreceptor cell (Fig. 1). In the cytosol of the retinula cell the precipitate is extremely dense so that mitochondria, multivesicular bodies and submicrovillar cisternae are not identifiable. In the region of the microvilli calcium antimonate is somewhat less dense than in the other parts of the retinula cells. In the region of the tapetum cell we find the fewest precipitates of all. Most of the pig-

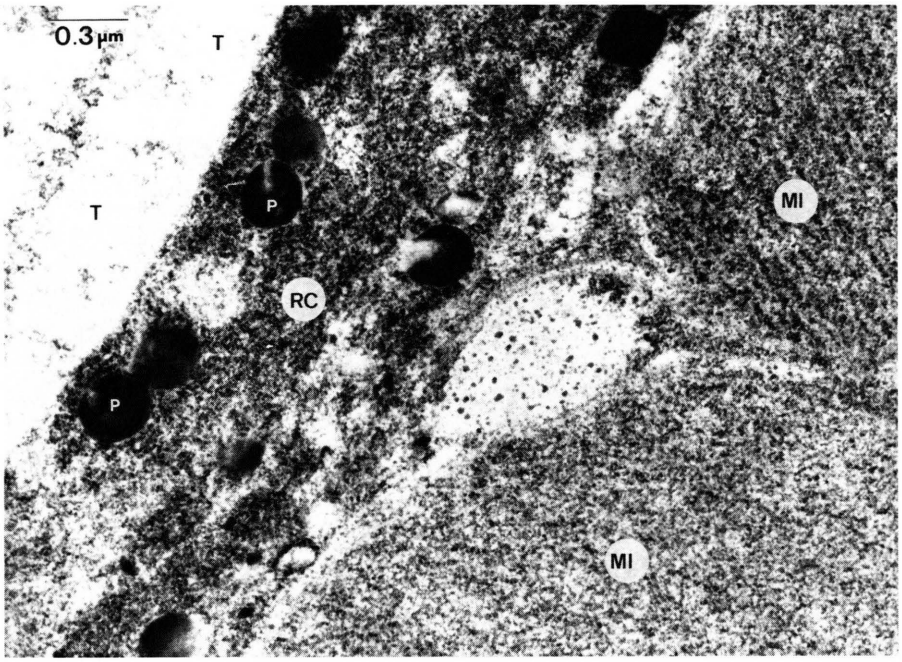

Fig. 1. Longitudinal section through the proximal region of the retina with three different cell regions: Microvilli (MI), retinula cell body (RC) with pigment granules (P) and tapetum cell (T). The retina was treated with oxalate and antimonate without other preincubation. The reaction product of calcium antimonate precipitates in fine granules.

ment granules are very densely covered by precipitate (Fig. 2).

2. Incubation in "calcium-free" saline

If the retinae were incubated before fixation in "calcium-free" saline, the antimonate precipitate in the cytoplasm of the retinula cell is reduced in the region of the microvilli (Fig. 3) as well as in the tapetum cell. Interestingly precipitate is also found within the multivesicular bodies. We found precipitate also in mitochondria and pigment granules. The amount of precipitate in the pigment granules is slightly reduced after incubation of the retina in "calcium-free" saline.

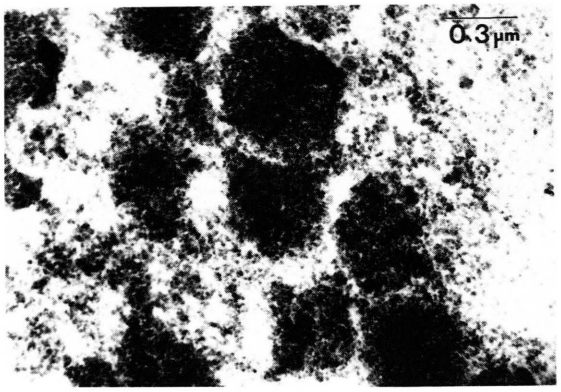

Fig. 2. A group of pigment granules in the proximal retinula cell region with the fine granular precipitate of calcium antimonate. The retina was treated in the same way as in Fig. 1.

3. Incubation in "calcium-free" saline after pre-treatment of the retinae with the plasma membrane permeabilizing β-escin

After treatment of the retinae with β-escin and incubating with "calcium-free" saline the amount of precipitate of calcium antimonate is even more reduced. In the cytoplasm of the retinula cell there is nearly no precipitate. In the region of the microvilli only a few granules of calcium antimonate are to be observed (Fig. 4). In mitochondria (Fig. 5), multivesicular bodies (Fig. 4), and in the submicrovillar cisternae (not shown) the precipitate is still to be seen. There is no precipitate found in the tapetum cell. Only a few pigment granules show the reaction product of calcium and antimonate, which is in addition strongly reduced compared to incubation in "calcium-free" saline without β-escin.

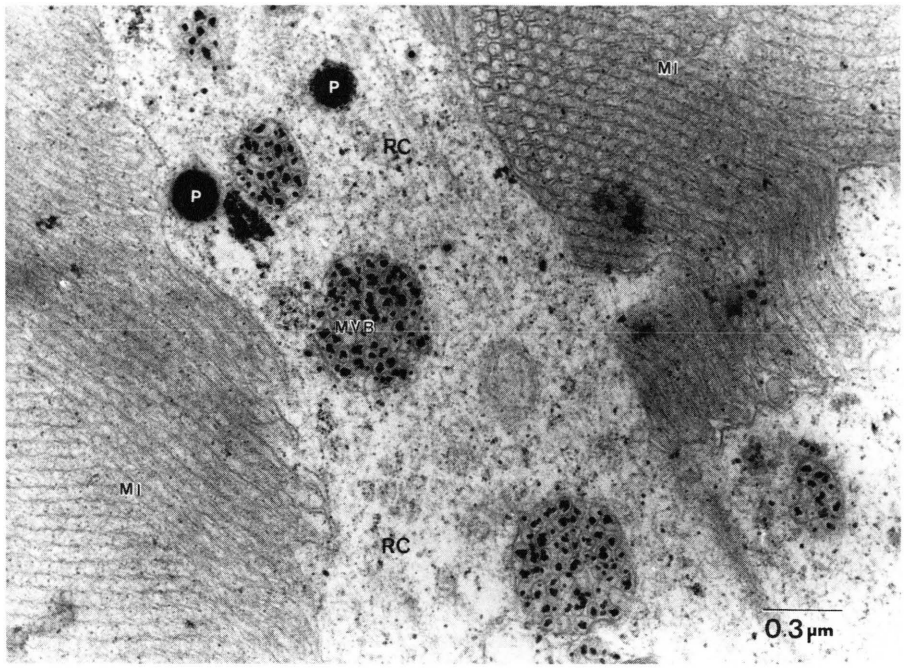

Fig. 3. Longitudinal section through the proximal region of the retina with some multivesicular bodies (MVB), filled with precipitate, retinula cell body (RC), pigment granules (PG) and microvilli (MI). The retina was incubated with "calcium free" saline (<1 µmol/l) and then treated with oxalate and antimonate. Compared with the retina in Fig. 1 the precipitate is reduced in the cytoplasm of the retinula cell as well as in the region of the microvilli.

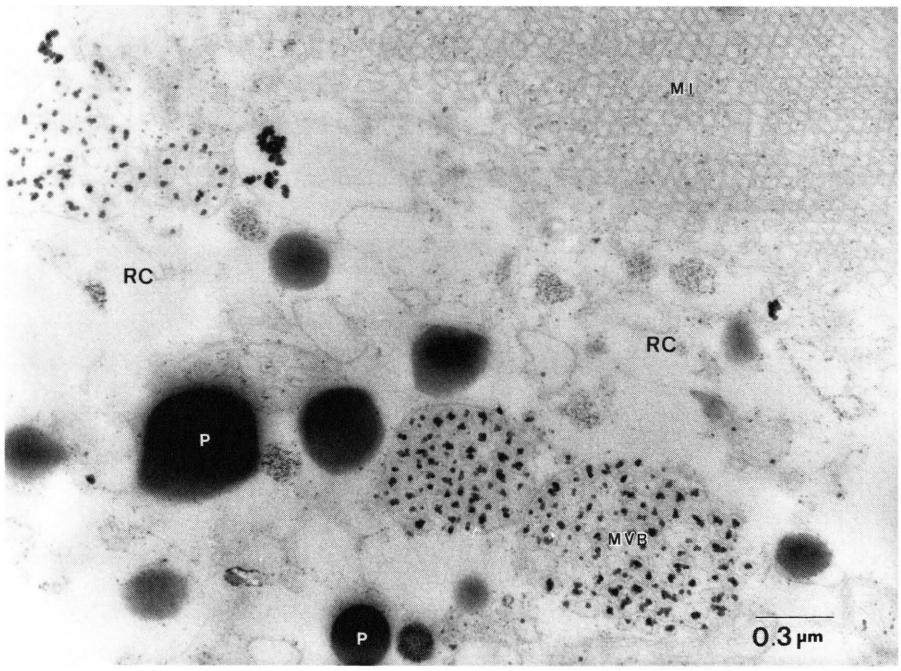

Fig. 4. Longitudinal section through the proximal region of the retina with some multivesicular bodies (MVB), filled with calcium antimonate, retinula cell body (RC), pigment granules (P), and microvilli (MI). In the retinula cell cytoplasm there is nearly no precipitate, and in the region of the microvilli only a few granules of calcium antimonate are visible. The plasma membrane of the retina was permeabilized with ß-escin, incubated with a lowered calcium solution and then treated with oxalate and antimonate.

X-ray microanalysis

The X-ray spectra of electron-dense precipitates obtained after pyroantimonate treatment verified that antimony and calcium are components of these precipitates. Fig. 6 shows the X-ray spectrum of a pigment granulum shown in Fig. 2. The calcium peaks are partially overlapping the anti-

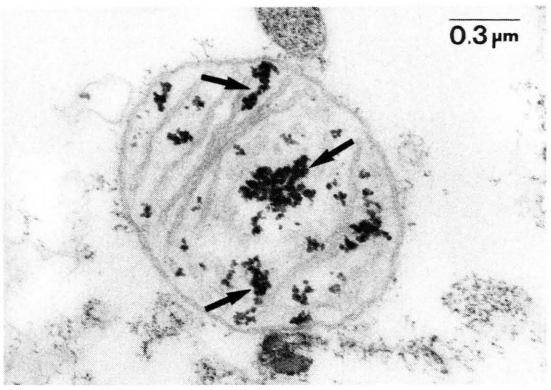

Fig. 5. Mitochondrium filled with calcium antimonate (arrows). The retina was treated in the same way as in Fig. 4.

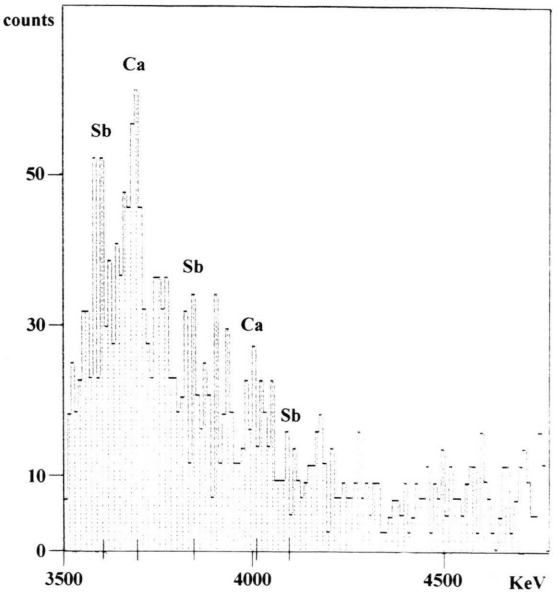

Fig. 6. X- ray spectrum obtained from a pigment granulum with precipitate shown in Fig. 2. Vertical scale = 72 counts, data acquired over a 3500–4780 keV energy range (horizontal scale). Identified elements are calcium and antimony (Ca/Sb, spectra overlap). Calcium spectrum: K_α = 3.691, K_β = 4.012; antimony spectrum: L_α = 3.605, L_β = 3.843, $L_{\beta 2}$ = 4.100.

monate peaks. From this analysis it can be virtually excluded that there are other cations which are precipitated by antimonate in these samples. All analyses (n = 3) of precipitates found in the cytoplasm of the retinula cell and in mitochondria deliver the same results (data not shown). The only ion precipitated with antimonate is calcium.

Discussion

Our results show that the cytosolic calcium content of crayfish photoreceptor cells depends upon the extracellular calcium concentration. Calcium is stored in several intracellular organelles: submicrovillar cisternae, mitochondria, and pigment granules (Jarminowski *et al.* 1993). Some of these stores can exchange their calcium with the cytosol. When the cytosolic calcium is reduced due to the omission of extracellular calcium, the pigment granules lose part of their calcium content; this effect is even greater after permeabilizing the plasma membrane with β-escin.

Electrophysiological experiments have shown that the photoreceptor cells of crayfish can tolerate very low external calcium concentration (< 1 µmol/l) much better than the ventral nerve photoreceptor cells of *Limulus* (Stieve *et al.*, 1986) unless very high concentrations of calcium chelators (10 mmol/l EDTA) are applied (Stieve and Claßen-Linke, 1980). Extracellulary lowered calcium concentration led to only a slight reduction of the light-induced transient cytosolic calcium increase in *Limulus*, while the light-induced rise in cytosolic calcium was nearly abolished in the photoreceptor cells of the crustacean *Balanus* under the same conditions (Brown and Blinks, 1974). This difference could be due either to the small size or capacity of the internal calcium stores (Werner *et al.*, 1992) and/or to differences in the regulation of the calcium content of the intracellular calcium stores.

There are only few publications dealing with the calcium content of the different cell compartments of photoreceptors. Somlyo and Walz (1985), using energy dispersion X-ray microanalysis in cryo sections, show the cytoplasmatic calcium concentration of the rod inner segment in the frog to be about three times larger than in the endoplasmic reticulum of the inner segment. Baumann *et al.* (1991) using the same method for honey bee photoreceptor cells, found that after illumination the amount of calcium in the endoplasmic reticulum is reduced from 47.5 mmol/kg dry weight to 22.4 mmol/kg. Schroeder *et al.* (1980), using laser microprobe mass spectroscopy, showed that the

distal pigment granules of the crayfish retina contain up to 100 mmol/l calcium.

We estimated the size of the intracellular stores in photoreceptor cells of *Orconectes* on ultrathin sections: In the area (ca. 800 μm^2) of a longitudinal section of a retinula cell 1650 pigment granules were counted. Their area adds up to ca. 320 μm^2. The area of the submicrovillar cisternae determined similarly in the same section is ca. 6 μm^2. These measurements, made by one retinula cell, yield ca. 40% of the area for the pigment granules, and ca. 0.75% for the submicrovillar cisternae, of The total area of a longitudinal section of a retinula cell (Jarminowski, 1994). Under the assumption that the volumes of cisternae and all pigment granules of a retinula cell are distributed uniformely, the volume of the calcium reservoir of the pigment granules from a light adapted cell must be at least 30 fold larger than that of the submicrovillar cisternae.

The regulation of the cytosolic calcium concentration in the photoreceptor cells of the crayfish *Orconectes* is quite different from that in *Limulus*. In *Limulus* ventral photoreceptor bathed in "calcium-free" saline the intracellular stores can only be exhausted by strong illumination, in crayfish "calcium-free" superfusate with EGTA has the same effect, regardless whether the photoreceptor cell is illuminated or kept in darkness for the same time.The submicrovillar cisternae in the retinula cell of *Orconectes* are much smaller and thus their calcium content is probably much lower than in *Limulus*. The ventral photoreceptor of *Limulus* does not have any pigment granules (Calman and Chamberlain, 1982); it has, however, dense bodies (Herman, 1991) and it is not known wether these contain calcium.

It has been argued that the mitochondria do not contribute to the regulation of the cytosolic calcium concentration because they release calcium only at much lower than physiological calcium concentrations (Meldolesi *et al.*, 1990). Here we have shown that at least the pigment granules contain less calcium when the cytosolic calcium concentration is reduced to values that are probably not much lower than the physiological values. In earlier studies we have demonstrated that the pigment granules can actively accumulate calcium in the presence of ATP (Jarminowski *et al.* 1993). Obviously they can release calcium under almost physiological conditions. We therefore suppose that the calcium-storing pigment granules may take part in the regulation of the cytosolic calcium concentration of the crayfish photoreceptor cells. They may serve at least as active calcium stores which supply the cytosol with calcium when the calcium concentration is lowered in a physiological range.

Acknowledgements

We are especially thankful to Marianne Dohms for excellent photographical assistance. We also thank Marlies Dorlöchter, Felix Gribakin, Birgit Hoh and Georg Stommel for critical reading of this manuscript and helpful comments and Helga Gaube for improving the English. We thank Hans-Joachim Klaar, Gemeinschaftslabor für Elektronenmikroskopie, RWTH Aachen, for doing the X-ray analysis.

Baumann O., Walz B., Somlyo A. V. and Somlyo A. P. (1991), Electron probe microanalysis of calcium release and magnesium uptake by endoplasmic reticulum in bee photoreceptors. Proc. Natl. Acad. Sci. **88**, 741–744.

Becker U. W., Stieve H. and Wendel H. (1988), Is desensitisation of crayfish retinula due to calcium depletion independent of stimulation ? Proceedings of the 16 th Göttingen Neurobiology Conference (Elsner N. and Barth F. G., eds.). Georg Thieme Verlag, Stuttgart, New York, p. 207.

Borgers M., Thone F. and van Nueten J. M. (1981), The subcellular distribution of calcium and the effects of calcium-antagonists as evaluated with a combined oxalate-pyroantimonate technique. Acta Histochem. Suppl. **24**, 327–332.

Brown J. E. and Blinks J. R. (1974), Changes in intracellular free calcium concentration during illumination of invertebrate photoreceptors. J. Gen. Physiol. **64**, 643–665.

Calman B. G.and Chamberlain S. C. (1982), Distinct lobes of *Limulus* ventral photoreceptors. II. Structure and ultrastructure. J. Gen. Physiol. **80**, 839–862.

Herman K. G. (1991), Light-stimulated rhabdom turnover in *Limulus* ventral photoreceptors maintained *in vitro*. J. Comp. Neurol. **303**, 11–21.

Ivens I. and Stieve H. (1984), Influence of the membrane potential on the intracellular light induced Ca^{2+}-concentration change of the *Limulus* ventral photoreceptor monitored by arsenazo III under voltage clamp conditions. Z. Naturforsch. **39c**, 986–992.

Jarminowski H. (1994), Elektronenmikroskopische Untersuchungen zur Verteilung von Calcium und zur Lokalisation von Calcium-speichernden Organellen in der Retina des Flußkrebses *Orconectes limosus*. Dissertation, Institut für Biologie II, RWTH Aachen.

Jarminowski H. and Stieve H. (1994), The subcellular distribution of calcium in the visual cells of crayfish, observed with the combined oxalate-pyroantimonate technique, depends on extracellular calcium. Proceedings of the 22nd Göttingen Neurobiology Conference 1994 (Elsner N. and Breer H., eds.), Vol. **I**, p. 22.

Jarminowski H., Schraermeyer U. and Stieve H. (1993), Calcium-accumulating organelles in the photoreceptor cells of the crayfish *Orconectes limosus*. Comp. Biochem. Physiol. **105**, 389–394.

Levy S. and Fein A. (1985), Relationship between light sensitivity and intracellular free Ca concentration in *Limulus* ventral photoreceptors. A quantitative study using Ca-selective microelectrodes. J. Gen. Physiol. **85**, 805–841.

Maaz G. and Stieve H. (1980), The correlation of the receptor potential with the light induced transient increase in intracellular calcium-concentration measured by absorption of arsenazo III injected into *Limulus* ventral nerve photoreceptor cell. Biophys. Struct. Mech. **6**, 191–208.

Meldolesi J., Madeddu L. and Pozzan T. (1990), Intracellular Ca^{2+} storage in non-muscle cells: heterogenity and functional assignment. Biochim. Biophys. Acta, **1055**, 130–140.

Nagy K. and Stieve H. (1983), Changes in intracellular calcium ion concentration in the course of dark adaptation measured by arsenazo III in *Limulus* photoreceptors. Biophys. Struct. Mech. **9**, 207–223.

Payne R., Walz B., Levy S. and Fein A. (1988), The localization of calcium release by inositol trisphosphate in *Limulus* photoreceptors and its control by negative feedback. Phil. Trans. Roy. Soc. Lond. **B 320**, 359–379.

Schroeder W., Frings D. and Stieve H. (1980), Measuring calcium uptake and release by invertebrate photoreceptor cells by laser microprobe mass spectroscopy. Scan. Electrom. Microsc. **11**, 647–652.

Slocum R. D. and Roux S. T. (1982), An improved method for the subcellular localization of calcium using a modification of the antimonate precipitation technique. J. Histochem. Cytochem. **7**, 617–629.

Somlyo A. P. and Walz B. (1985), Elemental distribution in *Rana pipiens* retinal rods: Quantitative electron probe analysis. J. Physiol. (Lond.) **358**, 183–195.

Stieve H. and Benner S. (1992), The light-induced rise in cytosolic calcium starts later than the receptor current of the *Limulus* ventral photoreceptor. Vision Res. **3**, 403–416.

Stieve H. and Claßen-Linke I. (1980), The effect of changed extracellular calcium and sodium concentration on the electroretinogram of the crayfish retina. Z. Naturforsch. **35c**, 308–318.

Stieve H., Gaube H. and Klomfass J. (1986), Effect of external calcium concentration on the intensity dependence of light-induced membrane current and voltage signals in two defined states of adaptation in the photoreceptor of *Limulus*. Z. Naturforsch. **41c**, 1092–1110.

Ukhanov K. Y., Flores T. M., Hsiao H. S., Mohapatra P., Pitts H. and Payne R. (1995), Measurement of cytosolic Ca^{2+} concentration in *Limulus* ventral photoreceptor using fluorescent dyes. J. Gen. Physiol. **105**, 95–116.

Werner U., Suss-Toby E., Rom A. and Minke B. (1992), Calcium is necessary for light excitation in barnacle photoreceptors. J. Comp. Physiol. **170**, 427–434.