Electron probe microanalysis of calcium release and magnesium uptake by endoplasmic reticulum in bee photoreceptors

(signal transduction/retina/invertebrates)

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Communicated by Robert M. Berne, October 19, 1990

ABSTRACT Honey bee photoreceptors contain large sacs of endoplasmic reticulum (ER) that can be located unequivocally in freeze-dried cryosections. The elemental composition of the ER was determined by electron probe x-ray microanalysis and was visualized in high-resolution x-ray maps. In the ER of dark-adapted photoreceptors, the Ca concentration was 47.5 ± 1.1 mmol/kg (dry weight) (mean ± SEM). During a 3-sec nonsaturating light stimulus, ~50% of the Ca content was released from the ER. Light stimulation also caused a highly significant increase in the Mg content of the ER; the ratio of Mg uptake to Ca released was ~0.7. Our results show unambiguously that the ER is the source of Ca release during cell stimulation and suggest that Mg can nearly balance the charge movement of Ca.

The mobilization of Ca from an intracellular store by a reaction cascade comprising a cell-surface receptor, a guanine nucleotide binding protein, a phospholipase C, and inositol 1,4,5-trisphosphate [Ins(1,4,5)P₃] is an important mechanism of signal transduction (1). A great deal of evidence indicates that the endoplasmic reticulum (ER) is the target of Ins(1,4,5)P₃ action (2–5) and the organelle involved in the physiological regulation of cytoplasmic Ca in non-muscle cells (6–8), although some authors proposed that the Ins(1,4,5)P₃-sensitive Ca pool is located in a separate organelle ("calciosome") (9, 10). Direct measurements of the Ca content and of Ca changes in the ER (or the calciosomes) have been difficult to obtain. Consequently, little is known about the ion movements that must occur during Ca release, to maintain charge balance across the ER membrane. The large sacs of ER in bee photoreceptors (11) are uniquely suitable for addressing these questions because the size makes them clearly identifiable in ultrathin freeze-dried cryosections and allows unambiguous experiments in which the analyzed area is completely within the ER cisternae. Therefore, we determined by electron probe x-ray microanalysis (EPMA) the elemental composition of the ER in situ, in the dark and during light stimulation, that, in invertebrates, is known to be followed by Ins(1,4,5)P₃-mediated (12, 13) intracellular Ca release (14, 15). We quantitated the amount of Ca released from the ER during photoresponse and demonstrate an associated uptake of Mg into the ER, suggesting that Mg movements make a major contribution toward maintaining electroneutrality during Ca release from the ER in bee photoreceptors. Preliminary reports of these findings have been published (16, 17).

MATERIALS AND METHODS

Honey bee drones (Apis mellifera) were kept in the dark at 32°C to 36°C for up to 10 days and fed a concentrated sugar solution. Decapitation and all subsequent manipulations were done under dark light (λ > 600 nm), which elicits no electrical response of the photoreceptors (18). Thick slices (600–1000 μm) of the drone head (19) remained in oxygenated physiological saline containing 170 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 1.6 mM CaCl₂, 10 mM Tris-HCl (pH 7.4), and 400 mM sucrose. Sucrose was added to increase the osmolarity (815 milliosmol compared to the 615 milliosmol conventionally used) and to act as the cryoprotectant. Intracellular recordings (data not shown) demonstrate that the incubation in hypertonic saline for ~20 min had no effect on the resting potential. The light intensity that elicits half-maximal response was not changed, but the maximum amplitude of the receptor potential was decreased by ~30% and the kinetics of the light response was slowed down.

After 20–30 min of incubation in physiological saline, the slices were rapidly frozen in a LifeCell CF 100 slam-freezer (LifeCell, The Woodlands, TX), either while dark-adapted or 3 sec after turning on a tungsten filament light source that delivered ~0.2 mW/cm² in the specimen plane and in the spectral region of 450–600 nm. This intensity, given in a 20-msec test flash, is 500 times dimmer than necessary to saturate the intracellularly recorded receptor potential. Thick sections (100–200 μm) were cut at ~130°C with a Reichert–Jung cryoultramicrotome and then freeze-dried (20). The sections were analyzed either by EPMA, in the spot mode, or by x-ray mapping. The instrumentation and methods for EPMA, including calibration, have been published (20–24). The individual spectra were collected until the measurement error was 1.2 mmol of Ca per kg (dry weight) for cytoplasm and mitochondria and until the measurement error was ±5% for Ca in the ER. The probe diameter was 200 nm in the mitochondria and 200–1000 nm in the cytoplasm. In the ER the probe diameter varied between 200 and 500 nm but excluded any overlap of cytoplasm. Quantitative x-ray maps were acquired by collecting an entire energy-dispersive x-ray spectrum in 15 sec at each pixel, by using a high-brightness field emission source. The scanning system was computer-controlled and included a dark-field-image cross-correlation feedback loop to compensate for specimen drift (23).

Ca²⁺ is used throughout the manuscript to designate the ionized element and Ca is used for total calcium, as measured by x-ray microanalysis.

RESULTS

The morphology of the bee photoreceptor, including the submicrovillar ER cisternae (11) that range from 0.2 to 1.0 μm in diameter, is illustrated in electron micrographs in Fig. 1; a freeze-dried cryosection is shown in Fig. 2. The large size and the significantly greater electron lucency of the ER than of

Abbreviations: ER, endoplasmic reticulum; EPMA, electron probe x-ray microanalysis; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate.

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the adjacent cytoplasm permit the unambiguous selection of a given region, ER or cytoplasm, for electron probe analysis.

The results of the elemental quantitation of the ER, the cytoplasm, and the mitochondria in dark-adapted and light-stimulated photoreceptors are summarized in Table 1. The quantitation is based on the ratio of the characteristic peak to the x-ray continuum counts from freeze-dried material (21, 22) and, therefore, the data are expressed as mmol/kg (dry weight). The cellular water content of bee photoreceptors is difficult to measure. By assuming that all K [823 mmol/kg (dry weight)] is in solution, and based on very accurate ion-selective microelectrode measurements (25) of intracellular K + [activity coefficient for potassium (ak) = 89 mM; activity coefficient = 0.7], we can estimate a cytoplasmic water content of ≈85%. Given this value, the calculated cytoplasmic concentrations of Na + (8 mmol/liter of cell H2O) and Cl − (15 mmol/liter of cell H2O) are in good agreement with published data obtained with ion-selective electrodes (25, 26); the K content is comparable to that obtained in a previous electron probe study (27).

The mean Ca concentration in the ER in dark-adapted photoreceptors was 47.5 ± 1.1 mmol/kg (dry weight) (mean ± SEM). X-ray maps (Fig. 3) of the area outlined in the cryosection shown in Fig. 2 illustrate the high Ca concentration, evenly distributed and confined within the cisternae. The relatively high Ca concentration of the cytoplasm [3.0 ± 0.2 mmol/kg (dry weight)] may reflect the inclusion of small ER tubules not imaged in unstained cryosections and/or Ca bound to proteins such as calmodulin (28). The low Ca concentration of the mitochondria [1.0 ± 0.2 mmol/kg (dry weight)] is comparable to that found in liver mitochondria [ref. 29; 0.8 mmol/kg (dry weight)].

A 3-sec continuous illumination with light at a nonsaturating intensity caused a large and highly significant (P < 0.001) reduction in the total Ca content of the ER by 52.8 ± 6.3% (confidence interval or 2 SD). We do not know whether more Ca can be released by saturating light intensities. Given the 0.5 mass ratio [measured as the x-ray continuum ratio (20) through paired analysis, with identical probe parameters, of ER and cytoplasm] and the 0.08 volume ratio (morphometric measurements in chemically fixed specimens) of ER/cytoplasm, the amount of Ca released should be sufficient to raise cytoplasmic Ca by ≈1 mmol/kg (dry weight), that is, above the detectable limits of analysis [2–3 SEM = 0.4–0.6 mmol/kg (dry weight)]. However, illumination causes a net extrusion of Ca2+ from photoreceptors (19) that is apparently
Table 1. Subcellular distribution of Ca and other elements in bee photoreceptors

<table>
<thead>
<tr>
<th>Region</th>
<th>n</th>
<th>Na (mmol/kg)</th>
<th>Mg (mmol/kg)</th>
<th>P (mmol/kg)</th>
<th>S (mmol/kg)</th>
<th>Cl (mmol/kg)</th>
<th>K (mmol/kg)</th>
<th>Ca (mmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ER</td>
<td>179</td>
<td>106 ± 5.4</td>
<td>66 ± 1.3</td>
<td>688 ± 9.0</td>
<td>1225 ± 21.0</td>
<td>131 ± 2.8</td>
<td>1353 ± 26.3</td>
<td>47.5 ± 1.1</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>63</td>
<td>44 ± 4.3</td>
<td>65 ± 1.8</td>
<td>522 ± 13.1</td>
<td>732 ± 14.6</td>
<td>83 ± 4.3</td>
<td>823 ± 17.0</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>70</td>
<td>52 ± 3.8</td>
<td>53 ± 1.4</td>
<td>553 ± 5.9</td>
<td>586 ± 7.1</td>
<td>61 ± 2.4</td>
<td>483 ± 9.4</td>
<td>1.0 ± 0.2</td>
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<tr>
<td>Light-stimulated</td>
<td></td>
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</tr>
<tr>
<td>ER</td>
<td>138</td>
<td>177 ± 6.3</td>
<td>83 ± 2.2</td>
<td>612 ± 10.3</td>
<td>1052 ± 24.3</td>
<td>137 ± 3.5</td>
<td>1116 ± 20.3</td>
<td>22.4 ± 1.0</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>67</td>
<td>102 ± 6.1</td>
<td>65 ± 1.6</td>
<td>485 ± 12.9</td>
<td>803 ± 16.4</td>
<td>111 ± 3.4</td>
<td>772 ± 15.8</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>67</td>
<td>88 ± 6.1</td>
<td>51 ± 1.6</td>
<td>528 ± 8.1</td>
<td>588 ± 9.2</td>
<td>90 ± 3.5</td>
<td>427 ± 9.7</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

Seven control animals and six light-stimulated animals were used. Data are mean ± SEM.

The concentrations [expressed in mmol/kg (dry weight)] of Na, Cl, and K, in both control and light-stimulated photoreceptors, were higher in the ER than in the cytoplasm. This finding is not necessarily indicative of a Na, Cl, or K concentration gradient across the ER membrane, because an "excess" of ions in solution is associated with the greater hydration of the ER and because EPMA measures total (free and bound) elemental concentrations, rather than activities.

During photostimulation the concentration of Na increased and that of K decreased in all probed compartments (ER, cytoplasm, and mitochondria). Except for the cytoplasmic K decrease, which is just statistically significant (P = 0.05), all these changes are highly significant (P < 0.001). They reflect the light-induced Na" influx and K" efflux in invertebrate photoreceptors (25) and suggest that the permeability of the ER membrane to these ions permits their rapid redistribution among the compartments. In addition, there was an increase in cytoplasmic Cl during illumination (P < 0.05), in agreement with ion-selective microelectrode measurements (26).

Light stimulation also caused Mg uptake into the ER (P < 0.001), suggesting that Mg++, moves as a counterion during Ca++ release to maintain the charge balance across the ER membrane. The amount of Mg uptake (16.7 ± 5.1 mmol/kg; confidence interval or 2 SD) was slightly lower than the amount of Ca release (25.1 ± 3.0 mmol/kg). Given the aforementioned ER/cytoplasm volume ratio, the amount of Mg moving out of the cytoplasm, about 1 mmol/kg (dry weight), would have been below the level of detectability (Table 1), even in the absence of an extracellular Mg influx that could occur.

**DISCUSSION**

The present study demonstrates directly that the well-characterized submicrovillar cisternae of ER in invertebrate photoreceptors (11, 29) are the source of Ins(1,4,5)P3-mediated (12, 13) Ca++ release during photostimulation. Evidence, although indirect, for this conclusion was also obtained in *Limulus* ventral photoreceptors (13, 30): In acquirin-injected *Limulus* photoreceptors, light flashes or brief pressure injections of Ins(1,4,5)P3 elicit acquirin luminescence that originates in the cell area where the submicrovillar ER cisternae are located. In addition, the studies on *Limulus* ventral photoreceptors suggest that, although all ER accumulates Ca++ actively, not all ER is competent to release Ca++ in response to Ins(1,4,5)P3.

The present results support the conclusion derived from EPMA studies on hepatocytes (31), retinal rods (32), and a variety of other cells (33) that the ER is the major intracellular Ca store in nonmuscle cells. However, the Ca concentrations obtained in those studies (5 and 13 mmol/kg (dry weight), respectively) were underestimated of the true Ca content of the ER, because the probe diameter was larger than the ER tubules. The Ca concentration in bee photoreceptor ER (47.5 mmol/kg (dry weight)) is lower than in the sarcoplasmic reticulum of toadfish muscle [ref. 34; 77 mmol/kg (dry weight)] or the terminal cisternae of frog muscle (ref. 20; 117 mmol/kg (dry weight)) but comparable to the steady-state Ca content of 32 nmol/mg of protein in liver microsomes under optimal conditions (35).
The finding that, in bee photoreceptors, Ca release is associated with Mg uptake suggests that Mg$^{2+}$ moves as a counterion during Ca$^{2+}$ release to maintain the charge balance across the ER membrane. In striated muscle, K$^+$ is the major counterion moving into the sarcoplasmic reticulum during Ca$^{2+}$ release (20), and monovalent cation movement may play a similar role during Ca$^{2+}$ release from the ER in permeabilized hepatocytes (36). However, in vivo measurements, comparable to those in the present study, are yet to be made on vertebrate cells. In the sarcoplasmic reticulum of tetanized skeletal muscle, Mg$^{2+}$ makes a relatively small contribution to counterion movement (20). In bee photoreceptors, Mg$^{2+}$ seems to be the major counterion providing charge neutralization. Further tests of this possibility will require measurements during the early phase of photostimulation, before the onset of the recovery process. However, it is already clear from this and previous studies (37) that not only the Ca$^{2+}$ but also the Mg$^{2+}$ content of cell organelles (mitochondria and ER) is subject to dynamic changes that may play a role in physiological regulation, and the possibility should be explored that, under physiological conditions in the ER of nonpermeabilized liver and other vertebrate cells, Mg$^{2+}$ is also the source of counterion movement during Ca$^{2+}$ release.

Thus, our results demonstrate unambiguously that the ER is the organelle involved in the regulation of cytoplasmic Ca$^{2+}$. Our data are consistent with the view that the ER contains a Ca$^{2+}$-ATPase (5–8) that maintains a high Ca$^{2+}$ gradient across the ER membrane, and a Ca$^{2+}$ release channel sensitive to Ins(1,4,5)P$_3$ (2–5). These findings do not support the view that an ER-independent cell organelle contains the Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ pool.

We thank Dr. Z. Shao for consultations in x-ray mapping and Mr. S. Majewski for writing the slow-scan mapping program and for help in data processing. We thank Mr. J. Silcox for technical assistance, Dr. R. Kretzinger, Dr. J. Herr, and Mrs. S. Cobey for supplying honey bees, and LifeCell for kindly providing the slam-freezer. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 4, 11), NATO, and National Institutes of Health Grant HL-15835 to the Pennsylvania Muscle Institute.