Caffeine- and Ryanodine-sensitive Ca^{2+}-induced Ca^{2+} Release from the Endoplasmic Reticulum in Honeybee Photoreceptors

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ABSTRACT Light stimulation of invertebrate microvillar photoreceptors causes a large rapid elevation in Ca_i, shown previously to modulate the adaptational state of the cells. Ca_i rises, at least in part, as a result of Ins(1,4,5)P_3-induced Ca^{2+} release from the submicrovillar endoplasmic reticulum (ER). Here, we provide evidence for Ca^{2+}-induced Ca^{2+} release (CICR) in an insect photoreceptor.

In situ microphotometric measurements of Ca^{2+} fluxes across the ER membrane in permeabilized slices of drone bee retina show that (*a*) caffeine induces Ca^{2+} release from the ER; (*b*) caffeine and Ins(1,4,5)P_3 open distinct Ca^{2+} release pathways because only caffeine-induced Ca^{2+} release is ryanodine sensitive and heparin insensitive, and because caffeine and Ins(1,4,5)P_3 have additive effects on the rate of Ca^{2+} release; (*c*) Ca^{2+} itself stimulates release of Ca^{2+} via a ryanodine-sensitive pathway; and (*d*) cADPR is ineffective in releasing Ca^{2+}.

Microfluorometric intracellular Ca^{2+} measurements with fluo-3 indicate that caffeine induces a persistent elevation in Ca_i. Electrophysiological recordings demonstrate that caffeine mimics all aspects of Ca^{2+}-mediated facilitation and adaptation in drone photoreceptors.

We conclude that the ER in drone photoreceptors contains, in addition to the Ins(1,4,5)P_3-sensitive release pathway, a CICR pathway that meets key pharmacological criteria for a ryanodine receptor. Coexpression of both release mechanisms could be required for the production of rapid light-induced Ca^{2+} elevations, because Ca^{2+} amplifies its own release through both pathways by a positive feedback. CICR may also mediate the spatial spread of Ca^{2+} release from the submicrovillar ER toward more remote ER subregions, thereby activating Ca^{2+}-sensitive cell processes that are not directly involved in phototransduction.

INTRODUCTION

Light stimulation of invertebrate microvillar photoreceptors causes a large rapid increase in the concentration of cytosolic free calcium (Ca_i) (Brown and Blinks, 1974;

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Brown, Brown, and Pinto, 1977; Maaz and Stieve, 1980; Stieve and Benner, 1992; Walz, Zimmermann, and Seidl, 1994). Although it is still an enigma whether the light-sensitive channels are activated by the rise in Ca_i, either directly or indirectly (reviewed in Hardie, 1993), firm evidence indicates that the light-induced rise in Ca_i mediates light adaptation (Bader, Baumann, and Bertrand, 1976; Lisman and Brown, 1972, 1975; reviewed in Brown, 1986) and facilitation (Walz, 1992). Moreover, the light-induced increase in Ca_i stimulates pigment granule migrations (Kirschfeld and Vogt, 1980), oxidative metabolism (Fein and Tsacopoulos, 1988), and membrane turnover (Martin and Hafner, 1986) in some invertebrate photoreceptors. Thus, Ca^{2+} ions play a key role in a variety of physiological processes in invertebrate photoreceptors, and an exact knowledge of the coordinated contribution of all mechanisms involved in the regulation of Ca_i is a prerequisite to understanding how these cells use Ca^{2+} ions for the spatio-temporal regulation of such diverse cell processes.

One Ca^{2+} regulatory mechanism that has been well documented in invertebrate microvillar photoreceptors is the inositol phosphate cascade. This reaction sequence is activated by light-activated rhodopsin and produces inositol 1,4,5-trisphosphate (Ins[1,4,5]P_3) via a G protein and phospholipase C (Brown, Rubin, Ghalayini, Tarver, Irvine, Berridge, and Anderson, 1984; Fein, Payne, Corson, Berridge, and Irvine, 1984; reviewed in Minke and Selinger, 1992; Payne, 1986). Ins(1,4,5)P_3 releases Ca^{2+} from the submicrovillar cisternae (SMC) of the endoplasmic reticulum (ER) (Baumann and Walz, 1989*b*; Baumann, Walz, Somlyo, Somlyo, 1991), and mimics the effect of light (Payne and Fein, 1987).

In the ventral photoreceptors of *Limulus*, the light-induced increase in Ca_i is mainly the result of Ins(1,4,5)P_3-induced Ca^{2+} release from internal stores (reviewed in Payne, 1990). In insect photoreceptors, however, there is also a considerable light-induced Ca^{2+}-influx from the extracellular space (Minke and Tsacopoulos, 1986; Sandler and Kirschfeld, 1988; Ziegler and Walz, 1989), most probably through Ca^{2+}-permeable light–sensitive channels (Hardie, 1991; Ranganathan, Harris, Stevens, and Zuker, 1991). There is, as yet, no information on whether any invertebrate photoreceptor has mechanisms besides Ins(1,4,5)P_3-induced Ca^{2+} release to cause the release of Ca^{2+} from the ER.

In muscle and many nonmuscle cells, such as neurons and sea urchin eggs, the Ca^{2+}-sequestering ER has, in addition to receptors for Ins(1,4,5)P_3, another calcium channel, the ryanodine receptor (RyR). The RyR responds to an increase in Ca_i, and mediates Ca^{2+}-induced Ca^{2+} release (CICR) (reviewed in Berridge, 1993; McPherson and Campbell, 1993*a*, *b*). In some preparations, it has been shown that the metabolite cyclic ADP-ribose (cADPR) is a natural modulator or agonist for CICR (e.g., Galione, Lee, and Busa, 1991; Galione, White, Willmott, Turner, Potter, and Watson, 1993; Mészáros, Bak, and Chu, 1993; reviewed in Berridge, 1993). A recent study has provided the first molecular evidence for a RyR gene (*dry*) in *Drosophila*; dry expression has been discovered in some muscles, the head, antennae, and the compound eyes (Hasan and Rosbash, 1992).

In this study, we have sought physiological evidence for CICR in an insect photoreceptor, by combining different techniques, viz, in situ microphotometric

measurements of Ca^{2+} fluxes across the ER membrane, electrophysiology, and microfluorometrical Ca^{2+} measurements with fluo-3.

Some of the results presented in this paper have been appeared previously in abstract form (Walz, Baumann, and Zimmermann, 1994).

METHODS

Animals, Preparation

Honeybees (*Apis mellifera* ♂, drones) were obtained either from local hives or from Daykel Apiaries, (Kaitaia, Northland, New Zealand). The drones were kept together with worker bees in the dark and fed a 50% sugar solution. All experiments were carried out on slices of the head of the drones, cut as described elsewhere (Bertrand, Fuortes, and Muri, 1979; Coles and Orkand, 1983), mounted in a glass-bottomed recording chamber, and continuously superfused with well-oxygenated physiological saline containing 270 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 1.6 mM $CaCl_2$, 10 mM TRIS, pH 7.4. The tissue slices were 200–300 μm thick when used for microphotometric measurements of calcium oxalate (Ca oxalate) accumulation, and 400–800 μm thick for microfluorometric measurements and intracellular recordings. During the preparation and mounting, the slices were only illuminated with red light (filter RG 610) so as not to excite the photoreceptors (Bertrand, Fuortes, and Muri, 1979). All experiments were performed at room temperature (20 ± 1°C).

Chemicals

Inositol 1,4,5-trisphosphate, ryanodine, and cyclopiazonic acid were purchased from Calbiochem Corp. (La Jolla, CA), heparin (MW ~6000), saponin, and β-escin from Sigma (Deisenhofen, Germany), caffeine from Fluka (Neu-Ulm, Germany). Cyclic adenosine 5′-diphosphoribose (cADPR) was from Amersham Buchler (Braunschweig, Germany). Fluo-3 pentapotassium salt was obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of analytical grade and supplied by Sigma, Fluka, and Serva (Heidelberg, Germany).

Microphotometric Recordings of Ca^{2+} Uptake into and Ca^{2+} Release from the Endoplasmic Reticulum

The rates of Ca^{2+} fluxes across the ER membrane were measured microphotometrically in situ in permeabilized tissue slices by means of the oxalate method described in detail previously (Walz, 1982*b*; Walz and Baumann, 1989; Baumann and Walz, 1989*b*). The retinal tissue slices were first permeabilized with either 200 μg/ml saponin or 40 μg/ml β-escin in 200 mM potassium aspartate, 5 mM $MgCl_2$, 2 mM K_2EGTA, 5 mM Na_2ATP, 20 mM HEPES, pH 7.0. Permeabilization proceeded for ~20 min at room temperature. The slices were then incubated in standard loading medium (LM) containing 180 mM potassium aspartate, 20 mM potassium oxalate, 5 mM $MgCl_2$, 2 mM K_2EGTA, 3 mM CaEGTA, 5 mM Na_2ATP, 20 mM HEPES, pH 7.0. The free Ca^{2+} concentration of the LM was 0.5 μM when measured fluorometrically with fluo-3 (Walz et al., 1994).

Incubation of permeabilized tissue slices in LM stimulates ATP-dependent Ca^{2+}-uptake by the ER. When the Ca-oxalate solubility product is exceeded in the ER lumen because of Ca^{2+} uptake (the ER membrane is permeable to the oxalate anion), Ca-oxalate precipitates within the ER (Baumann and Walz, 1989*a*; Walz, 1982*a b*; Walz and Baumann, 1989). Ca-oxalate is birefringent. Therefore, the rate of Ca-oxalate formation can be measured microphotometrically in a polarization microscope. The rate of change in the optical signal can be used as a reliable measure of the rate of Ca^{2+} uptake or release if (*a*) the size of the optical signal is a

linear function of the amount of Ca-oxalate in the field of measurement (in the preparation), and (*b*) a linear function exists between the rate of rise in the optical signal and the various rates of Ca^{2+} accumulation into the compartment in which its precipitation as Ca-oxalate occurs. Fig. 1, *A* and *B*, illustrate two experimental tests for linearity of the microphotometric method (data from Walz, 1982*b*; for methodological details see figure legend). These experiments provide evidence that the optical signal varies linearly with the formation of Ca-oxalate, and is adequate for monitoring the kinetics of the uptake and release of Ca^{2+}. An absolute calibration of the optical signal would be extremely difficult because of the cellular heterogeneity of our small tissue slices.

The reliability of the method is also documented by the requirement for ATP in producing Ca-oxalate precipitates in the ER (Baumann and Walz, 1989*a*,*b*; Walz, 1982*b*; Walz and Baumann, 1989). This excludes the possibility that the precipitate formation is the result of some spontaneous, unspecific and/or passive process.

When oxalate experiments are performed on SR membrane vesicles or leech photoreceptors on which the microphotometric method has been developed (Walz, 1982*b*), it is almost impossible to trigger Ca^{2+} release. In this and a previous study (Baumann and Walz, 1989*b*) significant Ca^{2+} release must have occurred to induce a change in the Ca-oxalate concentration within the ER. Why doesn't oxalate block Ca^{2+} release in the photoreceptor ER in the drone? Drone photoreceptors are a very favorable preparation because their ER is made up of extremely voluminous, vacuolelike sacs (Baumann and Walz, 1989*a*), and the Ca-oxalate precipitates occupy only a fraction of the total ER volume. In this volume, the free Ca^{2+} concentration is clamped by the Ca-oxalate solubility product ($K_{SP} = 2 \times 10^{-8}$ M^2; Beil, von Chak, Hasselbach, and Weber, 1977) and the free oxalate concentration. Ca-oxalate precipitates form only when the product of the activities of Ca^{2+} and $oxalate^{2-}$ is raised beyond K_{SP}. As soon as the free Ca^{2+} concentration in the free volume of the ER drops because of Ca^{2+} release, the amount of Ca-oxalate, and the optical signal, will decrease.

For the microphotometric measurements, the recording chamber was placed on the stage of a Zeiss UEM microscope (see below) equipped for polarization microscopy. We used a Zeiss Plan-Neofluar 16/0.5 water-immersion objective for the recordings. The plane polarizers were crossed, and the light transmitted by the analyzer was recorded by a photomultiplier tube (Zeiss photometer 03). The anode current of the photomultiplier tube was converted to a voltage signal and recorded on a chart recorder. The field of measurement was aligned with the field of illumination, and restricted to $\sim 100 \times 100$ μm by a variable rectangular diaphragm in the intermediate image plane. The solutions in the recording chamber were in ~100-fold excess of the tissue volume and were continuously stirred by a small rotating propeller. Recordings were made with red light (Schott RG 610), which does not elicit a light response in the photoreceptors (Bertrand et al., 1979).

Electrical Recordings

Intracellular recordings were made by standard techniques. Microelectrodes were filled with 3 M KCl and had resistances between 50 and 100 MΩ. Data were only accepted from cells having resting membrane potentials > -50 mV. After impalement, and before the beginning of an experiment, each preparation was dark adapted for 1 h. The light intensity of the 20-ms test flashes was chosen to be so dim that they gave linear responses (see also Bertrand et al., 1979). This also minimized the contribution of voltage-dependent conductances to the shape of the receptor potentials.

The membrane potential was continuously recorded on a pen recorder. The responses to the test flashes were digitized (sampling rate 1,000 Hz) and stored on floppy disk. A/D conversion, signal averaging, and data display and output were controlled by the software EASYEST (Asyst Software Technologies, Rochester, NY).

For light stimulation, the light from a 100-W halogen lamp was passed through an

electromechanical shutter, a heat absorbing filter (KG1), and neutral density filters, and illuminated the retinal slice from below, through the glass bottom of the recording chamber. The intensity of the unattenuated beam was ~1 mW/cm^2 in the specimen plane. However, the absolute light intensity at the recording site within the retinal slice was not precisely known, because the eyes contain screening pigments, and the slices varied in thickness. Therefore, relative light intensities are given as $\log_{10} I/I_o$, where I_o is the unattenuated light intensity.

Microfluorometric Measurements of Intracellular Ca^{2+} Changes

Ca_i was measured microfluorometrically using the Ca^{2+}-sensitive fluorescent indicator dye fluo-3 as previously described in detail (Walz et al., 1994): fluo-3 was introduced into the photoreceptor cells by pressure injections. The microelectrodes contained a column of 10 mM fluo-3 in a carrier solution consisting of 125 mM potassium aspartate, 10 mM HEPES, pH 7.0, in their tips. They were backfilled with carrier solution alone, and broken back before impalement until the electrode resistance dropped to 50–80 MΩ. Up to 10 cells per slice were impaled on the intracellular recording set up and injected. Successful injections were visible because of the transient pharmacological effect of fluo-3. As shown previously (Walz et al., 1994), the pharmacological effect, which resembles a weak EGTA injection, lasted for only 10–20 s because the dye concentration falls in the injected cell due to diffusion into the neighboring retinular cells of the same ommatidium. We kept the intracellular dye concentration between 20 and 50 μM. At these concentrations fluo-3 has no persistent pharmacological effects (Walz et al., 1994). After dye loading, the electrode was withdrawn from the preparation, and the specimen chamber mounted in the microfluorometer.

The microfluorometric set up consisted of an upright microscope (Zeiss UEM/UMSP-stand), equipped with UV-transmitting epifluorescence optics, a xenon lamp (XBO 75 W), an electromechanical shutter (Vincent Associates, Rochester, NY), and a filter cube containing the Zeiss fluorescein filter set (excitation filter BP485 nm, dichroic mirror FT510 nm, barrier filter LP515 nm). In addition, the excitation beam contained three heat absorbing filters (KG1) that together were 5 mm thick.

The photometer head (Zeiss MPM 03) contained a rectangular variable diaphragm in front of the photomultiplier tube (R 928), and an optical system permitting the direct observation of the specimen and the diaphragm in the eyepiece of the microscope. The diaphragm allowed the area from which fluorescence was collected to be limited to one injected cell or ommatidium. Measurements were made with a Zeiss Neofluar 16/0.5 water immersion objective.

The same light source was used to excite fluorescence and to stimulate the photoreceptors. The intensity of the unattenuated light beam was 357 mW/cm^2. This intensity is, in physiological terms, ~1.7 log units (factor 50) brighter than that necessary to saturate the transient component of a receptor potential elicited by a 1-s light flash (Walz et al., 1994). Light intensities were measured with a calibrated photodiode (OPHIR model PD2-A photodiode power meter, Ophir Optics, Jerusalem, Israel).

The anode current of the photomultiplier was converted to a voltage signal, digitized, and stored on floppy disk. The control of the electromechanical shutter, A/D-conversion, and data display and output were achieved by a program written in the ASYST programming language (Asyst Software Technologies, Rochester, NY).

RESULTS

Characterization of Caffeine-induced Ca^{2+} Release from the ER in Permeabilized Eye Slices

Caffeine decreases the rate of Ca-oxalate formation within the ER. Permeabilized photoreceptor cells in the compound eye of the honeybee drone accumulate Ca-oxalate when

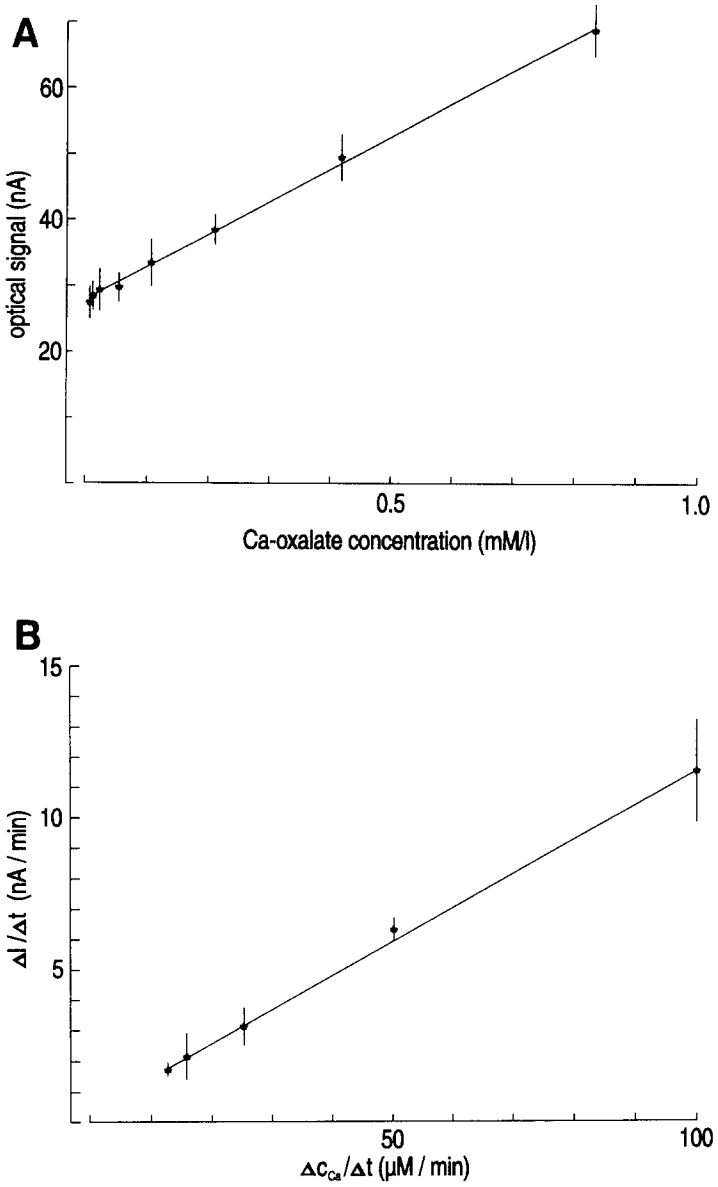

FIGURE 1. Experimental tests for linearity of the microphotometric method. The experimental arrangement for the in vitro measurements was identical to that used in the in situ Ca^{2+} accumulation and release experiments. (*A*) Optical signal as a function of the amount of Ca-oxalate in the field of measurement. A Ca-oxalate stock solution (0.83 mM/liter) was prepared by adding a known amount of a solution containing $CaCl_2$ (KCl 100 mM, Imidazol/HCl 20 mM, $CaCl_2$ 5 mM, pH 7.0) to a solution containing 75 mM KCl, 25 mM K-oxalate, 20 mM Imidazol/HCl, pH 7.0. Part of the resulting Ca-oxalate suspension was filtered, and the filtrate used to prepare a sequence of test solutions by diluting the stock solution. The figure demonstrates that linearity exists between the amount of Ca-oxalate in the light path and the

incubated in LM containing physiological free Ca^{2+} concentrations (Ca^{2+}-EGTA buffer), MgATP, and oxalate anions (Baumann and Walz, 1989*a*,*b*). Electron microscopic examination of Ca-oxalate–loaded eye slices has demonstrated that the Ca-oxalate deposits are localized exclusively within the ER (Baumann and Walz, 1989*a*). The ATP-dependent loading of the ER with Ca-oxalate causes a steady rise, and, depending on the experimental conditions, a linear rise over tens of minutes in the optical birefringence signal. Recently, we have shown that this approach is suitable not only for measuring active Ca^{2+} uptake into the ER, but also for the characterization of Ca^{2+}-release mechanisms; the opening of Ca^{2+}-permeable channels in the ER membrane causes Ca^{2+} efflux that competes with the precipitation, by oxalate, of Ca^{2+} pumped into the ER, and thus reduces the rate of Ca-oxalate accumulation (see Materials and Methods and Baumann and Walz 1989*b*).

Honeybee drone photoreceptors contain an extensive ER composed of several distinct domains. Electron microscopic examination of permeabilized Ca-oxalate–loaded photoreceptors visualizes most precipitates within the vacuolelike submicrovillar ER cisternae (Baumann and Walz, 1989*a*). We thus conclude that the birefringence signal is dominated by Ca-oxalate within the submicrovillar ER, and that the measurements reflect Ca^{2+} uptake and Ca^{2+} efflux from this ER domain.

Fig. 2 shows the birefringence signal recorded from a slice preparation permeabilized with β-escin, then incubated in LM, and subsequently superfused with LM containing 50 mM caffeine. The ER accumulated Ca-oxalate at a constant rate when bathed in LM. Addition of caffeine caused a biphasic depression in the rate of Ca-oxalate accumulation. The initial almost complete depression of Ca-oxalate accumulation lasted for ~5 min, and was followed by Ca-oxalate accumulation at constant rate lower than the control rate. Furthermore, the effect of caffeine on the rate of Ca-oxalate accumulation by the ER was reversible (Fig. 2); the rate recovered to 88% of the control rate after withdrawal of caffeine (see Fig. 8 *A* for the range of recovery in five different preparations). These effects of caffeine on the rate of Ca-oxalate accumulation were consistently observed in seven out of eight preparations. After an initial phase lasting 2–5 min with no Ca-oxalate accumulation or even loss of accumulated Ca-oxalate, there was a second phase of Ca-oxalate accumulation with a constant but lower rate than in the absence of caffeine. This second phase persisted until the caffeine was washed out, e.g., in one experiment for 85 min.

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recorded optical signal. The line is drawn by a least-squares fit: $y = 49.97\ x + 27.96$ ($r^2 = 1.0$). Each point gives the mean value (±SD) from 12 determinations. (*B*) Rate of rise in the optical signal ($\Delta I/\Delta t$) as a function of the rate of rise of Ca^{2+} concentration ($\Delta c_{Ca}/\Delta t$) in the perfusion chamber. The chamber contained 2 ml of a solution consisting of 75 mM KCl, 25 mM K-oxalate, and 20 mM Imidazol/HCl, pH 7.0. This solution was vigorously stirred and titrated with a test solution (KCl 100 mM, $CaCl_2$ 5 mM, Imidazol/HCl 20 mM, pH 7.0) at a sequence of constant rates. $\Delta I/\Delta t$ was plotted as a function of $\Delta c_{Ca}/\Delta t$. Each symbol gives the mean ±SD from four determinations. The line was fitted by eye. In both types of experiments the size and the density of the Ca-oxalate precipitates produced in the field of measurement mimicked fairly well these two parameters in the preparation. The figure illustrates that a linear function exists between $\Delta I/\Delta t$ and $\Delta c_{Ca}/\Delta t$. Both experiments provide evidence that the optical signal varies linearly with the formation of Ca-oxalate.

Transition between the two phases was either abrupt (Fig. 2) or gradual (not shown). In one out of eight preparations, there was a reduced constant rate of Ca-oxalate accumulation during the entire time of caffeine exposure (20 min). In all further Ca-oxalate accumulation experiments, we regarded only the initial phase of caffeine exposure.

The effect of caffeine on the rate of Ca-oxalate accumulation was dose dependent (Fig. 3). The threshold for a statistically significant effect was 10 mM caffeine ($n = 7$, $P < 0.01$). Ca-oxalate accumulation was completely suppressed in the presence of 50 mM caffeine, the highest concentration tested.

In the following experiments, we used 25 mM caffeine.

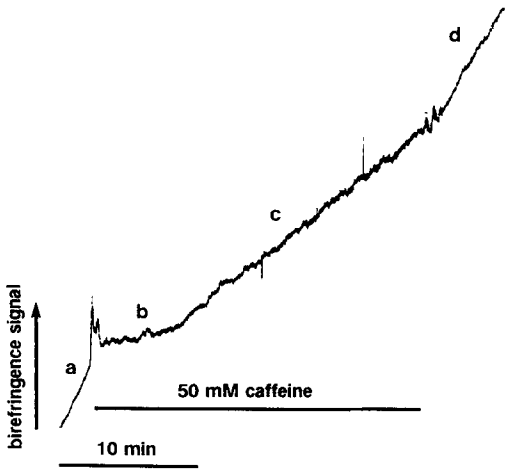

FIGURE 2. Effect of caffeine on the ATP-dependent accumulation of Ca-oxalate by the ER. The retinal tissue slice was permeabilized with β-escin and then incubated in LM containing 0.5 μM free Ca^{2+}, 5 mM ATP, 20 mM oxalate; the optical birefringence signal was continuously recorded by a microphotometer. (*a*) The birefringence signal rose linearly as the ER accumulated Ca-oxalate. (*b*) In LM containing 50 mM caffeine, the rate of Ca-oxalate accumulation was depressed, and measured 11% for ~5 min when normalized to the control rate *a*. (*c*) Later, in the presence of caffeine, the rate of Ca-oxalate accumulation recovered to 43% of the control rate. (*d*) Washing out of caffeine and incubation in normal LM caused a complete recovery of the rate of Ca-oxalate accumulation. The deflections (*arrows*) are artifacts attributible to exchange of media.

Caffeine Causes Loss of Ca-Oxalate in the Absence of Ca^{2+} Uptake

The caffeine-dependent depression in the rate of Ca-oxalate accumulation may be the result of caffeine-induced Ca^{2+} release from the ER. Alternatively, caffeine may block the Ca^{2+} transport into the ER. To distinguish between these two possibilities, we loaded permeabilized eye slices with Ca-oxalate, completely blocked further Ca^{2+} uptake by the ER, and then examined the effect of caffeine on the birefringence signal. Under these conditions, caffeine should not effect the birefringence signal if it targets and inhibits the Ca^{2+} pump, but should cause loss of Ca^{2+}-oxalate and a decrease in the birefringence signal if caffeine increases the Ca^{2+} permeability of the ER membrane.

The conditions required for complete blockage of Ca^{2+} uptake by the ER were first examined. Fig. 4 shows the birefringence signal recorded from a preparation preincubated in normal LM, and then exposed to LM containing cyclopiazonic acid

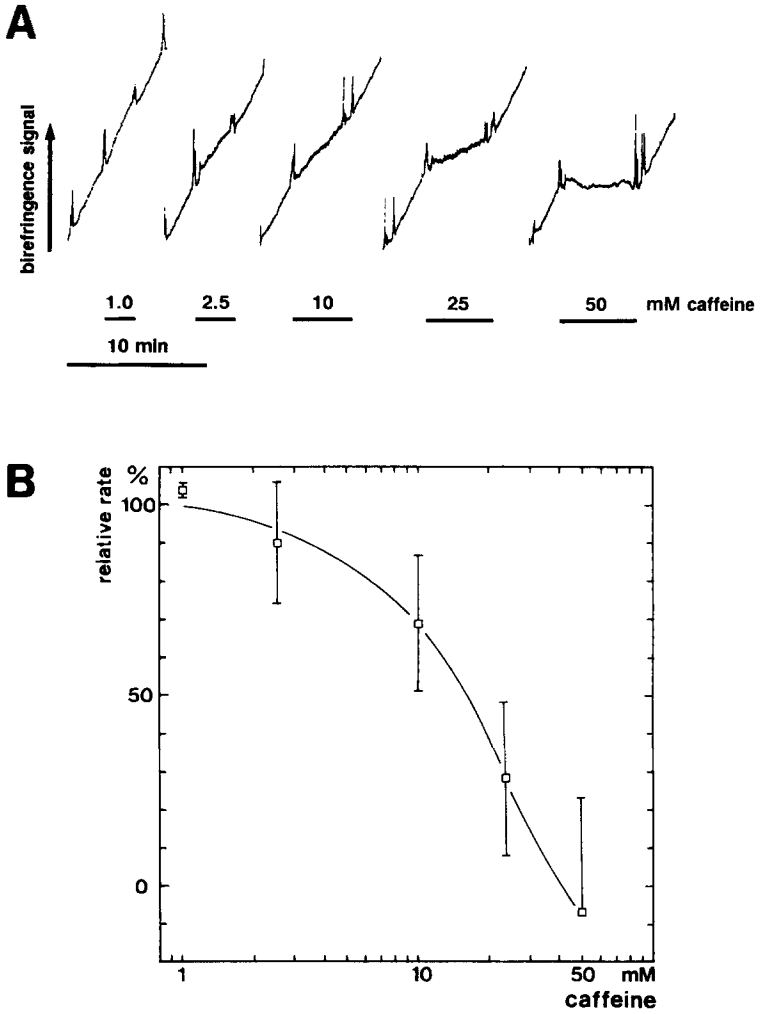

FIGURE 3. Effects of increasing caffeine concentrations on the rate of Ca-oxalate accumulation by the ER. (*A*) The permeabilized retinal tissue was incubated in loading medium and exposed to various concentrations of caffeine. The rate of Ca-oxalate accumulation was depressed with ≥2.5 mM caffeine in this experiment. Ca-oxalate accumulation was completely abolished with 50 mM caffeine. Data are from a single experiment. (*B*) Dose-response relation for the effect of caffeine on the rate of Ca-oxalate accumulation. Each symbol gives the average ±SD of 5–10 measurements, each having been normalized to the mean of the control rates before and after caffeine administration. The curve was fitted by eye. Significance levels (paired *t* test): 1 mM, 2.5 mM, $P > 0.05$; 10 mM, $P \approx 0.002$; 50 mM, $P < 0.0001$.

(CPA), which has been shown to be a specific inhibitor of the Ca^{2+}-ATPase of the ER/SR in vertebrate tissues (Seidler, Jona, Vegh, and Martonosi, 1989; Mason, Garcia-Rodriguez, and Grinstein, 1991; Demaurex, Lew, and Krause, 1992). In permeabilized honeybee retina, CPA blocked the accumulation of Ca-oxalate by the ER in a concentration-dependent manner. Half-maximal depression of Ca-oxalate

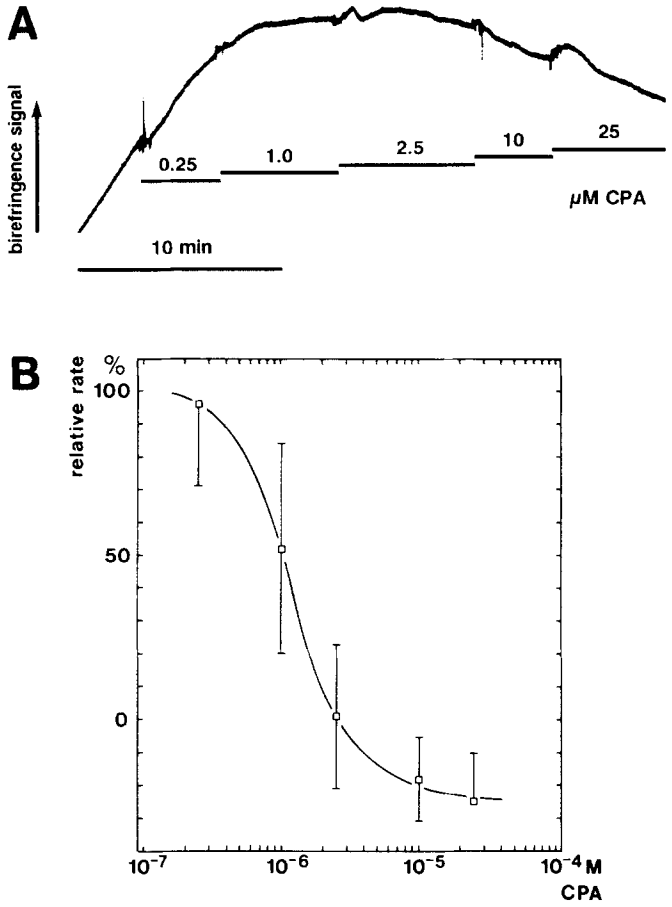

FIGURE 4. The Ca^{2+}-ATPase inhibitor CPA blocks the accumulation of Ca-oxalate in a concentration-dependent manner. (*A*) Saponin-permeabilized retinal tissue was incubated in loading medium and exposed to increasing concentrations of CPA. Addition of CPA caused a depression in the rate of Ca-oxalate accumulation. The slow decrease of the birefringence signal in ≥2.5 mM CPA indicates a degradation of Ca-oxalate precipitates as a result of Ca^{2+} leakage from the ER. (*B*) Dose-response curve for the CPA effect on the rate of Ca-oxalate accumulation. Each symbol gives the average ±SD of three (0.25 mM) to five independent measurements, normalized to the rate of Ca-oxalate formation just before exposure to CPA. The curve was fitted by eye. Significance levels: 0.25 μM, $P > 0.5$; 1 μM, $P \sim 0.02$; 2.5, 10, 25 μM, $P < 0.0001$.

accumulation was obtained with ~1.5 μM CPA; the maximal effect was observed with 10 μM CPA. Upon treatment with CPA concentrations ≥10 μM, there was a slow loss of Ca-oxalate, indicating some Ca^{2+} leakage from the ER. Depression of Ca-oxalate accumulation persisted for more than 30 min after replacing CPA-containing LM by standard LM. Thus, complete persistent blockage of Ca^{2+} uptake by the ER required exposure to ≥10 μM CPA for only a few minutes.

The birefringence signal from a preparation loaded with Ca-oxalate, and then treated with LM containing 10 μM CPA can be seen in Fig. 5. After CPA treatment,

there was a slow decrease of the biregringence signal because the ER slowly lost its accumulated Ca-oxalate as a result of Ca^{2+} leakage. The relative rate of the birefringence change after CPA treatment was −28% ± 18% (mean ± SD; n = 5) when normalized to the rate of rise of the optical signal before CPA treatment. Exposure of the CPA-treated preparation to 25 mM caffeine resulted in a rapid decline of the optical signal (Ca-oxalate content of the ER) lasting 2–5 min. The relative rate of the birefringence change during the initial 2 min of caffeine exposure was −114% ± 35% (mean ± SD; n = 7; seven different preparations) when normalized to the control rate before CPA treatment. The rate of the caffeine-induced loss of Ca-oxalate was calculated by subtracting the rate of passive Ca-oxalate loss, and amounted to (−114%) − (−28%) = −86% ± 15% (mean ± SEM). This caffeine-induced loss of Ca-oxalate from the ER demonstrated that caffeine increased the Ca^{2+} permeability of the ER membrane. Moreover, the relative effect of caffeine

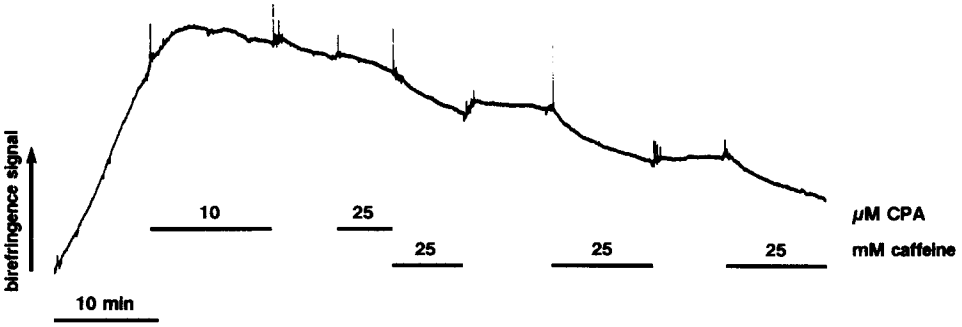

FIGURE 5. Original recording demonstrating that the caffeine-induced depression in the rate of Ca-oxalate accumulation is the result of Ca^{2+} release from the ER. Permeabilized retinal tissue was loaded with Ca-oxalate, and then exposed to a saturating CPA concentration. Inhibition of the Ca^{2+} pump by CPA caused a slow decrease in the optical signal, and persisted for >30 min after CPA withdrawal. When the CPA-treated retinal tissue was exposed to caffeine, there was a rapid decline in the Ca-oxalate content lasting for 3–5 min.

on the birefringence change was of a similar magnitude when measured during the inhibition of Ca^{2+} uptake (−86% ± 15%) or during ongoing Ca^{2+} uptake (Fig. 3 *B*: −72% ± 7%; mean ± SEM). Caffeine-induced Ca^{2+} release from the ER may thus account entirely for the caffeine-dependent depression of Ca-oxalate accumulation shown in Figs. 2 and 3.

Caffeine and Ins(1,4,5)P_3 Open Distinct Ca^{2+} Release Mechanisms

In a previous study (Baumann and Walz, 1989*b*), we have characterized the Ins(1,4,5)P_3-induced Ca^{2+} release from the ER in honeybee drone photoreceptors. The following experiments address the question of whether caffeine acts on a distinct Ca^{2+} release mechanism.

First, we used heparin, an antagonist of Ins(1,4,5)P_3 binding to the Ins(1,4,5)P_3 receptor (Worley, Baraban, Supattapone, Wilson, and Snyder, 1987) and a specific inhibitor of Ins(1,4,5)P_3-induced Ca^{2+} release from intracellular stores (Hill, Berggren, and Boynton, 1987; Kobayashi, Somlyo, and Somlyo, 1988). When 1 mg/ml

heparin was applied to permeabilized drone retina, it completely blocked the Ins(1,4,5)P_3-induced depression of Ca-oxalate accumulation ($n = 5$; $P \approx 0.002$), but did not significantly affect the caffeine-dependent depression ($n = 5$; $P > 0.1$) (Fig. 6).

Secondly, we examined the effect of ryanodine on the rate of Ca-oxalate accumulation (Figs. 7, 8). The plant alkaloid ryanodine binds to the caffeine-dependent Ca^{2+}-release channel of skeletal and cardiac muscle (Imagawa, Smith, Coronado, and Campbell, 1987; Inui, Saito, and Fleischer, 1987; Anderson, Lai, Liu, Rousseau, Erickson, and Meissner, 1989) and the brain (McPherson and Campbell, 1990; McPherson, Kim, Valdivia, Knudson, Takekura, Franzini-Armstrong, Coronado, and Campbell, 1991) and locks the release channel in an open state (Fleischer, Ogunbunmi, Dixon, and Fleer, 1985; Meissner, 1986). Gating of the Ins(1,4,5)P_3-dependent Ca^{2+} channel, however, is not influenced by ryanodine (Taylor and Marshall, 1992). In permeabilized drone photoreceptors, addition of neither 10 nM (results not shown) nor 10 μM ryanodine (Fig. 7 *B*) to the LM significantly affected the rate of Ca-oxalate accumulation ($n = 5$; $P > 0.5$). When 10 μM ryanodine were added together with 25 mM caffeine, the depression in the rate of Ca-oxalate accumulation was also not significantly different from the depression with caffeine alone ($n = 5$; $P > 0.2$). However, subsequent to the simultaneous treatment with caffeine and ryanodine, the rate of Ca-oxalate accumulation remained depressed and amounted to only 23% ± 16% (mean ± SD; $n = 5$; $P < 0.001$) when normalized to the control rate before drug treatment. For a control, the rate of Ca-oxalate accumulation recovered to 102 ± 22% ($n = 5$; Figs. 7 *A* and 8 *A*) after exposure to only caffeine. This result is in line with the observation that ryanodine requires the opening of the Ca^{2+} channels to bind to them and lock them in the open state (Meissner, 1986). The persistent depression in the rate of Ca-oxalate accumulation was observed only after ryanodine/caffeine treatment, but not after ryanodine/Ins(1,4,5)P_3 treatment (Figs. 7 *C* and 8 *B*).

Finally, we examined whether caffeine and Ins(1,4,5)P_3 had additive effects on the rate of Ca-oxalate accumulation. As shown in Fig. 9, this was the case. Exposure to both 25 mM caffeine and 6 μM Ins(1,4,5)P_3 caused a significantly larger depression in the rate of Ca-oxalate accumulation than exposure to either agonist alone ($n = 5$; $P < 0.01$). Because the Ins(1,4,5)P_3-dependent Ca^{2+} release mechanism is saturated at ≥ 1 μM Ins(1,4,5)P_3 (Baumann and Walz, 1989*b*), the additional depression in the rate of Ca-oxalate accumulation may be accounted for by the efflux of Ca^{2+} through distinct caffeine-sensitive channels.

In conclusion, these results demonstrate that the caffeine-sensitive Ca^{2+} release mechanism in honeybee photoreceptors is distinct from the Ins(1,4,5)P_3-dependent Ca^{2+} release mechanism.

Ca^{2+} Stimulates Release of Ca^{2+} via a Ryanodine-sensitive Pathway

The ryanodine-sensitive channels are thought to mediate CICR (Tsien and Tsien, 1990). The following experiments address the question of whether elevations in cytosolic Ca^{2+} can also stimulate Ca^{2+} efflux through the caffeine/ryanodine-sensitive Ca^{2+} channels in honeybee photoreceptors. Fundamental to these experiments is the ability of ryanodine to bind only to the open Ca^{2+} channel, and to keep it in the open state even after withdrawal of the agonist (Ca^{2+} in the experiments to be described).

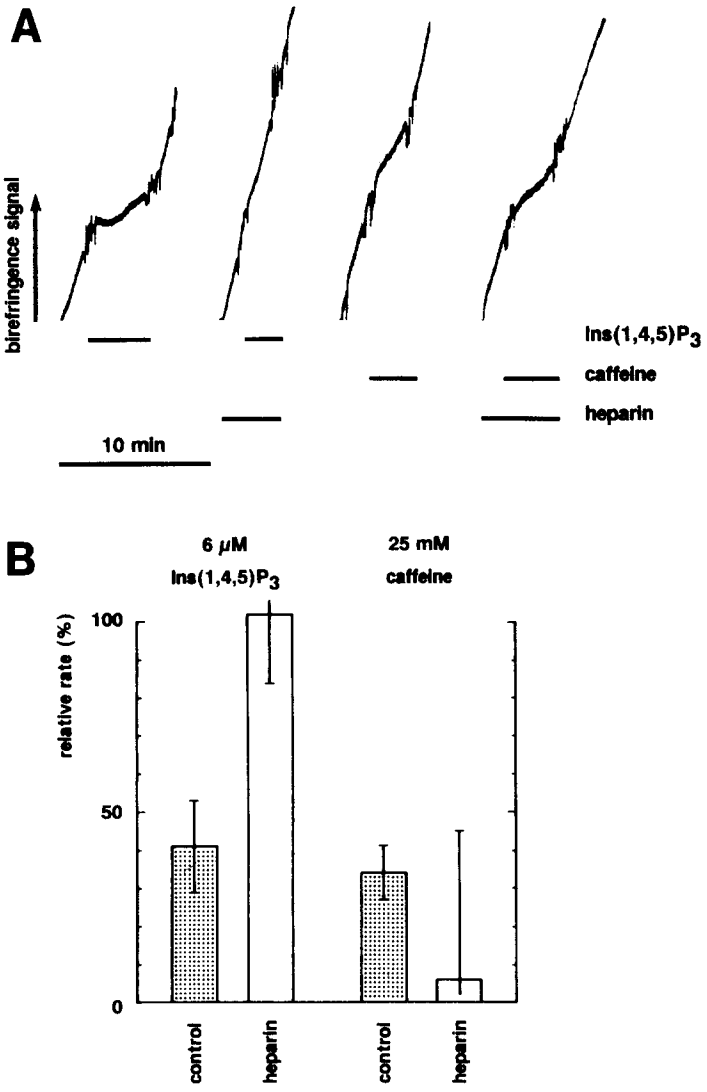

FIGURE 6. Heparin inhibits $Ins(1,4,5)P_3$-induced Ca^{2+} release but not caffeine-induced Ca^{2+} release from the ER. (*A*) The permeabilized retinal tissue was incubated in LM and successively exposed to 6 μM $Ins(1,4,5)P_3$ and 25 mM caffeine, first without, then together with 1 mg/ml heparin. Recordings are from a single representative experiment. (*B*) Quantitative summary of five independent experiments. Each bar gives the mean value ±SD, normalized to the mean of the control rates before and after $Ins(1,4,5)P_3$ or caffeine exposure. Heparin inhibits $Ins(1,4,5)P_3$-induced depression of Ca-accumulation ($P \approx 0.002$), but has no significant effect on caffeine-induced depression of Ca-oxalate accumulation ($P > 0.1$).

The rationale and the protocol of these experiments are explained in Fig. 10*A*. After permeabilization, the retinal tissue slice is successively incubated in (*a*) a low-Ca^{2+} LM; (*b*) a high-Ca^{2+} LM; and (*c*) again in low-Ca^{2+} LM. Free Ca^{2+} in the low-Ca^{2+} LM is kept so low (0.1 μM) that it (presumably) does not stimulate CICR.

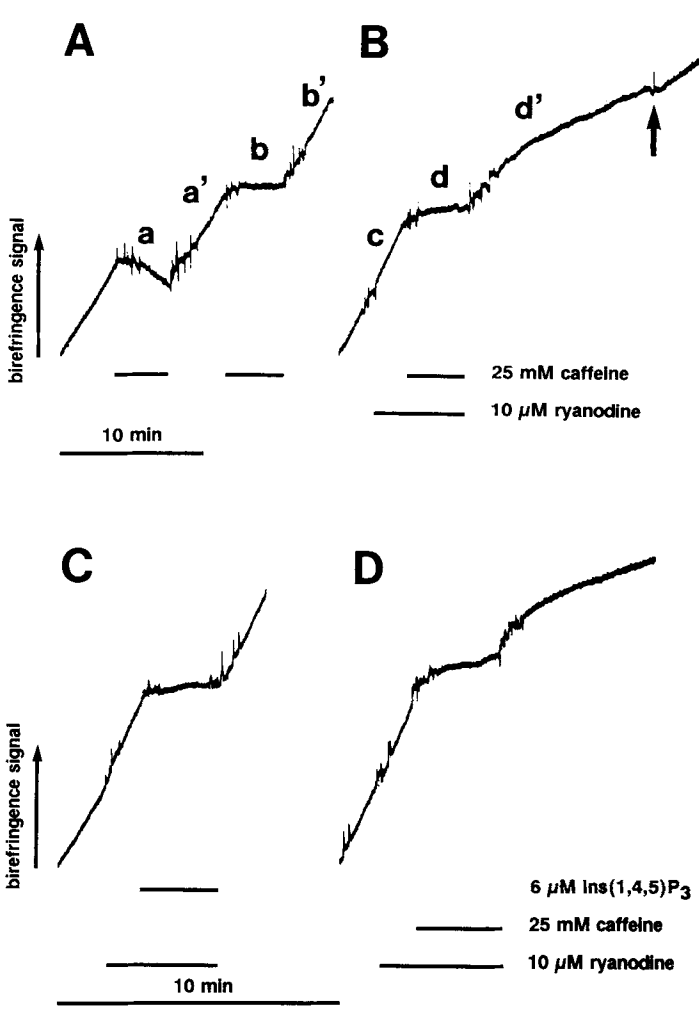

FIGURE 7. Caffeine-induced Ca^{2+} release is sensitive to ryanodine. (*A*) Control experiment demonstrating that the rate of Ca-oxalate accumulation completely recovers (*a'*, *b'*) after repetitive exposure to 25 mM caffeine (*a*, *b*). (*B*) The same retinal tissue as in *A* was exposed to ryanodine (10 μM; *c*), then to both ryanodine and caffeine (*d*), and finally superfused with normal LM (*d'*). The rate of Ca-oxalate accumulation did not completely recover after ryanodine/caffeine treatment, indicating that the caffeine-sensitive channels remained open. (*Arrow*) Loading medium was changed once more to ensure complete washing out of caffeine. (*C*) Retinal tissue was exposed to the same experimental protocol as shown in *B*, but with $Ins(1,4,5)P_3$ instead of caffeine. The rate of Ca-oxalate accumulation fully recovered after ryanodine/$Ins(1,4,5)P_3$ treatment. (*D*) Same retinal slice as in *C*, but exposed to ryanodine/caffeine.

To detect whether the high-Ca^{2+} LM (free Ca^{2+} concentration = 2.5 μM) induces opening of caffeine/ryanodine-sensitive channels, this solution contains ryanodine to bind to open channels, and to keep them open after switching back to low-Ca^{2+} LM. Thus, provided that the channels are sensitive to the Ca^{2+} concentration of the

high-Ca^{2+} LM, the rate of Ca-oxalate accumulation should be lower during the second exposure to low-Ca^{2+} LM than during the first exposure.

An original recording of an experiment according to above protocol can be seen in Fig. 10 *B*. Incubation in LM containing 0.1 μM free Ca^{2+} causes a slow rise in the birefringence signal. Superfusion with the test solution containing 2.5 μM free Ca^{2+} and 10 μM ryanodine causes an increase in the rate of rise in the birefringence signal because the Ca^{2+} uptake rate is increased in this medium. After switching back to low-Ca^{2+} LM, Ca-oxalate accumulation not only drops to a rate lower than the control rate (in 0.1 μM Ca^{2+} LM), but also decreases because of Ca-oxalate loss from

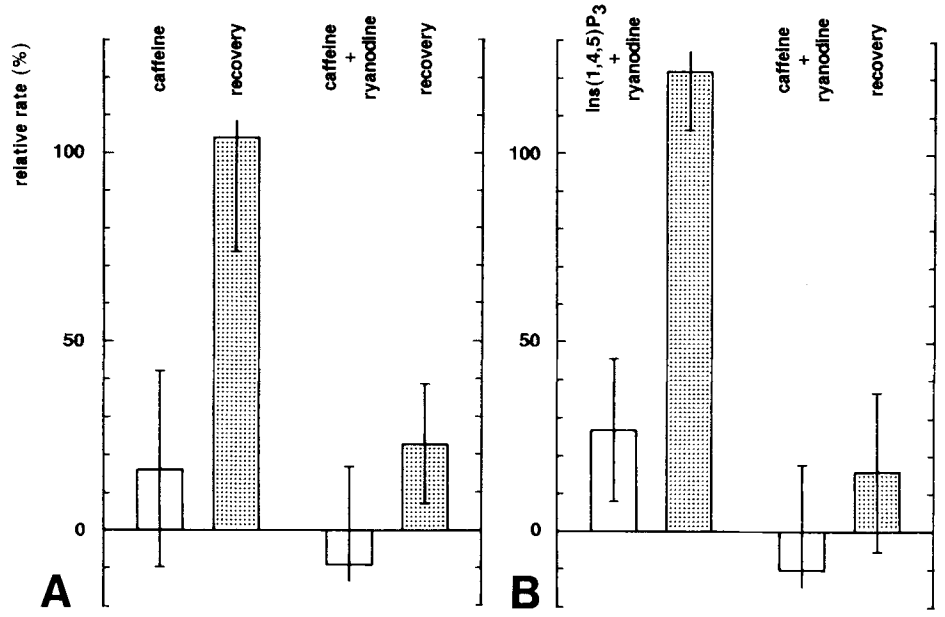

FIGURE 8. Quantitative comparison of the effect of ryanodine on the rate of Ca-oxalate accumulation by the retinal ER. The recovery of the rate of Ca-oxalate accumulation is shown after agonist treatment; for experimental protocol see Fig. 6. Each bar gives the mean ±SD of five independent measurements, normalized to the control rate before drug exposure. (*A*) The rate of Ca-oxalate accumulation recovers to the control rate after caffeine withdrawal ($P > 0.2$) but stays permanently depressed after ryanodine/caffeine treatment ($P < 0.001$). (*B*) The rate of Ca-oxalate accumulation recovers after ryanodine /Ins(1,4,5)P_3 treatment ($P > 0.02$) but stays depressed after ryanodine/caffeine treatment ($P < 0.001$).

the ER. Fig. 11 shows more quantitatively that this CICR occurs via the ryanodine-sensitive channels. A brief Ca^{2+} elevation in the absence of ryanodine does not significantly effect the rate of Ca-oxalate accumulation in low-Ca^{2+} LM ($n = 6$; $P > 0.1$). After a Ca^{2+} elevation in the presence of ryanodine, however, the rate of Ca-oxalate accumulation was significantly lowered ($n = 8$; $P < 0.01$). The variability in the amount of the suppression of Ca-oxalate formation after ryanodine washout in low Ca^{2+} LM can be explained by the fact that at a free Ca^{2+} concentration of only 0.1 μM, which is close to the threshold for the activation of the Ca^{2+}-pump, the rate of Ca-oxalate formation shows already a large variability (Baumann and Walz, 1989).

This variability can be expected to become even larger in low-Ca^{2+} LM after ryanodine treatment.

We thus conclude that treatment with high-Ca^{2+} concentrations opens a Ca^{2+}-release mechanism.

cADPR Has No Effect on Ca^{2+} Fluxes across the ER Membrane

Recent experiments have provided evidence that cADPR acts as a physiological regulator of the nonskeletal muscle-type RyR Ca^{2+} channel and that it releases Ca^{2+}

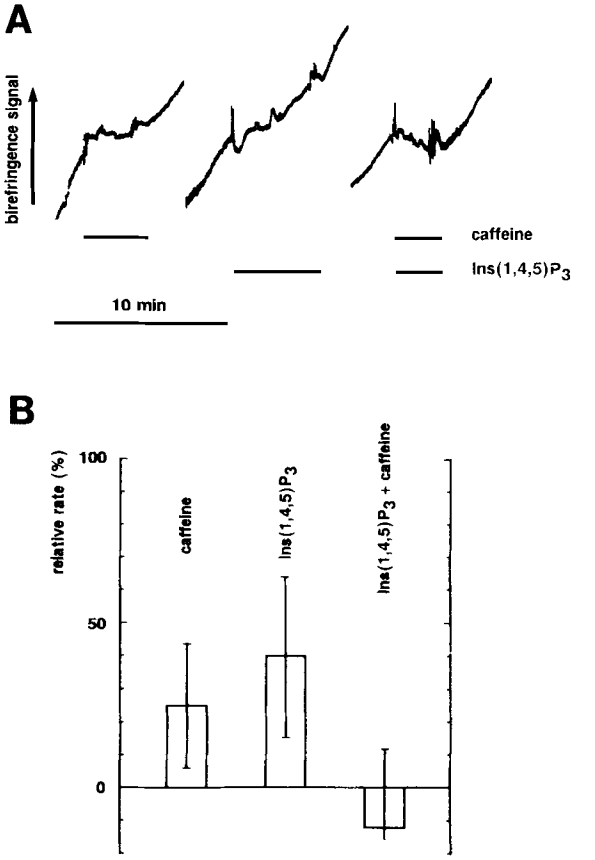

FIGURE 9. Caffeine and Ins-(1,4,5)P_3 have additive effects on the rate of Ca-oxalate accumulation by the ER. (*A*) Permeabilized retinal tissue was incubated in LM and subsequently superfused with LM containing caffeine (25 mM), Ins(1,4,5)P_3 (6 μM), and both, caffeine and Ins(1,4,5)P_3. Recordings are from a single representative experiment. (*B*) Data summarize the mean ± SD of five independent experiments, each normalized to the mean control rate before and after agonist treatment. Exposure to both caffeine and Ins(1,4,5)P_3 caused a significantly ($P < 0.01$) larger depression in the rate of Ca-oxalate accumulation (≡ larger rate of Ca^{2+} release) than exposure to either agonist alone. Note the negative rate of Ca-oxalate accumulation with both caffeine and Ins(1,4,5)P_3, indicating that Ca^{2+} release exceeds Ca^{2+} uptake under these conditions.

from internal stores (Galione et al.,1991; Mészaros et al., 1993). We have thus examined whether cADPR also affects Ca^{2+} fluxes across the ER membrane in honeybee drone photoreceptors.

Permeabilized eye slices were incubated with LM and then with LM containing 1 or 4 μM cADPR; these concentrations were ≥cADPR concentrations used in other preparations to induce Ca^{2+} release (Galione et al., 1991; Koshiyama, Lee, and Tashjian, 1991; Mészaros et al., 1993). As shown in Fig. 12, neither concentration had a visible effect on the rate of Ca-oxalate accumulation. This rules out the

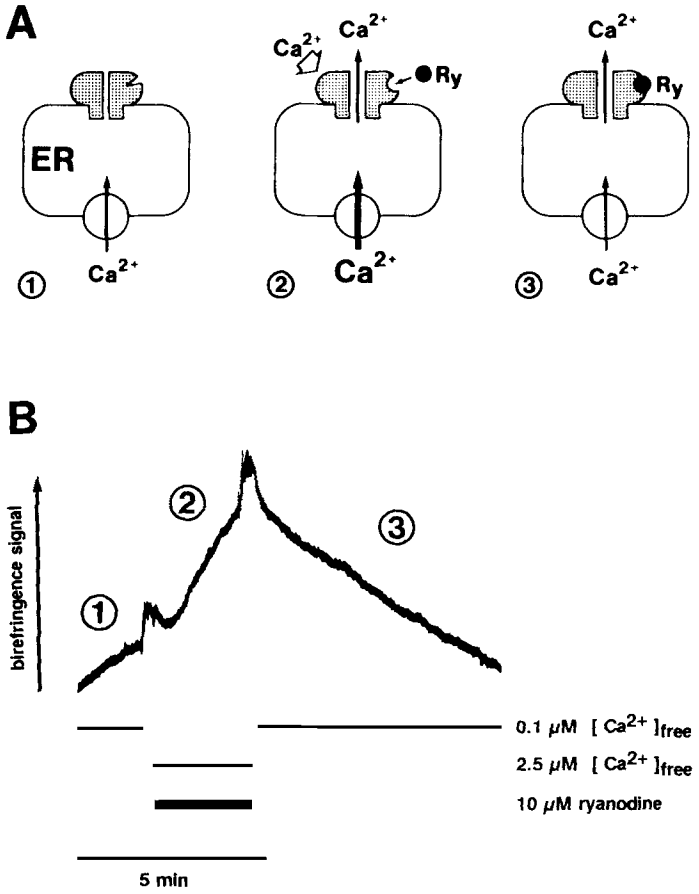

FIGURE 10. CICR from the ER of permeabilized drone retina. (*A*) Schematic representation of the experimental paradigm. The retinal tissue slice is first (*1*) incubated with low-Ca^{2+} medium, then (*2*) with test solution containing a higher free Ca^{2+} concentration and ryanodine (*Ry*), and finally (*3*) again in low-Ca^{2+} medium. Provided that Ca^{2+} in (*2*) induces the opening of the ryanodine-sensitive channels, ryanodine should bind to the channels and keep them open, even in low-Ca^{2+} medium (*3*). (*B*) Original recording of an experiment that demonstrates Ca^{2+}-induced Ca^{2+}-release. Elevation of free Ca^{2+} from 0.1 μM to 2.5 μM in the presence of ryanodine (*2*) causes an opening of a release mechanism that remains open after switching back to 0.1 μM free Ca^{2+} (*3*).

possibility that cADPR alone is sufficient to induce Ca^{2+} release from the ER in honeybee photoreceptors under our experimental conditions.

Elevation of Intracellular-free Ca^{2+} by Caffeine

The preceding experiments show that caffeine induces Ca^{2+} release from the ER in permeabilized photoreceptors. To find out whether caffeine is able to elevate Ca_i in intact cells, we have attempted to detect (qualitatively) caffeine-induced changes in

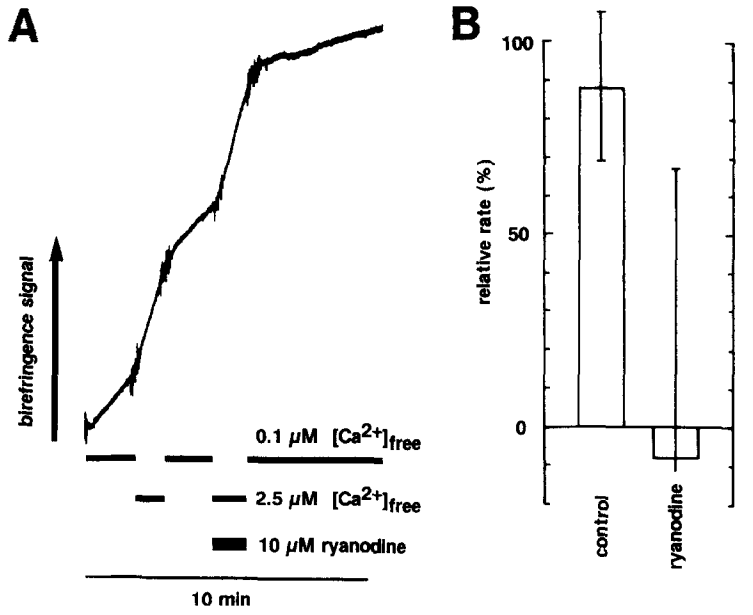

FIGURE 11. CICR occurs via the RyR. (*A*) The preparation was first exposed to the test solution in the absence of ryanodine; then, the preparation was exposed to the protocol given in Fig. 9. (*B*) The relative rate of Ca-oxalate accumulation after a brief Ca^{2+} elevation in the absence (*control*) and presence of ryanodine, both normalized to the pretreatment rate. After high-Ca^{2+}/ryanodine exposure, the rate of Ca-oxalate accumulation was completely depressed ($P < 0.01$), whereas in the control, the rate was not significantly different from the pretreatment rate ($P > 0.1$).

Ca_i with the Ca^{2+}-sensitive fluorescent dye fluo-3. Light-induced changes in Ca_i have been characterized previously, both quantitatively and qualitatively, in honeybee drone photoreceptors by means of fura-2, fluo-3, and Ca-green 5N (Walz et al., 1994). Our previous study indicated that a whole ommatidium was loaded with fluo-3

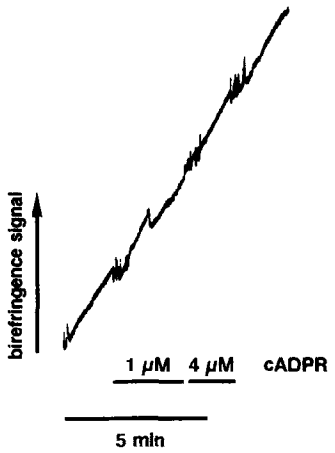

FIGURE 12. cADPR effect on Ca^{2+} fluxes across the ER membrane. Addition of cADPR to the LM does not elicit a Ca^{2+} release from the ER as there is no depression in the rate of Ca-oxalate accumulation.

when the dye was pressure injected into a single retinula cell, because the retinula cells within an ommatidium are dye coupled. Therefore, we collected the fluorescence from a whole fluo-3-loaded ommatidium. Fluorescence was excited periodically by opening the shutter in the epifluorescence light path for 50 ms every 15 s. The computer delivered a pulse for the opening of the shutter, and started A/D conversion of the fluorescence signal (voltage) after a delay of 10 ms. Over the next 30 ms, 100 voltage signals were sampled and averaged; then the shutter was closed. The averaged voltage signals were plotted over time (Fig. 13). To minimize bleaching of the dye and photodynamic damage, the intensity of the excitation light was attenuated 100-fold. Ca_i rose immediately after the first few test flashes (Fig. 13) because these excited the fluorescence of fluo-3 and stimulated the photoreceptors (beginning of the trace). After ~5 min, the preparation was superfused with drone Ringer containing 25 mM caffeine. This caused a persistent elevation in Ca_i, whereby

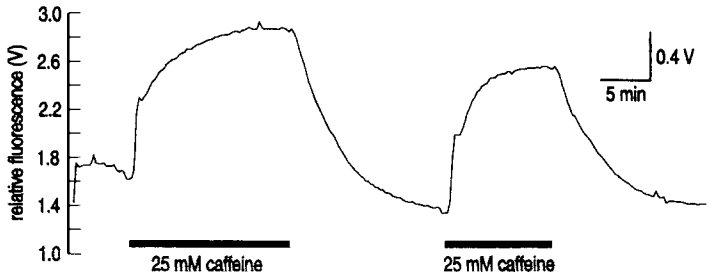

FIGURE 13. Caffeine produces a persistent increase in Ca_i. Ca_i was measured microfluorometrically with fluo-3. Fluorescence was measured periodically by opening the shutter in the epifluorescence beam every 15 s for 50 ms only. The relative fluorescence (V) of fluo-3 is plotted over time. The fast rising edge at the beginning of the trace is attributible to the elevation of Ca_i caused by the light flashes that excite the fluorescence and stimulate the photoreceptors. The caffeine-induced elevation of Ca_i exhibits an initial fast rise, followed by a slower elevation. The caffeine-induced rise in Ca_i is reversible. Similar records were obtained from five preparations.

the time course of the rising edge showed an initial rapid rise, followed by a slower elevation in Ca_i. Ca_i decreased within several minutes after washing out the caffeine. Similar results were obtained from five different preparations.

Photoreceptor Facilitation and Desensitization by Caffeine

As in other invertebrate photoreceptor cells, intracellular Ca^{2+} concentration modulates the adaptational state of honeybee drone photoreceptors (Bader et al., 1976; for a review see Brown, 1986). Drone photoreceptors have a bell-shaped relationship between sensitivity and adapting background lights (Walz, 1992); dim backgrounds increase the sensitivity (facilitation) of deeply dark-adapted cells up to four times, whereas brighter backgrounds produce desensitization (light adaptation). Both phenomena are caused by the background light-induced elevation of Ca_i (Walz, 1992).

We examined whether caffeine and the rise in Ca_i produced by caffeine mimic the sensitizing and/or desensitizing effects of background lights. We did this by studying the pharmacological effect of caffeine on deeply dark-adapted cells, and, alternatively, on cells sensitized by a moderately bright background light.

Fig. 14 shows the pharmacological effect of caffeine on a deeply dark-adapted photoreceptor cell. The cell was impaled, and then dark adapted for 45 min. The

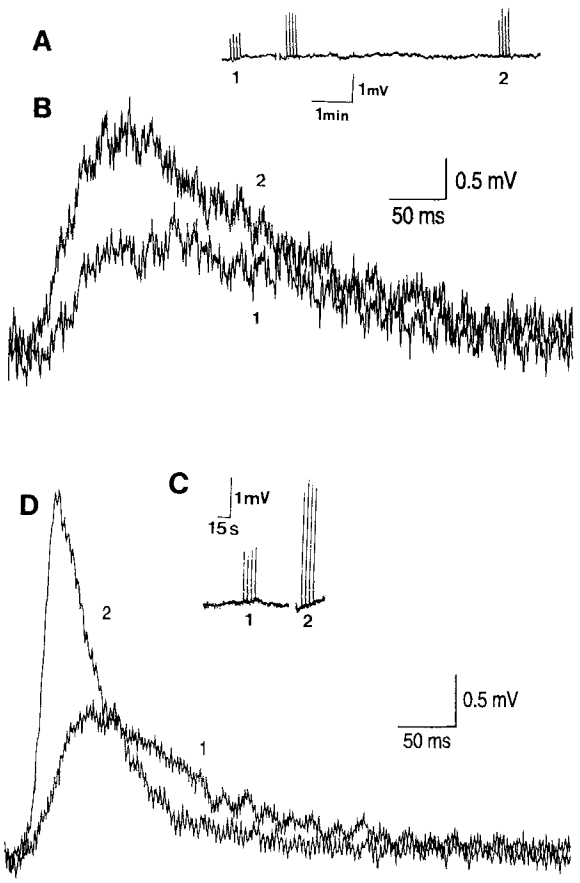

FIGURE 14. Enhancement of sensitivity (facilitation) produced by 5 and 50 mM caffeine in a deeply dark-adapted cell. (*A*) The cell was dark adapted for 45 min, and then stimulated with a series of four dim 20-ms test flashes (1 flash/5 s). The preparation was then superfused with 5 mM caffeine, and after 10 min in darkness, again stimulated with the same light flashes. The light flashes labeled *2* were given 2 min later. The traces labeled *1* and *2* in *B* are averages of the corresponding light responses in *A* shown on an expanded time scale. *C* and *D* are from a similar experiment on another cell. This cell was dark adapted for 1 h before the four control flashes were given. The preparation was then superfused with 50 mM caffeine, and after 5 min in darkness, again stimulated with the test flashes. The traces labeled *1* and *2* in *C* and *D* are averages of the corresponding light responses in *C* shown on an expanded time scale. Both caffeine concentrations increased the sensitivity of the deeply dark-adapted cells and accelerated the light responses.

sensitivity was subsequently probed with four dim test flashes (20 ms, 1 flash delivered every 5 s), producing receptor potentials of only ~1 mV. The preparation was then superfused with 5 mM caffeine in darkness, and 10 and 12 min later, the sensitivity was again probed with the same test flashes. The light responses became almost twice as big and slightly faster in the presence of caffeine (Fig. 14, *A* and *B*). These effects were usually more pronounced at higher caffeine concentrations (50

mM caffeine; Fig. 14, *C* and *D*), in particular with respect to the speed of the light responses. However, the amount of facilitation produced by caffeine, as expressed in the amplitude of the responses, varied greatly between the five preparations tested in these experiments.

The effect of caffeine on a slightly light-adapted cell is illustrated in Fig. 15. After impalement, the cell was deeply dark adapted for 1 h, and its sensitivity was probed with four dim test flashes (20 ms, 1 flash delivered every 5 s). Background lights of increasing intensities were then applied, and the sensitivity of the cell was probed

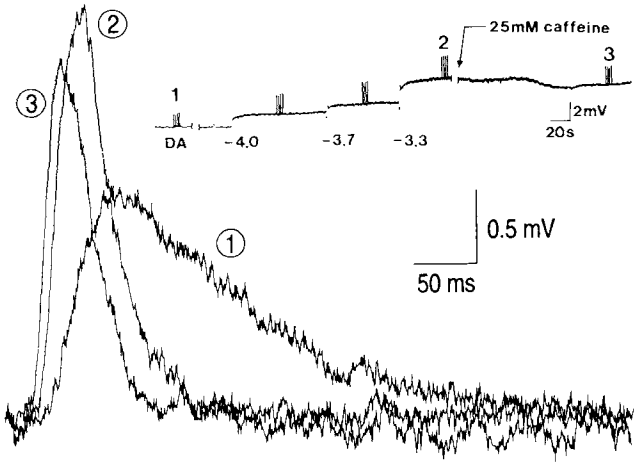

FIGURE 15. Decrease in sensitivity produced by 25 mM caffeine in a slightly light-adapted cell. (*A*) The cell was dark adapted for 1 h, and then stimulated with a series of four dim 20-ms test flashes (1 flash/ 2 s, log. rel. int. −4.0) in order to probe the sensitivity of the cell. Subsequently, background lights of increasing intensity (log. rel. int. −4.0, −3.7, −3.3) were applied, and the sensitivity of the cell probed with the same four test flashes superimposed on the backgrounds. After the test flashes on the −3.3 background, the preparation was superfused with 25 mM caffeine, and 3 min later, the test flashes were given again. The traces labeled *1*, *2*, and *3* in *B* are averages of the corresponding light responses in *A*. The gaps cut from the original recording in *A* are 25 and 40 s. The figure illustrates that the dim background lights increased the sensitivity of the cell (facilitation; compare responses *1* and *2*), because the responses became larger and faster. Increasing the background intensity from log. rel. int. −3.7 to −3.3 produced no further enhancement in sensitivity; 25 mM caffeine, however, made the test flash responses smaller and faster (response *3*), thus mimicking all aspects of light adaptation.

with the same test flashes superimposed on the backgrounds. The background lights enhanced the sensitivity of the cell (facilitation) to its maximum. At the highest background intensity, the preparation was superfused with 25 mM caffeine, and 3 min later, the test flashes were again given. The responses to these flashes were now smaller (desensitized) and faster with respect to the facilitated light responses. This caffeine-induced decrease in the sensitivity (by 19 ± 9%, mean ± SD) and speeding of the light responses in slightly light adapted cells was reproducibly observed in six different preparations.

Caffeine, like other methylxanthines such as theophylline and isobuthyl-methylxanthine (IBMX), is known to be a phosphodiesterase (PDE) inhibitor. These methylxanthines, and the nonxanthine PDE inhibitor papaverine have been shown to affect the electrical activity of vertebrate rods (e.g., Capovilla, Cervetto, and Torre, 1983) and the photoreceptor of the ventral nerve of *Limulus* (Corson, Fein, and Schmidt, 1979), presumably because they affect cyclic nucleotide concentrations (e.g., Cohen, 1981; Miki, Keirns, Marcus, Freemen, and Bitensky, 1973). To determine whether the sensitizing and/or desensitizing effect of caffeine is attributible to its pharmacological effect as a PDE inhibitor, we tested whether IBMX and papaverine mimic the pharmacological effects of caffeine. We chose IBMX and papaverine concentrations that were effective in rods and *Limulus* ventral photoreceptors (IBMX: 0.5 mM; papaverine: 100 μM).

The experiment illustrated in Fig. 16 shows that 0.5 mM IBMX affects neither the sensitivity nor the kinetics of the electrical responses of a deeply dark adapted photoreceptor cell to dim light flashes (three cells tested). The same result was

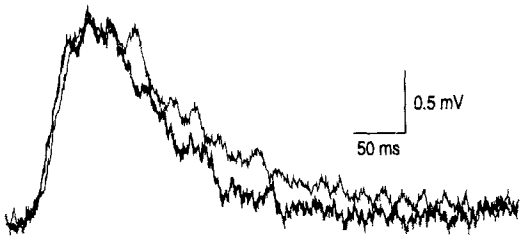

FIGURE 16. Effect of 0.5 mM IBMX on the sensitivity of a deeply dark-adapted cell. The preparation was dark adapted for 1 h. The sensitivity of the cell was then probed with four dim 20-ms test flashes (log. rel. int. −4.3; 1 flash/2 s) and the responses averaged (*light trace*). The preparation was then superfused with IBMX. The heavy trace is the average response to the same four test flashes after 30 min in IBMX.

obtained with 100 μM papaverine in two other cells (data not shown). We thus conclude, that the pharmacological effects seen with caffeine are not caused by its action as a PDE inhibitor.

DISCUSSION

Most invertebrate photoreceptors have an extensive network of palisadelike arranged smooth ER cisternae closely juxtaposed to the bases of their photoreceptive microvillar membrane (for a review see Whittle, 1976). These submicrovillar cisternae (SMC) of the ER function as a Ca^{2+} store (Baumann and Walz, 1989*a*,*b*; Walz and Baumann, 1989), and release Ca^{2+} in response to Ins(1,4,5)P_3 and light (Baumann and Walz, 1989*a*,*b*; Baumann et al., 1991; Payne, Walz, Levy, and Fein, 1988). In this study, we provide physiological evidence that the ER of bee photoreceptors contains, besides the Ins(1,4,5)P_3-sensitive release pathway, an Ins(1,4,5)P_3-independent, caffeine- and ryanodine-sensitive release mechanism. We also show that this mechanism is able to mediate CICR. Because expression of a RyR gene (*dry*) has recently been shown in the compound eye of *Drosophila* (Hasan and Rosbash, 1992), CICR may be a general property of the ER in insect photoreceptors.

The CICR Mechanism in Drone Photoreceptors

Our experiments on permeabilized drone retina show that caffeine stimulates Ca^{2+} release from the ER in a reversible and dose-dependent manner. Similarly, caffeine causes in vivo a reversible increase in Ca_i. Finally, our electrophysiological recordings are also consistent with the finding that caffeine is responsible for a maintained rise in Ca_i. These experiments demonstrate that the caffeine-induced elevation in Ca_i is sufficiently large to cause either facilitation or light adaptation depending on the adaptational state of the cell. In this respect, caffeine mimics the effects of background lights: dim backgrounds sensitize deeply dark-adapted cells (facilitation), whereas brighter backgrounds desensitize them. This behavior had been shown to be mimicked by Ca^{2+} injections (Walz, 1992).

Measurements of Ca^{2+} fluxes across the ER membrane further show that the caffeine-induced Ca^{2+} release is distinct from the well-documented $Ins(1,4,5)P_3$–dependent Ca^{2+} release; it is not inhibited by heparin but is sensitive to ryanodine, and the effects of caffeine and $Ins(1,4,5)P_3$ are additive at saturating $Ins(1,4,5)P_3$ concentrations. We thus conclude that the submicrovillar ER of bee photoreceptors contains, in addition to the $Ins(1,4,5)P_3$-receptor Ca^{2+} channel, a Ca^{2+} release mechanism with the key pharmacological criteria of a RyR Ca^{2+} channel.

We have also tried to study the effects of ryanodine (applied to the experimental bath) on the electrical light responses, with and without caffeine under various light regimens (data not shown). However, ryanodine produces no obvious pharmacological effect under any of the tested conditions. This could either mean that ryanodine-induced Ca^{2+} efflux from the ER does not raise Ca_i effectively because ryanodine locks the channels in only a subconductance state (Fleischer et al., 1985; Meissner, 1986) and Ca^{2+} uptake mechanisms compete well with the Ca^{2+} release, or that ryanodine does not reach/enter the intact cells within the tissue slices.

We also show that the caffeine- and ryanodine-sensitive Ca^{2+} release mechanism in drone photoreceptors differs from that in sea urchin eggs and some nervous tissues because it lacks sensitivity to cADPR, a recently described modulator/activator of the nonskeletal muscle-type RyR Ca^{2+} channel (Galione et al., 1991; Lee, Aarhus, Graeff, Gurnack, and Walseth, 1993; Mészáros et al., 1993; White, Watson, and Galione, 1993). In the latter preparations, cADPR alone was sufficient to induce Ca^{2+} release, whereas in drone photoreceptors it is not. We can not exclude the possibility that cADPR, together with some unidentified factors, such as calmodulin (Lee, Aarhus, Graeff, Gurnack, and Walseth, 1994), is able to modulate Ca^{2+} release, or that an inhibitor of cADPR-induced Ca^{2+} release is present in the cytosol (Sitsapesan, McGarry, and Williams, 1994). Pressure injections of 80 μM cADPR into the ventral photoreceptors of *Limulus*, however, does not exert any pharmacological effects (R. Payne, personal communication). This makes it at least unlikely, that cADPR-induced Ca^{2+} release, if present at all, plays an important role in Ca^{2+} handling by invertebrate photoreceptors. Our study indicates that Ca^{2+} itself is able to release Ca^{2+} from the ER, and thus we conclude that Ca^{2+} is the physiological agonist of the RyR Ca^{2+} channel in honeybee photoreceptors.

Because of this property, the caffeine- and ryanodine-sensitive release mechanism seems most closely to resemble the RyR Ca^{2+} release channel in cardiac muscle cells

(RYR-2). RYR-2 has also been localized within various vertebrate neurons (McPherson and Campbell, 1993*a*,*b*; McPherson et al., 1991). In skeletal muscle, the cytoplasmic domain of the RYR-1 contacts the dihydropyridine (DHP) receptor of the plasma membrane to form "feet structures," a structural linkage between terminal sarcoplasmic reticulum and plasma membrane (Franzini-Armstrong, 1970; Lai, Erickson, Rousseau, Liu, and Meissner, 1988; Saito, Inui, Radermacher, Frank, and Fleischer, 1988). The DHP receptor may act as a voltage sensor, and the information on voltage change across the plasma membrane may be transmitted by way of conformational changes of these feet structures to the RyR. So far, we have no information whether similar structures occur in invertebrate photoreceptors. Although the submicrovillar ER cisternae in the photoreceptors of the honeybee and other invertebrates approach the plasma membrane closely, we have never observed morphological equivalents to the feet structures of skeletal muscle (Baumann and Walz, 1989*a*; Walz, 1979, 1982*a*). Interestingly, *Drosophila* photoreceptors possess a plasma membrane Ca^{2+} channel with close homology to the DHP receptor, the *trp* (transient receptor potential) gene product (Philips, Bull, and Kelly, 1992). This channel is necessary for a maintained receptor potential during stimulation with long steps of light (Hardie and Minke, 1992). The *trp* gene product may physically interact with the Ins(1,4,5)P_3 receptor of the submicrovillar ER membrane, and the latter may activate the *trp* channel to induce Ca^{2+} influx for the refilling of the Ca^{2+} depleted ER (Minke and Selinger, 1992). The presence of several different Ca^{2+}-regulating mechanisms in the microvillar/submicrovillar transduction compartment warrants further investigations of their functional interactions.

Caffeine induces an increase in Ca_i in intact drone photoreceptors. This could be expected on the basis of the results obtained with permeabilized slices of drone retina. The observation that caffeine produces a persistent increase in Ca_i is however, unexpected. In several cell types, such as sympathetic neurons and smooth muscle cells, the caffeine-induced elevation of Ca_i is transient, despite the continued presence of caffeine, presumably because Ca^{2+} is rapidly extruded from the cells (Friel and Tsien, 1992; Ganitkevich and Isenberg, 1992). We note, however, that caffeine elevates Ca_i in two phases in drone photoreceptors; an initial rapid increase is followed, after a brief inflection, by a slower rise toward a maintained elevated intracellular Ca^{2+} concentration. In addition to a Ca^{2+} release, we suspect that in intact cells, caffeine also causes directly or indirectly a Ca^{2+} influx from the extracellular space, with some delay. Future experiments should address the question of whether caffeine leads to storage depletion, and whether this in turn is signaled to a plasma membrane Ca^{2+} channel, leading to a Ca^{2+} influx for storage repletion. This (hypothetical) scenario is closely related to the "capacitative Ca^{2+} entry model" put forward by Putney (1986), and modified by Minke and Selinger (1992) (see above) in order to explain electrophysiological properties of the *trp* mutation in *Drosophila*. However, these questions have yet to be resolved.

Functional Implications of CICR

The functional significance of the RyR Ca^{2+} channel critically depends on its localization in the ER. Because the optical signal in our Ca-oxalate measurements is largely dominated by Ca-oxalate within the submicrovillar cisternae, we conclude that

the RYR resides within this domain of the ER. However, the size and surface of the submicrovillar ER cisternae are large (Baumann and Walz, 1989*a*), and the RYR may only be localized to a subdomain of the submicrovillar ER membrane, e.g., those areas that face the microvillar bases. In the vertebrate Purkinje cell, where the distribution of Ca^{2+}-regulating proteins has been investigated most thoroughly by immunocytochemical techniques, both the RYR and the Ins(1,4,5)P_3 receptor are not evenly distributed within the ER membrane (Nori, Villa, Podini, Witcher, and Volpe, 1993; Walton, Airey, Sutko, Beck, Mignery, Südhof, Deerinck, and Ellisman, 1991). Thus, information on the precise localization of the RYR, and other Ca^{2+}-regulating mechanisms in invertebrate photoreceptors is required. Moreover, we do not yet know, whether and to what extent light stimulation causes activation of both release pathways, viz, Ins(1,4,5)P_3-induced release and CICR.

Insect photoreceptors share the redundancy of two release mechanisms with, for example, vascular smooth muscle cells, sea urchin eggs, chromaffin cells, and some neurons, such as cerebellar Purkinje cells (Galione et al., 1993; Lee et al., 1993; for a review see Berridge, 1993). The functional implications of this redundancy of release mechanisms is not completely understood in any of these cells. Both Ins(1,4,5)P_3-induced release and CICR are probably involved in the generation of complex patterns of Ca^{2+} signaling, such as propagating Ca^{2+} waves and oscillations (reviews: Berridge, 1993; Tsien and Tsien, 1990). What are the spatiotemporal aspects of Ca^{2+} signaling in invertebrate photoreceptors?

Light stimuli probably do not induce propagated Ca^{2+} waves in a longitudinal direction along the photoreceptors. Several studies have shown that Ca^{2+}-mediated light adaptation is localized to the area of illumination (Hagins, Zonana, and Adams, 1962; Hamdorf, 1970; Fein and Lisman, 1975; Payne and Fein, 1983; Walz, Coles, Poitry, and Levy, 1986). Ultrastructural investigations have demonstrated that the Ca^{2+}-sequestering ER in invertebrate photoreceptors is a morphological continuum (Baumann and Walz, 1989*a*; Feng, Carson, Morgan, Walz, and Fein, 1994; Walz, 1979). If there were a spatial spread of Ca^{2+} release from the ER along the photoreceptor, light adaptation should not be restricted spatially. However, CICR may facilitate Ca^{2+} release over distances of a few micrometers. Therefore, Ca^{2+} release initiated at the submicrovillar portions of the ER in insect photoreceptors may propagate along the ER radially toward the bulk cytoplasm, a distance of only 5–10 μm. There, a rise in Ca_i has been shown to stimulate oxidative metabolism (Fein and Tsacopoulos, 1988).

Under physiological conditions, Ca_i does not oscillate in invertebrate photoreceptors. Long steps of light produce, after an initial large transient increase, a smooth, maintained elevated intracellular Ca^{2+} concentration (e.g., Brown and Blinks, 1974; Levy and Fein, 1985; Maaz and Stieve, 1980; Walz et al., 1994). Ca_i oscillates in *Limulus* ventral photoreceptors only after pressure injections of the nonmetabolizable Ins(1,4,5)P_3 analogue inositol trisphosphorothioate (Payne and Potter, 1991). These oscillations can be explained, at least in part, by the positive and negative feedback that Ca^{2+} exerts on its own release through the Ins(1,4,5)P_3 receptor Ca^{2+} release channel alone (Baumann and Walz, 1989*b*; Bezprozvanny, Watras, and Erlich, 1991; Finch, Turner, and Goldin, 1991; Payne, Flores, and Fein, 1990; Payne et al., 1988), or in concert with the CICR mechanism as discussed by various authors (e.g.,

Berridge, 1993; Tsien and Tsien, 1990). However, this does not elucidate the mechanisms that suppress oscillations of Ca_i during continuous light stimulation (Payne and Potter, 1991).

The redundancy of release pathways may have the advantages of (*a*) allowing the separate modulation of the two channels by different agonists, and (*b*) keeping ready two release pathways with different Ca^{2+} sensitivities (Bezprozvanny et al., 1991) as discussed for example in sea urchin eggs, which also express both the Ins(1,4,5)P_3 and RyR (Galione et al., 1993; Lee et al., 1993). The light-induced Ca^{2+} increase is extremely fast in drone photoreceptors. After switching on a bright light, Ca_i rises, after a latency of only 9–10 ms, to an initial peak within only 150–200 ms. The peak intracellular Ca^{2+} concentration saturates the fluorescent Ca^{2+} indicator dyes fura-2 and fluo-3 in the submicrovillar transduction compartment. Thus, Ca_i locally exceeds a concentration of several micromolar (Walz et al., 1994). In the rhabdomeric lobe of the *Limulus* ventral nerve photoreceptor, Ca_i rises, because of Ca^{2+} release from the ER, up to 80 μM after stimulation by a bright light step (Levy and Fein, 1985; R. Payne, personal communication). We consider it likely that coexpression of both receptors is advantageous or a prerequisite for the production of such rapid large Ca^{2+} elevations, because the Ins(1,4,5)P_3-induced Ca^{2+} release is inhibited by negative feedback at Ca^{2+} concentrations above 0.3–3 μM (Baumann and Walz, 1989*b*; Bezprozvanny et al., 1991; Finch et al., 1991), whereas the ryanodine-sensitive Ca^{2+} release channels are maximally activated by Ca^{2+} concentrations between 1 and 100 μM (Bezprozvanny et al., 1991).

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