Caffeine-Sensitive Calcium Stores Regulate Synaptic Transmission from Retinal Rod Photoreceptors

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We investigated the role of caffeine-sensitive intracellular stores in regulating intracellular calcium ([Ca2+]i) and glutamatergic synaptic transmission from rod photoreceptors. Caffeine transiently elevated and then markedly depressed [Ca2+]i, to below prestimulus levels in rod inner segments and synaptic terminals. Concomitant with the depression was a reduction of glutamate release and a hyperpolarization of horizontal cells, neurons postsynaptic to rods. Caffeine did not affect the rods’ membrane potentials indicating that caffeine likely acted via some mechanism(s) other than a voltage-dependent deactivation of the calcium channels. Most of caffeine’s depressive action on [Ca2+]i, on glutamate release, and on I_{Ca} in rods can be attributed to calcium release from stores: (1) caffeine’s actions on [Ca2+]i, and I_{Ca} were reduced by intracellular BAPTA and barium substitution for calcium, (2) other nonxanthine store-releasing compounds, such as thymol and chlorocresol, also depressed [Ca2+]i, and (3) the magnitude of [Ca2+]i depression depended on basal [Ca2+]i, before caffeine. We propose that caffeine-released calcium reduces I_{Ca} in rods by an as yet unidentified intracellular signaling mechanism. To account for the depression of [Ca2+]i below rest levels and the increased fall rate of [Ca2+]i, with higher basal calcium, we also propose that caffeine-evoked calcium release from stores activates a calcium transporter that, via sequestration into stores or extrusion, lowers [Ca2+]i, and suppresses glutamate release. The effects of store-released calcium reported here operate at physiological calcium concentrations, supporting a role in regulating synaptic signaling in vivo.

Key words: photoreceptor; rod; calcium; caffeine; intracellular calcium stores; glutamate release

It is now generally recognized that intracellular calcium concentration ([Ca2+]i) is an important regulator of neurotransmitter release. Extensive studies have delineated a role for calcium influx in gating transmitter release (Katz and Miledi, 1969; Matthews, 1997; Neher, 1998). Much less study has been directed at a possible role of intracellular calcium stores in exocytosis, particularly in glutamatergic neurons. In chromaffin cells, Ca2+ released from caffeine-sensitive intracellular storage compartments can both enhance and reduce exocytosis of catecholamines. The stimulation of exocytosis by caffeine may occur in the absence of extracellular calcium (Cheek et al., 1990). However, caffeine treatment can also reduce the size of depolarization-evoked release of catecholamines from these same cells (Lara et al., 1997). On the basis of the finding that caffeine reduced the depolarization-evoked rises of [Ca2+]i, in bullfrog neurons, Fried and Tsien (1992) articulated the hypothesis that caffeine-induced depletion of stores stimulated uptake back into these compartments and that this sequestration acted as a sink for incoming calcium. Cseresnyes et al. (1997) concluded that in frog sympathetic ganglion neurons there is a release-activated calcium transport (RACT) that significantly increases sequestration into stores when calcium is released from caffeine-sensitive compartments. When activated, RACT transports calcium at rates 1.6 and 4 times faster than the conventional sarcoplasmic–endoplasmic calcium ATPases (SERCA) and the plasma membrane extrusion pumps, respectively. This indicates that RACT can play a significant role in controlling [Ca2+]i, and possibly exocytosis as well. In this present study we used the rod–horizontal cell synapse of the amphibian retina to examine the possibility that caffeine-sensitive Ca2+ stores contribute to the regulation of glutamate exocytosis.

A hallmark feature of synaptic transmission at the photoreceptor and bipolar cell synapses of the retina is the continuous release of glutamate. It is generally accepted that the release from rods is directly controlled by an influx of Ca2+, predominantly through L-type calcium channels located in inner segments and synaptic terminals of these cells (Copenhagen and Jahl, 1989; Rieke and Schwartz, 1996; Schmitz and Witkovsky, 1997; Witkovsky et al., 1997). In general, caffeine-sensitive stores are localized to smooth endoplasmic reticulum (ER) (Golovina and Blaaustein, 1997; Meldolesi and Pozzan, 1998), an intracellular compartment widely distributed throughout the cell body, dendritic trees, and synaptic terminals of neurons (Walton et al., 1991; Krijnse-Locker et al., 1995). Although there are no reports of caffeine-sensitive stores in photoreceptors, cisterneae of smooth ER have been noted in synaptic nerve terminals of photoreceptors (Mercurio and Holtzman, 1982; Ungar et al., 1984). We studied the effects of caffeine on both presynaptic and postsynaptic cells at the photoreceptor synapse. We investigated

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the effects of caffeine on \([\text{Ca}^{2+}]_i\) in rods using fur-2, on L-type calcium currents in rods using whole-cell recording methods, on rod and horizontal cell membrane potentials using microelectrode techniques, and on the release of endogenous glutamate using a newly developed preparation consisting of a sheet of photoreceptors separated from the rest of the retina (Cahill and Besharse, 1992; Schmitz and Witkovsky, 1996). Our data strongly suggest that calcium release from caffeine-sensitive calcium stores can modulate synaptic transmission from these glutamatergic neurons.

Parts of this study have been published previously in abstract form (Krizaj et al., 1997).

MATERIALS AND METHODS

The experiments were performed on rod photoreceptors from two amphibian species: the clawed frog (Xenopus laevis) and the tiger salamander (Ambystoma tigrinum). All imaging experiments were performed on salamander photoreceptors because of their large size and experimental tractability; we used the Xenopus retina because it can be used as a "reduced preparation" to study glutamate release (see below) (Schmitz and Witkovsky, 1996). Intracellular recording was performed on both frog retinae and Ambystoma horizontal cells (HC). We found no significant species differences in relation to the light-evoked responses or membrane potential of HCs when exposed to caffeine.

Preparation of isolated cells. Larval stage tiger salamanders were decapitated and pithed. Retinas were dissected at room temperature (20–22°C) in room light, incubated on a shaker in 0 Ca²⁺ and papain (7 U/ml; Worthington, Freehold, NJ) saline for 25 min, and triturated with a BSA-coated Pasteur pipette. The outer segments of many rods were sheared during the isolation procedure, resulting in the absence of the dark current and hyperpolarization of their membrane potentials. Dissociated cells were kept at 4°C in 80% L-15 medium supplemented with 10 mM HEPES, 20 mM glucose, 1 mM pyruvic acid, 1 mg/ml bovine serum albumin, and 1 μM Liquid Media Supplement containing transferrin and selenium (Sigma, St. Louis, MO). Cells were plated on acid-cleaned glass coverslips coated with IgG and/or IgM (Jackson ImmunoResearch, West Grove, PA) and the Sal-1 antibody [a kind gift from Dr. Peter MacLeish (MacLeish et al., 1983)]. The control saline solution contained (in mM): 97 NaCl, 2 KCl, 2 CaCl₂, 2 MgCl₂, 10 HEPES, 20 glucose, 1 pyruvic acid, 0.3 ascorbic acid, and 1 glutathione at 240 mOsm. pH was adjusted to 7.6 with NaOH. In the high (20 mM) potassium saline, the concentration of NaCl was correspondingly reduced. The volume around cells was superfused using either a Y-tube mechanism in which solution exchange was completed within 10 sec or a multibarrel quartz perfusion system that allowed solution exchange within 100 msec.

\([\text{Ca}^{2+}]_i\) measurement and image acquisition. These measurements are described in more detail elsewhere (Krizaj and Copenhagen, 1998). Briefly, photoreceptors were loaded for 10 min with 3–5 μM fura-2 AM (Molecular Probes, Eugene, OR) supplemented with 0.005% Pluronic F-127 in L-15 and then washed for 20 min in L-15. The fluorescence signals were acquired at 0.3–1 Hz by a cooled 12-bit digital CCD camera (PXL; Photometrics, Tucson, AZ) controlled by commercial software (MetaFluor; Universal Imaging Corporation, West Chester, PA). Ratios between the 340 and 380 nm excitation wavelengths were calculated after subtraction of the background fluorescence. Free Ca²⁺ levels were calibrated in vivo with 10 μM ionomycin using the standard relationship developed by Grzynkiewicz et al. (1985). The \(K_0\) values for Ca²⁺ (224 nm) and Ba²⁺ (780 nm) binding to fura-2 were taken from the literature (Grzynkiewicz et al., 1985; Schilling et al., 1989; Neher, 1995). The actual calcium concentration should be considered estimates, because a standardized value for the \(K_0\) of fura-2 was used throughout these experiments. Most measurements were taken in a region encompassing most of the inner segment of isolated rods. Loss of synaptic terminals during dissociation and low signal/noise ratios made it difficult to test routinely every protocol on the terminals. The waveforms of the transient elevations and depressions of calcium were not discernibly different from those in the outer segments. The lower signal/noise ratio prevented us from quantitatively assessing whether the responses were faster in the terminals, as we had reported previously for potassium-evoked rises of calcium (Krizaj and Copenhagen, 1998).

Patch-clamp recording. Recording electrodes were pulled in four steps on a horizontal pipette puller (P97; Sutter Instruments, Novato, CA) from 1.7 mm borosilicate capillary glass (TW 150; World Precision Instruments, Sarasota, FL). Electrodes were filled with (in mM): 75 Cs MeSO₄, 5 EGTA, 30 HEPES, 0.5 CaCl₂, 2 Mg-ATP, 0.5 NaGTP, and 20 TEA-Cl, with pH adjusted to 7.5 with CsOH. The electrode resistance was 10–20 MΩ. The extracellular solution contained (in mM): 55 NaCl, 2.5 KCl, 10 CaCl₂, 1 MgCl₂, 8 glucose, 10 HEPES, and 30 TEA-Cl, with pH buffered to 7.6 with NaOH. In some experiments, 10 mM BaCl₂ was replaced by 10 mM BaCl₂. Seal resistance ranged from 1 to 20 GΩ. Series resistances were typically 4–12 MΩ. The membrane current was measured with an Axopatch 2-D amplifier (Axon Instruments, Foster City, CA). Capacitance currents were canceled electronically. Current records were low-pass filtered at 2 kHz (−3 dB) and digitized at 5× the filter cutoff frequency. Data were acquired via an analog-to-digital interface (Indec Systems, Sunnyvale, CA). Junction potentials were 1–2 mV, as measured using a low-resistance 3 M KCl reference electrode in the bath; membrane potentials were not corrected for junction potentials. Leak subtraction was performed on whole-cell currents as follows. Ca²⁺ currents were blocked by adding 100 μM Cd²⁺, and the remaining leak currents were subtracted from the equivalent experimental records from the same cell. The leak currents usually were obtained immediately after each set of experimental records. The protocol-patch recordings were performed as described above, except that the electrode was filled with 2 ng/ml gramicidin.

Rods were held at −70 mV. In most experiments, Ca²⁺ currents were induced by depolarizing the cells with voltage ramps (−70 to +50 mV) scanned at a rate of 1.02 V/sec. Ramps were given at 20 sec intervals throughout the experiments, and the drugs under study were applied onto cells after Ca²⁺ currents stabilized, which typically occurred within a few minutes after break in. Solution changes were made by gravity-driven multibarrel micropipette perfusion system positioned within 1 mm of the tested cell.

The percentage inhibition of Ca²⁺ currents was expressed as \([1−I_{\text{Ca}}(\text{test})/I_{\text{Ca}}(\text{control})] \times 100\). Cadmium-subtracted currents were used, whenever possible, to calculate the peak amplitude. Student’s t-tests were performed for paired or unpaired groups, whereas one-way ANOVA was done for three or more groups.

Intracellular recording. Eyecups were prepared as described previously (Krizaj et al., 1994). Microelectrodes were backfilled with 4 mM potassium acetate and had an average resistance of 150–200 MΩ. After isolation in room light, the eyecups were dark adapted in the superfusion chamber for >1 hr before recording. The eyecups were superfused continuously at 1.5 ml/min and stimulated with diffuse 200 msec steps of light emitted by a green light-emitting diode (λmax = 567 nm). Light intensity was controlled by a neutral density wedge. Data were stored on digital tape for off-line analysis with SPIKE software ( Modular Instruments, Taunton, MA). Isolated cells were obtained as described above and imaged with borosilicate microelectrodes attached to a motorized manipulator (MP-285; Sutter Instruments). Under these conditions photoreceptors are held at a potential of −55 to −60 mV (Bider and Hille, 1989) (D. Krizaj and D. R. Copenhagen, unpublished observations) that is close to the light-evoked plateau potentials measured in intact rods in vivo (Fain, 1976; Witkovsky et al., 1997). Because we were interested in the modulation of glutamate release, whose magnitude is higher at more depolarized membrane potentials, we usually depolarized the test rod by elevating extracellular \([K^+]_o\). In isolated rods, 20 mM KC1 raised Vm from approximately −60 mV to approximately −40 mV, a value that is close to dark potentials recorded in intact rods.

Glutamate release. The preparation of the reduced retina was performed as described previously (Schmitz and Witkovsky, 1996). Briefly, the cornea, the inner ring of the iris, and the lens were removed, and the eyeball was flushed successively with 0.5% Triton X-100 in distilled water, distilled water alone, and culture medium. After ~1 hr of incubation in culture medium, the retina split apart in the inner nuclear layer, permitting the inner retinal layers to be removed with fine forceps and thereby preserving a laminar sheet of photoreceptor cells. The preservation of physiological function in this preparation is documented in Schmitz and Witkovsky (1996) and Witkovsky et al. (1997).

All experiments were done in room light (40 μW/cm²) in the plane of the retina. The reduced retinas were maintained in aerated chambers (95% O2/5% CO2) and superfused at 1 ml/hr with culture medium containing (in mM): 82 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 35 NaHCO₃, and 1 NaHPO₄, pH 7.55. The basic salt medium was supplemented with 5 mM glucose, 100 μM ascorbic acid, and a mixture of 14 amino acids including 5 μM glutamine. Dihydrokainate (2 mM) was added to all media.
Dilutions were made fresh with final concentrations of DMSO, dissolved directly into the internal pipette solution. 4-Chloro-

1995). The caffeine-evoked release of Ca$^{2+}$ stores is typically observed as a transient increase in [Ca$^{2+}$]$_i$. We observed caffeine-evoked transient [Ca$^{2+}$]$_i$ increases in inner segments and synaptic terminals but not in outer segments of rods. Figure 1A illustrates, in the inner segment of a rod, transient increases of [Ca$^{2+}$]$_i$, produced by brief puffs of caffeine (50 mM in the pipette). In control saline containing 2 mM KCl, [Ca$^{2+}$]$_i$ was elevated transiently by ~50 nM. Caffeine-evoked increases of [Ca$^{2+}$]$_i$, ranged from 20 to 80 nM under these same conditions (n = 10 cells). Responses to repeated puffs of caffeine in calcium-free saline gradually diminished over time but began to increase after a return to control saline (Fig. 1A). These findings are not only indicative of a caffeine-sensitive store in the rods but demonstrate that the stores can be depleted if calcium influx from the extracellular milieu is diminished. The major influx pathway for calcium influx into rods is via L-type calcium channels (Corey et al., 1984; Krizaj and Copenhagen, 1998). In agreement with a requirement for influx through these channels to fill the caffeine-sensitive stores, we found that repeated caffeine puffs in nifedipine, an L-type channel antagonist, caused the transient [Ca$^{2+}$]$_i$ elevations to run down (data not shown) similar to the rundown in 0 Ca$^{2+}$ conditions shown in Figure 1A.

The magnitude of caffeine-evoked [Ca$^{2+}$]$_i$ transients was positively correlated to the basal calcium concentration in the rods. Figure 1B plots the peak amplitude of the caffeine transient versus prestimulus [Ca$^{2+}$]$_i$. This finding agrees with previous studies indicating that the releasable pool of calcium from stores is enhanced in proportion to the amount of calcium in the cytosol (Sitsapesan and Williams, 1990; Hua et al., 1993; Garaschuk et al., 1997).

**RESULTS**

Caffeine is a xanthine that releases [Ca$^{2+}$]$_i$ from intracellular stores by increasing the affinity of the ryanodine receptor for cytoplasmic Ca$^{2+}$. The caffeine-evoked release of Ca$^{2+}$ from intracellular stores is typically observed as a transient increase in [Ca$^{2+}$]$_i$. We observed caffeine-evoked transient [Ca$^{2+}$]$_i$ increases in inner segments and synaptic terminals but not in outer segments of rods. Figure 1A illustrates, in the inner segment of a rod, transient increases of [Ca$^{2+}$]$_i$, produced by brief puffs of caffeine (50 mM in the pipette). In control saline containing 2 mM KCl, [Ca$^{2+}$]$_i$ was elevated transiently by ~50 nM. Caffeine-evoked increases of [Ca$^{2+}$]$_i$, ranged from 20 to 80 nM under these same conditions (n = 10 cells). Responses to repeated puffs of caffeine in calcium-free saline gradually diminished over time but began to increase after a return to control saline (Fig. 1A). These findings are not only indicative of a caffeine-sensitive store in the rods but demonstrate that the stores can be depleted if calcium influx from the extracellular milieu is diminished. The major influx pathway for calcium influx into rods is via L-type calcium channels (Corey et al., 1984; Krizaj and Copenhagen, 1998). In agreement with a requirement for influx through these channels to fill the caffeine-sensitive stores, we found that repeated caffeine puffs in nifedipine, an L-type channel antagonist, caused the transient [Ca$^{2+}$]$_i$ elevations to run down (data not shown) similar to the rundown in 0 Ca$^{2+}$ conditions shown in Figure 1A.

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Caffeine also evokes a prolonged [Ca$^{2+}$]$_i$ depression in rods

At membrane potentials equivalent to the resting potential of rods in darkness (approximately -40 mV), caffeine evoked a prolonged depression in [Ca$^{2+}$]$_i$ after the initial peak (Fig. 2A). Basal [Ca$^{2+}$]$_i$ in isolated cells in 2 mM KCl was 49 ± 3 nM
(mean ± SE; n = 70). We found that 20 mM KCl raised the membrane potential from approximately −55 mV to approximately −40 mV and elevated the resting [Ca^{2+}] to 325 ± 16 mM (n = 54). At 20 mM KCl caffeine elicited a stereotyped two-phase response in [Ca^{2+}]. The first phase was a transient increase in [Ca^{2+}], peaking at ~5–30 sec, whereas the second phase was a prolonged [Ca^{2+}] undershoot. Figure 2A shows an example from an inner segment of a rod in which caffeine was applied in the presence of 20 mM KCl. Here [Ca^{2+}] rose transiently from ~480 to ~1450 mM and then rapidly dropped to a level around 250 nM, well below the precaffeine level. Figure 2B shows an example of a caffeine-evoked increase and subsequent depression in a synaptic terminal of a rod. These results demonstrate that for rods maintained near their dark resting potential in these cells, caffeine evoked a transient rise, followed by a prolonged depression of [Ca^{2+}], in inner segments and synaptic terminals. Caffeine did not evoke a transient peak or depression in rod outer segments.

Higher basal levels of [Ca^{2+}], not only increased the size of the transient peak but increased the speed of the falling phase after the peak and the magnitude of the depression. Figure 3A–C shows a caffeine response recorded from the same rod at [Ca^{2+}], of ~50, ~350, and ~530 mM, respectively. The magnitudes of the transient peaks and the size of the depression were larger at higher basal [Ca^{2+}]. Notably, the fall of calcium after the peak was significantly faster for higher basal [Ca^{2+}]. Single-exponential fits to the falling phase revealed time constants of 97 sec in 2 mM KCl and 42 and 40 sec, respectively, in 30 and 90 mM KCl. The depression below the prestimulus levels and the faster decline at raised [Ca^{2+}], suggest the activation of a second process, subsequent to the transient peak, that actively lowers calcium in the cytosol.

In some types of cell, ryanodine can block caffeine-induced release of Ca^{2+} from intracellular stores (McPherson et al., 1991). However, there is also precedent for caffeine releasing calcium from ryanodine-insensitive stores (Schmid et al., 1990; McNulty and Taylor, 1993; Orkand and Thomas, 1995). We tested the effects of ryanodine on the caffeine-induced rise and depression of [Ca^{2+}], and found that, by itself, ryanodine slightly raised baseline [Ca^{2+}], in 7 of 21 rods. It also irreversibly eliminated caffeine-mediated elevations of [Ca^{2+}], in the great majority of cells (n = 19/21). Ryanodine, however, only reduced and did not completely eliminate the caffeine-induced suppression of [Ca^{2+}]. In the control experiments caffeine suppressed [Ca^{2+}], by 75 ± 5% of prestimulus levels. After exposure to 20–100 μM ryanodine, [Ca^{2+}] was reduced to 51 ± 4% of baseline (n = 12 rods). In Figure 4, the left trace shows the response to caffeine in control saline. The middle and right traces show the caffeine response 12 and 30 min after ryanodine application, respectively. This differential effect of ryanodine on the two phases suggests that the transient elevation may be from a predominantly

![Figure 2](image1.png)

Figure 2. Caffeine also evokes prolonged depression in the inner segments and synaptic terminals of rods when basal [Ca^{2+}], is higher. A, Response in a rod inner segment to a puff of caffeine (50 mM in the pipette) in 20 mM KCl saline. [Ca^{2+}], started at ~480 nm, peaked at ~1450 nm, and dropped to ~250 nm. [Ca^{2+}], recovered to the prestimulus level ~5 min after the puff. B, Response to caffeine measured in the synaptic terminal of a rod. Calibration with ionomycin was not completed in this cell; therefore only the relative ratio of 340/380 nm fluorescence, known to be directly proportional to [Ca^{2+}], is shown.

![Figure 3](image2.png)

Figure 3. Caffeine-induced depression is higher for increased basal calcium. A–C, [Ca^{2+}], responses of a rod inner segment to bath-applied caffeine in 2 mM (A), 30 mM (B), and 90 mM (C) KCl. The magnitudes of the transient peak and depression were positively correlated to basal [Ca^{2+}], before caffeine. The decay from the initial peaks was fitted with single-exponential functions having time constants of 97, 42, and 40 sec.
ryanodine-sensitive store but the depressive action might involve caffeine effects on both ryanodine-sensitive and -insensitive stores. Accordingly, the depression may result from the activation of a pump or transporter that is triggered by the release of calcium from both types of stores (Schmid et al., 1990; Friel and Tsien, 1992; McNulty and Taylor, 1993; Orkand and Thomas, 1995).

In the following experiments we studied the effects of caffeine on synaptic transmission from rods to second-order cells.

**Caffeine hyperpolarizes horizontal cells but not rods**

In dark-adapted amphibian retinas, the membrane potentials of HCs in darkness reflect the activation of non-NMDA glutamate receptors by the tonic release of glutamate from photoreceptors (Krizaj et al., 1994). We tested the effects of caffeine on synaptic transmission by recording from HCs and rods in dark-adapted eyecup preparations of both *Xenopus* and tiger salamander retinas. The waveform and the chromatic sensitivity of HC light-evoked responses indicated that the HCs were driven solely by rods. The membrane potentials of rods in darkness were monitored during exposure to 10 mM caffeine. The initial dark membrane potentials varied from $-239$ to $-245$ mV, and they were essentially unchanged throughout the exposure to caffeine-containing saline (Fig. 5A, open circles). Figure 5B shows that the light-evoked responses of rods were altered little by exposure to 10 mM caffeine ($n = 6$). In HCs, caffeine elicited a brief period of depolarization followed by a more prolonged period of hyperpolarization. The resting potentials of HCs were $-43 \pm 4$ mV. Figure 5A (filled circles) shows that the HC membrane began to
depolarize within 2 min after the switch to caffeine-containing saline. After reaching a mean peak depolarization of 4 mV, the HCs hyperpolarized by 26 ± 2.5 mV over the next 4–5 min. The filled circles (Fig. 5A) show the mean and SE of seven HCs. These data indicate that caffeine has little effect on the rod membrane potential in darkness but strongly modulates the membrane potential of HCs. Given that the depolarization and subsequent hyperpolarization of HCs have a time course similar to the caffeine-induced rise and depression of [Ca$^{2+}$]$_i$ measured in the rods, we hypothesize that caffeine-evoked changes of calcium in the presynaptic neurons modulates the release of neurotransmitter from these cells. Because the membrane potentials of rods are not affected, the data tend to rule out a caffeine-dependent, voltage-induced suppression of transmitter release in rods.

**Caffeine inhibits glutamate release**

We tested directly whether caffeine could modulate endogenous glutamate release from photoreceptors. These experiments were performed using a photoreceptor sheet preparation (Schmitz and Witkovsky, 1996). We approximated the same conditions used for isolated rods (Fig. 2) by suppressing the dark current with light and then adding 20 mM [K$^+$]$_o$ to increase glutamate release. Figure 6 shows that 35 min after a switch from normal to 20 mM KCl, glutamate release stabilized after 35 min in 20 mM KCl, which is when the first measurements were made. Glutamate release was markedly suppressed by caffeine added at $t = 45$ min (to 47% of control; black bars). Each bar represents the glutamate content of 5 min samples of the perfusate. The effect of caffeine was reversible; during the washout (at $t = 65$ min) a consistent rebound increase in glutamate release was observed ($n = 15$).

**Caffeine inhibits calcium current in isolated rods**

We tested that hypothesis. Figure 8 illustrates an I–V curve for an L-type calcium current recorded from a rod in whole-cell mode. The following experiments tested that hypothesis. Figure 8 illustrates an I–V curve for an L-type calcium current recorded from a rod in whole-cell mode. The data presented so far suggest that caffeine affects exocytosis by modulating Ca$^{2+}$ influx through the voltage-dependent Ca$^{2+}$ channels in rods. The following experiments tested that hypothesis. Figure 8 illustrates an I–V curve for an L-type calcium current recorded from a rod in whole-cell mode. The current, $I_{Ca}$, peaked at $-0$ mV (Fig. 8A) and showed little inactivation during 120 msec voltage steps when the pipette solution contained 10 mM EGTA (see Fig. 8A, inset). $I_{Ca}$ was completely blocked by Cd$^{2+}$ (100 μM; $n = 26$; data not shown). Caffeine (5 and 10 mM) reversibly suppressed peak $I_{Ca}$ by 35.4 ± 3.1 and 50.0 ± 2.7%, respectively ($n = 29$; Fig. 8A,B). Caffeine had no detectable effect on the voltage range over which $I_{Ca}$ was activated. Often during caffeine washout, a rebound increase in Ca$^{2+}$ currents was observed (Fig. 8B, arrow), with the Ca$^{2+}$...
The amplitude of the idea that by 10 mM Ba2+ receptors (Corey et al., 1984; Tsien et al., 1988), several features of BAPTA-loaded cells showed faster kinetics of binding (Tsien, 1980). In these BAPTA-loaded cells, the inhibitory effect of caffeine on the peak amplitude (at +10 mV) but did not affect the activation voltage. Note the rebound increase in ICa after washout of caffeine. B, Time course of caffeine action on ICa is shown. The cell was held at −70 mV and stepped for 120 msec to 0 mV. Test pulses were applied every 20 sec. Sequential application of 10 mM caffeine reversibly reduced ICa. After the first washout, ICa rebounded to levels above control.

Figure 8. Caffeine reduces ICa in rods. Calcium current was recorded with whole-cell patch pipettes. A, Calcium currents were evoked with voltage-clamp ramps from −70 to +50 mV and with voltage-clamp steps (inset, −70 to +50 mV in 10 mV increments). Caffeine (CAF; 10 mM) reduced the peak amplitude (at −10 mV) but did not affect the activation voltage. Note the rebound increase in ICa after washout of caffeine. B, Time course of caffeine action on ICa is shown. The cell was held at −70 mV and stepped for 120 msec to 0 mV. Test pulses were applied every 20 sec. Sequential application of 10 mM caffeine reversibly reduced ICa. After the first washout, ICa rebounded to levels above control.

current returning to its precaffeine level after a 4–6 min wash (Fig. 8A,B).

The predominant action of caffeine on ICa in rods requires changes in [Ca2+].

The suppression of ICa by caffeine could result from either a direct caffeine action on the Ca2+ channel or an indirect one mediated by release of Ca2+ from intracellular stores (Pacaud et al., 1987; Kramer et al., 1994; Adachi-Akahane et al., 1996) or both. The indirect action of caffeine should be reduced or eliminated by blockers of Ca2+ release from stores. Figure 9A shows that in the presence of ryanodine the inhibitory action of 10 mM caffeine on peak ICa was reduced by approximately one-half. In ryanodine the mean reduction of peak current was 25.7 ± 5.8% (n = 8) versus 50.0 ± 2.7% in control. These data indicate that more than one-half of the caffeine-induced suppression of ICa results from release from ryanodine-sensitive stores.

The inhibitory effect of caffeine on ICa was much more pronounced in the presence of intrapipette EGTA compared with BAPTA, a Ca2+ chelator with a higher affinity for Ca2+ and faster kinetics of binding (Tsien, 1980). In the rod shown in Figure 9B, intracellular BAPTA blocked virtually all the caffeine-induced suppression of ICa. In summary, we found that when rods were loaded with 10–20 mM BAPTA, the average peak amplitude of ICa was significantly larger than that in controls (186.6 ± 74.5 pA; n = 5). This larger current is consistent with the idea that ICa, when tonically inhibited by cytosolic calcium. In these BAPTA-loaded cells, the inhibitory effect of caffeine on Ca2+ current was substantially reduced. Caffeine reduced ICa by 20.3 ± 7.7%. The difference between control and caffeine’s action was not significant statistically. These experiments demonstrate that a major proportion of caffeine’s suppression of ICa can be attributed to effects of caffeine on intracellular calcium.

Further indication that Ca2+ released by caffeine acts to inhibit ICa was provided by experiments in which [Ca2+]o was replaced by 10 mM [Ba2+]o. Although Ba2+ currents are well supported by neuronal L-type calcium channels, including those of photoreceptors (Corey et al., 1984; Tsien et al., 1988), several features of cellular Ba2+ regulation differ from the regulation of Ca2+. Ba2+, for example, is not recognized by SERCA’s and is thus not sequestered into intracellular stores (Schilling et al., 1989; Kwan and Putney, 1990). Moreover, in contrast to Ca2+, cytoplasmic Ba2+ ions do not inactivate calcium channels (Haack and Rosenberg, 1994) and may inhibit release of Ca2+ through ryanodine receptor-gated channels, thus allowing one to differentiate between direct caffeine effects on the conductance or open probability of the L-type calcium channel and its indirect effects on the channel via Ca2+ released from intracellular stores. In rod photoreceptors, peak ICa elicited by a depolarizing voltage ramp was 98.7 ± 13.1 pA. After a switch of the superfusate from [Ca2+]o, to [Ba2+]o, peak ICa increased to 300.8 ± 39.8 pA. Caffeine inhibited the Ba2+ current by 27.6 ± 6.6% (n = 8; data not shown) compared with 50 ± 2.7% in the Ca2+-containing saline. This result suggests that the upper bound for caffeine-mediated inhibition of ICa by direct block of the channels is 55%. In these experiments, if there were residual calcium in the stores after the change to Ba2+, this calcium could have been released by caffeine to inhibit the channels via the indirect pathway (Przywara et al., 1993), resulting in an overestimate of caffeine’s direct effect on the channels.

As an alternative method to test whether calcium influx via ICa was suppressed in caffeine, we measured [Ca2+]i, with fura-2 while recording under voltage clamp with perforated-patch recording pipettes. We found that a depolarization-induced influx of Ca2+ was evident in the presence of caffeine. Figure 9C illustrates that [Ca2+]i, decreased when the membrane potential was lowered from −30 to −65 mV, a reflection of deactivation of ICa. After the caffeine-evoked transient increase in [Ca2+]i, the membrane potential was switched back to −30 mV, [Ca2+]i, rose to a slightly higher level than the precaffeine level at −30 mV. These data illustrate that depolarization-induced calcium influx is not suppressed by caffeine, indicating that caffeine did not significantly block the L-type channels directly. These data, taken as a whole, indicate that a large proportion of caffeine’s effect is a result of caffeine acting inside the rods, presumably by releasing...
calcium from stores. Experiments discussed below add support to the idea that it is calcium release from stores that suppresses 

\[ \text{Ca}^{2+} \]


Chlorocresol, a nonxanthine store-releasing compound, and spontaneous calcium spikes produce a depression in \[ \text{Ca}^{2+} \].

Xanthines, such as caffeine, inhibit phosphodiesterases (PDEs) in addition to releasing calcium from stores (Nehlig et al., 1992). To test whether the caffeine-induced changes in \[ \text{Ca}^{2+} \] were separable from any inhibition of PDEs, we used 4-CmC, a nonxanthine compound that releases calcium from stores (Zorzato et al., 1993; Cseresnyes et al., 1997). Figure 10 shows the action of 4-CmC on \[ \text{Ca}^{2+} \], in a rod. 4-CmC elevated \[ \text{Ca}^{2+} \] transiently and evoked a prolonged decrease, similar to the undershoot elicited by caffeine (\( n = 13 \)). 4-CmC was shown to release \[ \text{Ca}^{2+} \] from the stores at a much slower rate than caffeine (Cseresnyes et al., 1997); hence the transient rise in calcium might be expected to be smaller than that in caffeine. Similar depressions in \[ \text{Ca}^{2+} \] were observed in thymol, another nonxanthine compound known to release \[ \text{Ca}^{2+} \] from stores (\( n = 4 \); data not shown).

Spikes of \[ \text{Ca}^{2+} \], observed in neuronal and non-neuronal cells primarily result from the spontaneous release of calcium from ryosoridine-sensitive stores (Berridge, 1998). We observed that spontaneous spikes in rod \[ \text{Ca}^{2+} \], often were followed by a depression of \[ \text{Ca}^{2+} \] below the resting level. Figure 10B illustrates caffeine-evoked increases and depression of \[ \text{Ca}^{2+} \], recorded from two rods simultaneously. In between caffeine applications, a spontaneous increase in \[ \text{Ca}^{2+} \], termed a calcium spike, occurred in one of the rods but not in the other. The increase (Fig. 10B, thick open arrow) was followed by an undershoot (thin filled arrow). The existence of the depressive phases after a spike suggests that even a noncaffeine-mediated release can cause a depression of \[ \text{Ca}^{2+} \], supporting the hypothesis that it is the transient release of calcium that triggers the prolonged decrease in intracellular calcium concentration.

As a further test of a possible role for caffeine-evoked calcium release in the depression of \[ \text{Ca}^{2+} \], we used fura-2 to monitor intracellular \[ \text{Ba}^{2+} \] (Schilling et al., 1989). As mentioned above, because barium is sequestered poorly into stores (Kwan and Putney, 1990; Adachi-Akahane et al., 1996), the effects of caffeine on \[ \text{Ba}^{2+} \] provide a measure of how caffeine affects influx through the calcium channels. Figure 11 shows that replacement of extracellular \[ \text{Ca}^{2+} \] by \[ \text{Ba}^{2+} \] increased fura-2 ratios. This increase reflects the higher permeability of \( I_{\text{Ca}} \) to barium. However, \[ \text{Ba}^{2+} \] substitution for \[ \text{Ca}^{2+} \] resulted in a block of the caffeine-evoked transient increase and a significant decrease of \[ \text{Ba}^{2+} + \text{Ca}^{2+} \], depression (16 ± 4% relative to the baseline in 2 mm \[ \text{Ba}^{2+} \], compared with control 82 ± 3% relative to the baseline in 2 mm \[ \text{Ca}^{2+} \]; \( n = 6 \)). This blockage of the initial transient increase is consistent with a diminished release of \[ \text{Ca}^{2+} \] from the stores. The small degree of caffeine-induced depression in barium indicates that direct effects of caffeine on \( I_{\text{Ca}} \) were <19% (=16/82) of the depression mediated by calcium released from the stores.

**DISCUSSION**

We report here that caffeine transiently raises and then depresses \[ \text{Ca}^{2+} \] in rod photoreceptors. Exposure to caffeine also reduces the L-type calcium current. These presynaptic events are paralleled by a transient depolarization and subsequent hyperpolarization of the membrane potential of a second-order retinal neuron, the horizontal cell. Release of endogenous glutamate from rods is also depressed by caffeine. The poor temporal resolution of our glutamate measurements did not allow us to observe a transient increase in release. Our data provide novel evidence that intracellular calcium stores play a role in mediating glutamate release. The main question raised by our findings is the mechanism(s) by which caffeine modulates \[ \text{Ca}^{2+} \] and the rate of transmitter release. Figure 12 schematizes the probable mechanisms. These are discussed in detail below.
Caffeine-evoked transient $[Ca^{2+}]_i$ increases reflect discharge of calcium from ryanodine-sensitive stores

The caffeine-mediated transient increase in $[Ca^{2+}]_i$ observed in rods has been reported in a multitude of other cell types and reflects immediate calcium release from the stores (Friel and Tsien, 1992; Orkand and Thomas, 1995; Garaschuk et al., 1997). The observation that the magnitude of the transient depended on the basal level of $[Ca^{2+}]_i$ (Fig. 1B) is consistent with greater amounts of calcium being pumped into the caffeine-sensitive stores by the constitutively active SERCA pumps. Supporting evidence of store release comes from our finding that the transient increase in $[Ca^{2+}]_i$ is substantially reduced when calcium is replaced with barium (Fig. 11), a divalent cation that is poorly pumped into stores (Kwan and Putney, 1990). In addition, ryanodine blocked the transient increases of $[Ca^{2+}]_i$, suggesting that caffeine acts on an intracellular store expressing a conventional class of ryanodine receptors (Pozzan et al., 1994; Berridge, 1998). It is noteworthy that ryanodine receptors have been reported recently in photoreceptor synaptic terminals (Gabriel et al., 1998). Caffeine-sensitive stores in rods may coexist with the other major class of calcium stores, gated by the inositol trisphosphate receptor (Peng et al., 1991) (Krizaj and Copenhagen, unpublished observations). $Ca^{2+}$ release from these stores is inhibited by caffeine (Ehrlich et al., 1994) and thus is unlikely to contribute to caffeine-mediated changes in rod $[Ca^{2+}]_i$.

Caffeine-evoked calcium release contributes significantly to the reduction of $I_{Ca}$ and the depression of $[Ca^{2+}]_i$

We show that caffeine-induced calcium release suppresses $I_{Ca}$ and depresses $[Ca^{2+}]_i$. We propose that a portion of the depression of $[Ca^{2+}]_i$ can be attributed to the reduction of calcium entry via $I_{Ca}$; however there must be additional mechanisms by which $[Ca^{2+}]_i$ is depressed. First we will discuss the suppression of $I_{Ca}$ by caffeine. Intracellular BAPTA, ryanodine, and the substitution of calcium by barium significantly reduced the suppressive action of caffeine on $I_{Ca}$ (Fig. 9). These experiments can be taken as strong evidence that an intracellular calcium-dependent mechanism, triggered by caffeine, leads to suppression of $I_{Ca}$. A smaller component of caffeine’s action on $I_{Ca}$ could be attributed to a direct effect in the channels (Hughes et al., 1990). Mechanistically, $I_{Ca}$ could be inactivated by a localized and restricted rise in caffeine-released calcium near the channel (Adachi-Akahane et al., 1996; Sham, 1997) or by a release-triggered activation of a second messenger cascade. In consideration of the first mechanism, L-type voltage-gated calcium channels are inhibited by
The caffeine-evoked depression of $[\text{Ca}^{2+}]_i$ is also unlikely to result from an inhibitory action of caffeine on a phosphodiesterase that reduces intracellular cyclic nucleotides. We found that exposure to caffeine did not markedly affect either the membrane potential or the light responses of dark-adapted rods. This strongly suggests that the cGMP-dependent phosphodiesterases in rod outer segments were not inhibited significantly by 10 mM caffeine. Furthermore, we found that 100 $\mu$M IBMX, a dose that should block PDEs, did not affect release of $\text{Ca}^{2+}$ from caffeine-sensitive channels (Capovilla et al., 1983; Cer-qetto and McNaughton, 1986; Cseresnyes et al., 1997) and had no effect on the steady-state $[\text{Ca}^{2+}]_i$, in rods ($n = 6$). It should be noted that our findings contrast to a previous report by Capovilla et al. (1983) in which 0.3 mM caffeine increased the amplitude of rod light responses in toad. Finally, the experiments with 4-CmC (0.5 mM) and thymol (0.1 mM), two nonxanthine compounds not known to interfere with phosphodiesterases, mimicked caffeine by generating a depression of $[\text{Ca}^{2+}]_i$.

Voltage-clamp and microelectrode recordings eliminate the possibility that caffeine depressed $[\text{Ca}^{2+}]_i$, by activating $\text{Ca}^{2+}$-dependent conductances that hyperpolarized the rods to decrease the activation of the L-type calcium channels and hence calcium influx (Akaike et al., 1983; Sah and Mclachlan, 1991; Marrion and Adams, 1992; Sridhar et al., 1996). Salamander rods possess calcium-activated chloride and potassium conductances (Bader et al., 1982). Although these conductances may have increased after caffeine-mediated release of $[\text{Ca}^{2+}]_i$ from the stores, they could not have contributed significantly to the reduction in $I_{\text{Ca}}$, that persisted under voltage clamp (Fig. 8). Moreover, intracellular recording from rods showed no effect of caffeine on rod membrane potential, whereas both glutamate release and horizontal cell membrane potential were markedly depressed under those conditions (Figs. 5, 6). This indicates that a change in the rod membrane potential is not likely to be responsible for generating the depression.

Yet another explanation might be proposed that caffeine suppresses the $P_3$ receptor, the inner segment ER (Peng et al., 1991; Ehrlich et al., 1994) and thus inhibits a possible depolarization-activated tonic $P_3$-gated release of $\text{Ca}^{2+}$ (Gan and Iuvone, 1997). However, an effect similar to that of caffeine was obtained with nonxanthine compounds, such as 4-CmC, that are not thought to inhibit $P_3$ receptors.

**A release-activated stimulation of calcium uptake or extrusion likely contributes to caffeine-evoked depression of $[\text{Ca}^{2+}]_i$**

It has been postulated that caffeine-induced calcium release from stores stimulates calcium uptake into ER or into mitochondria (Friel and Tsien, 1992; Orkand and Thomas, 1995; Cseresnyes et al., 1997; Lara et al., 1997; Golovina and Blaustein, 1998). Our finding that 1 mM caffeine slightly potentiated glutamate release whereas 10 mM caffeine inhibited it is similar to a previous report on the effect of caffeine on $[\text{Ca}^{2+}]_i$, in sympathetic neurons (Friel and Tsien, 1992). Friel and Tsien were the first to propose that “depending on its $\text{Ca}^{2+}$ content, the caffeine-sensitive store can either attenuate or potentiate responses to depolarization.” Recently, Cseresnyes et al. (1997) reported that in sympathetic neurons caffeine greatly potentiates calcium removal from the cytosol. After being triggered by caffeine-induced release, this mechanism continues removing calcium from the cytosol, resulting in a substantial lowering of $[\text{Ca}^{2+}]_i$. Our findings in rods that caffeine evokes a depression of $[\text{Ca}^{2+}]_i$, to values well below...
prestimulus levels and that the falling phase of the caffeine-evoked Ca\textsuperscript{2+} signal is faster (Fig. 3) are consistent with release-stimulated sequestration or extrusion.

**Why did ryanodine not completely eliminate the decreases in [Ca\textsuperscript{2+}], and glutamate release?**

In isolated cells, ryanodine eliminated caffeine-induced increases in [Ca\textsuperscript{2+}], consistent with its action as a blocker of the ryanodine receptor. Ryanodine also reduced the caffeine-mediated decrease in [Ca\textsuperscript{2+}], and I_{Ca}, suggesting that at least part of the undershoot in [Ca\textsuperscript{2+}], and glutamate release is mediated by Ca\textsuperscript{2+} release from ryanodine-gated channels. The finding that ryanodine did not completely block the [Ca\textsuperscript{2+}], undershoot suggests that at least part of the Ca\textsuperscript{2+} release, activated by caffeine, may be from another, ryanodine-insensitive, class of calcium stores, similar to those described in several other cell types (Marrion and Adams, 1992; McNulty and Taylor, 1993; Orkand and Thomas, 1995; Pessah et al., 1997). This release may be missed by the global fura-2 measurements yet significant enough to evoke a depression in [Ca\textsuperscript{2+}], (e.g., Fig. 4). Surprisingly, ryanodine had no effect on the caffeine-mediated reduction of glutamate release. Possibly this result was a consequence of poor tissue permeability of ryanodine, as has been hypothesized for tissue slices (Llano et al., 1994), than in the light, when inner segment [Ca\textsuperscript{2+}], and Thomas, 1995; Cseresnyes et al., 1997; Lara et al., 1997). Thus, the compounded action of Ca\textsuperscript{2+}-induced inactiva-

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**The role of calcium stores in the regulation of transmitter release from rods**

Our results demonstrate that intracellular stores in rods are capable of storing significant amounts of calcium. This finding complements the cytochemical and EM studies that localized the highest concentrations of calcium in amphibian and teleost rods to the inner segment ER (Mercerio and Holtzman, 1982; Ungar et al., 1984; Somlyo and Walz, 1985). Our finding that the pool size of caffeine-releasable intracellular calcium depends on the basal levels of [Ca\textsuperscript{2+}], suggests that the participation of calcium stores in synaptic signaling will be more significant in darkness, when [Ca\textsuperscript{2+}], is high (Ratto et al., 1988; Gray-Keller and Detwiler, 1994), than in the light, when inner segment [Ca\textsuperscript{2+}], drops to levels as low as 20–50 nM (Krizaj and Copenhagen, 1998). In vivo, calcium is released from ryanodine-sensitive stores by cytosolic calcium, by a process termed calcium-induced calcium release. The concentration range over which calcium activates the ryanodine receptor, an approximately sigmoidal activation by Ca\textsuperscript{2+} at concentrations between 10 nM and 10 \mu M (Bezprozvanny et al., 1991; Hernandez-Cruz et al., 1995), indicates that calcium stores in rod inner segments and synaptic terminals could be activated in both dark and light.

In darkness, steady influx of Ca\textsuperscript{2+} through the L-type Ca\textsuperscript{2+} channels (Rieke and Schwartz, 1996) would trigger Ca\textsuperscript{2+} release from the stores. The high density of ryanodine receptors, the long open times and high single-channel conductances of ryanodine receptor-gated channels [100 to ~400 pS (Bezprozvanny et al., 1991; Hernandez-Cruz et al., 1995)], and the close proximity of Ca\textsuperscript{2+} stores to the plasma membrane (Sridhar et al., 1996; Sham, 1997) would create a local negative feedback loop. Ca\textsuperscript{2+} released from the stores would inactivate the L-type channels and thus reduce calcium influx. The resulting depression of [Ca\textsuperscript{2+}], is accompanied by facilitation of Ca\textsuperscript{2+} uptake into the stores (Orkand and Thomas, 1995; Cseresnyes et al., 1997; Lara et al., 1997). Thus, the compounded action of Ca\textsuperscript{2+}-induced inactiva-


