Molecular Vision **1999; 5:1** <http://www.molvis.org/molvis/v5/p1>
Received 20 November 1998 | Accepted 1 February 1999 | Published 10 February 1999

Intracellular Ca^{2+} concentrations in cultured chicken photoreceptor cells: Sustained elevation in depolarized cells and the role of dihydropyridine-sensitive Ca^{2+} channels

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Purpose: Retinal photoreceptor cells are tonically depolarized in darkness. Ca^{2+} influx in darkness plays a critical role in the regulation of neurotransmitter release and melatonin synthesis in these sensory cells. The purpose of the present study was to examine the dynamic changes of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_{in}$) in response to a tonic depolarizing stimulus and to determine the role of dihydropyridine-sensitive calcium channels in the response.
Methods: Photoreceptor cells were prepared from embryonic chick retina and cultured for 6-12 days. Cells were depolarized by exposure to 35 mM extracellular K^+. $[Ca^{2+}]_{in}$ of individual photoreceptor cell bodies/synaptic terminals was determined by ratiometric fura-2 image analysis.
Results: Chemical depolarization with 35 mM $[K^+]_{out}$ greatly increased $[Ca^{2+}]_{in}$ of inner segment/synaptic terminal regions of photoreceptors. The increase usually reached a plateau after the first few minutes of stimulation and was sustained for prolonged periods (>2 h) in the presence of high K^+. When the extracellular K^+ concentration was reduced, the $[Ca^{2+}]_{in}$ rapidly returned to the basal level. Substitution of 1 mM $CoCl_2$ for $CaCl_2$ in the superfusion medium rapidly and reversibly reduced the $[Ca^{2+}]_{in}$ of depolarized photoreceptor cells. Antagonists of L-type Ca^{2+} channels, nitrendipine and nifedipine, inhibited the K^+-evoked increase of $[Ca^{2+}]_{in}$. Bay K 8644, a dihydropyridine Ca^{2+} channel agonist, potentiated the increase of $[Ca^{2+}]_{in}$ elicited by high K^+. In some cells, Bay K 8644 alone increased $[Ca^{2+}]_{in}$ under basal conditions.
Conclusions: The increase of $[Ca^{2+}]_{in}$ elicited by depolarization with 35 mM extracellular K^+ is due to influx of calcium through the dihydropyridine-sensitive voltage-gated channels. Intracellular $[Ca^{2+}]$ remains elevated for extended periods of time during tonic depolarization. This sustained response requires continuous Ca^{2+} channel activity.

The plasma membrane of vertebrate retinal photoreceptor cells is relatively depolarized in darkness, and hyperpolarizes in a graded fashion in response to light of increasing intensity [1,2]. Inner segments and synaptic terminals of photoreceptor cells have non-inactivating, voltage-gated Ca^{2+} channels [3-6]. These channels open upon depolarization and mediate the Ca^{2+} influx required for release of the synaptic neurotransmitter, glutamate [7], and the synthesis of a retinal neuromodulator, melatonin [8]. Calcium influx through voltage-gated channels also regulates cyclic AMP formation [9] and inositol phosphate accumulation [10] in retinal photoreceptor cells and may mediate a variety of effects on photoreceptor cell metabolism and gene expression.

The activity of serotonin *N*-acetyltranferase (arylalkylamine *N*-acetyltransferase), a key regulatory enzyme in the biosynthesis of melatonin [11-13], requires sustained Ca^{2+} influx in retinal photoreceptor cells. Serotonin *N*-acetyltransferase activity decreases rapidly when calcium channels are closed by light exposure or by pharmacologically inhibiting channel activity [14-17]. Ca^{2+}influx appears to be sustained through most of the night, the period of active melatonin synthesis [11].

In order to understand the mechanisms that underlie Ca^{2+}-dependent regulation of melatonin synthesis and, possibly, glutamate release in photoreceptor cells, we have examined the dynamic changes of $[Ca^{2+}]_{in}$ elicited by tonic depolarization in individual cultured chicken photoreceptor cells. In this study we describe a multi-phasic response that involves continuous calcium influx through voltage-gated, dihydropyridine-sensitive channels leading to a large, sustained increase of $[Ca^{2+}]_{in}$ of the photoreceptor inner segment/cell body and synaptic terminal that lasts for as long as the cell remains depolarized.

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METHODS

Cell cultures: Cultures of retinal photoreceptor cells were prepared from embryonic day 6 chick (*Gallus domesticus*) retina as described by Adler et al. [18], with minor modifications [14,19]. Cells were seeded into 35 mm glass bottom microwell dishes (MatTec Co, Ashland, MA, USA) precoated with polyornithine, at an initial density of 1.5 x 10^6 cells per dish. Cells were cultured in 3 ml of medium 199 supplemented with 10% fetal bovine serum, linoleic acid-BSA (110 µg/ml), 2 mM glutamine, and penicillin G (100 U/ml) for 6 to 12 days at 37 °C under a humidified atmosphere of 5-6% CO_2 in air.

Intracellular calcium measurement: Estimates of intracellular free Ca^{2+} concentrations were obtained by fura-2 image analysis of individual cells. On the day of an experiment, a stock solution of fura-2AM (Molecular Probes, Eugene, OR, USA; 0.67 mM), prepared in DMSO containing 6.7% pluronic F-127, was added to the culture medium to give a final concentration of 2 µM fura-2AM. Cells were incubated

at 37 °C for 60 min to accumulate fura-2AM in the cytoplasm, washed with basal salt solution (BSS; 125 mM NaCl; 3.6 mM KCl; 1.1 mM $CaCl_2$; 1.2 mM $MgCl_2$; 10 mM D-Glucose; 25 mM Tris/HCl at pH 7.2), and post-incubated in BSS at room temperature to allow the hydrolysis of cytoplasmic fura-2AM. All experiments were performed at room temperature (~22 °C). The culture dish was mounted on the stage of a Nikon TMS-F microscope. Cells were exposed to high K^+ and drugs by one of two methods. In Protocol I, the Ca^{2+} signals of the cells were first recorded in under static conditions in 2 ml of BSS. After a stable baseline was recorded for 3-5 min, 0.667 ml of modified BBS (129.2 mM KCl; 0 mM NaCl) was added to bring the final K^+ concentration to 35 mM while continuing to record. In Protocol II, cells were continuously superfused at a rate of 0.5 ml/min with a culture well volume of ~0.2 ml. The solution in the well was exchanged within ~1 min. Cells were initially superfused with BSS followed by stimulation with a salt solution containing 35 mM K^+ (93.6 mM NaCl; 35 mM KCl; 1.1 mM $CaCl_2$; 1.2 mM $MgCl_2$; 10 mM D-Glucose; 25 mM Tris/HCl at pH 7.2). Following acquisition of baseline and control stimulation data, cells were exposed to various drugs, which were added to the superfusion solutions 10 min before and during stimulation with 35 mM KCl. The KCl stimulation lasted for 6 min, unless noted otherwise. The difference between the averaged basal level of $[Ca^{2+}]_{in}$ for 3 min before the control KCl stimulation and the averaged level of the last 3 min of each KCl stimulation period was defined as the response to depolarization. The effects of drugs were assessed by determining the ratio of the response in the presence of test compounds to the control (first) response. The recovery from the drug's effect was checked 10 min after removal of the test compound from the superfusate.

Excitation light was provided by a 300 W xenon lamp (ORC, Azusa, CA, USA). Excitation wavelengths of 340 nm and 380 nm were applied for 0.3-0.5 s, and fluorescence emitted by fura-2 was collected by a Nikon Fluor 40 Ph 3DL objective and detected with a Cohu 4915 CCD camera (San Diego, CA, USA). Four signals at each wavelength were aver-

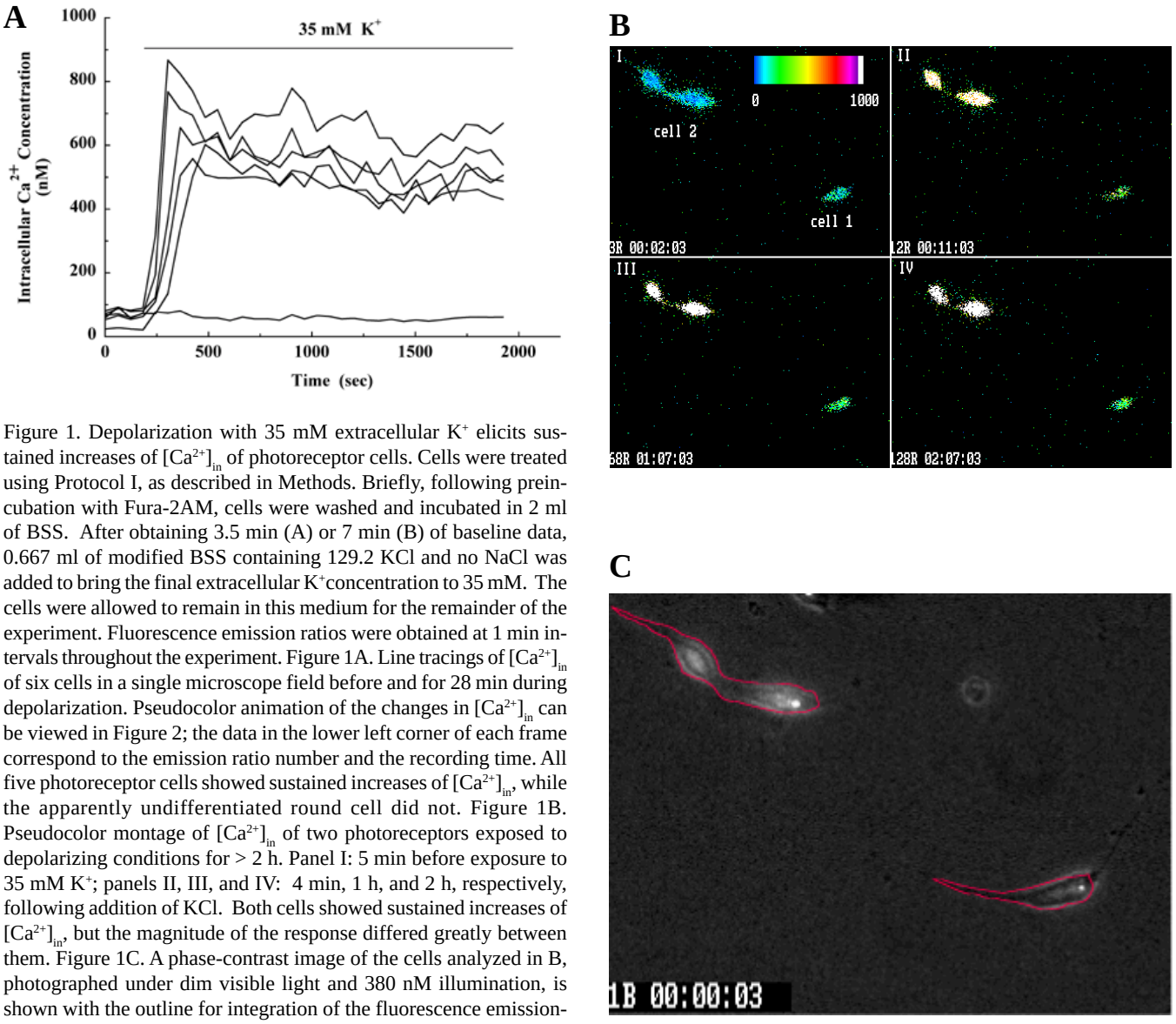

Figure 1. Depolarization with 35 mM extracellular K^+ elicits sustained increases of $[Ca^{2+}]_{in}$ of photoreceptor cells. Cells were treated using Protocol I, as described in Methods. Briefly, following preincubation with Fura-2AM, cells were washed and incubated in 2 ml of BSS. After obtaining 3.5 min (A) or 7 min (B) of baseline data, 0.667 ml of modified BSS containing 129.2 KCl and no NaCl was added to bring the final extracellular K^+concentration to 35 mM. The cells were allowed to remain in this medium for the remainder of the experiment. Fluorescence emission ratios were obtained at 1 min intervals throughout the experiment. Figure 1A. Line tracings of $[Ca^{2+}]_{in}$ of six cells in a single microscope field before and for 28 min during depolarization. Pseudocolor animation of the changes in $[Ca^{2+}]_{in}$ can be viewed in Figure 2; the data in the lower left corner of each frame correspond to the emission ratio number and the recording time. All five photoreceptor cells showed sustained increases of $[Ca^{2+}]_{in}$, while the apparently undifferentiated round cell did not. Figure 1B. Pseudocolor montage of $[Ca^{2+}]_{in}$ of two photoreceptors exposed to depolarizing conditions for > 2 h. Panel I: 5 min before exposure to 35 mM K^+; panels II, III, and IV: 4 min, 1 h, and 2 h, respectively, following addition of KCl. Both cells showed sustained increases of $[Ca^{2+}]_{in}$, but the magnitude of the response differed greatly between them. Figure 1C. A phase-contrast image of the cells analyzed in B, photographed under dim visible light and 380 nM illumination, is shown with the outline for integration of the fluorescence emission-ratio signal.

aged to calculate an emission ratio. Unless otherwise noted, emission ratios were sampled once every 10 s. For each experiment, a standard curve (0 - 1,350 nM) was constructed using Calcium Calibration Buffer Kit #2 and Fura-2 pentapotassium (Molecular Probes, Eugene, OR, USA), which was used for estimation of calcium concentrations. The concentrations reported represent averages of the digitized signals of the area of the cell outlined with an image analysis program, which included all visible structures from the connecting cilium to the synaptic terminal; putative outer segments and filopodial extensions from the cilium were not included. A typical outline of the cell area analyzed is shown in Figure 1C. The majority of the signal was derived from the inner segment/cell soma. Analysis of the video signals and filter-changer control were carried out using Inca++ and InCyt Im2 software (Intracellular Imaging Inc., Cinncinati, OH, USA).

Identification of photoreceptor cells in culture: Cultured embryonic photoreceptor cells were identified by morphological criteria established previously [18], including: (a) a highly polarized cell body; (b) a single, short, usually unbranched neurite; and (c) a refractile lipid droplet characteristic of chick cones. Cells having these morphological characteristics express immunoreactivity for visual pigments and binding sites for peanut lectin [6,18,20], which labels cone photoreceptors in chicken retina [21].

Data analysis: Data are expressed as mean ± standard error of the mean (s.e.m.), and were analyzed for statistical significance by one-way analysis of variance (ANOVA) or one-way repeated ANOVA. Pair-wise comparisons of groups following one-way ANOVA were made with the Student-Newman-Keuls test; pair-wise comparisons following repeated measures ANOVA were made with the Tukey test.

Materials: Poly-L-ornithine (MW 30,000-70,000), linoleic acid/bovine serum albumin, and L-glutamine were purchased from Sigma Chemical Co. (St.Louis, MO, USA); medium 199, penicillin G, and trypsin were from GIBCO (Grand Island, NY, USA); defined fetal bovine serum was from Hyclone (Logan, UT, USA) or Atlanta Biologicals (Atlanta, GA, USA); nitrendipine, nifedipine, and Bay K 8644 were from Research Biochemicals Inc. (Natick, MA, USA). Fura-2AM, Fura-2 pentapotassium, pluronic F127, and Calcium Calibration Kit 2 were from Molecular Probes Inc. (Eugene, OR, USA).

RESULTS

Application of a depolarizing concentration of extracellular K^+(35 mM $[K^+]_{out}$) induced a large, persistent increase of $[Ca^{2+}]_{in}$ of photoreceptor cells. Figure 1A shows the response to 35 mM K^+ of 5 photoreceptors and one apparently undifferentiated round cell. A pseudocolor animation of the cells is presented in Figure 2. Depolarization with 35 mM K^+ resulted in sustained (>25 min) increases of $[Ca^{2+}]_{in}$ of all 5 photoreceptors, but not in the round cell. In the photoreceptors, $[Ca^{2+}]_{in}$ increased from below 100 nM under basal conditions to ~500 nM in the presence of 35 mM K^+. In some cells, the changes of $[Ca^{2+}]_{in}$ did not appear to occur simultaneously in both ends of the cell body, but no consistent pattern was discernable. The magnitude of the $[Ca^{2+}]_{in}$ response to depolarization was similar among these 5 photoreceptor cells, but this was not

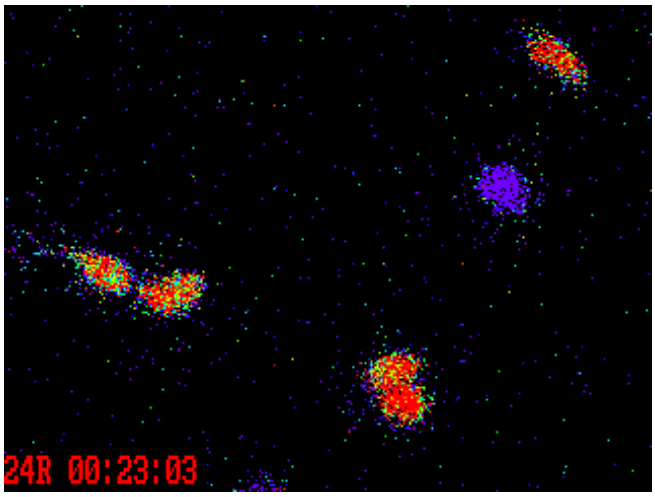

Figure 2. A pseudocolor animation of the responses of five photoreceptor cells to tonic depolarization with 35 mM K^+. After obtaining 3.5 min of baseline data in the presence of 3.6 mM K^+, the concentration of K^+ was increased to 35 mM, as described in the legend to Figure 1A. Emission-ratio images were acquired at 1 min intervals. The ratio number and time are displayed in the lower left corner of each image frame. Note that there is a quicktime movie of this figure in the online version at the following URL: http://www.molvis.org/molvis/v5/p1/uchida-fig2.html

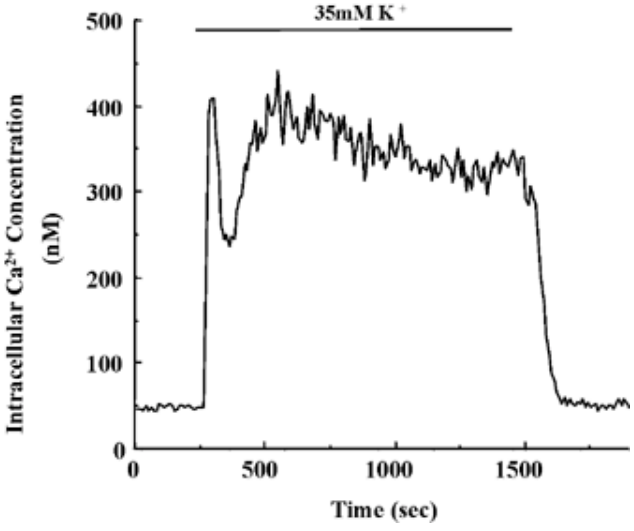

Figure 3. Effect of a 20 min pulse of 35 mM KCl on $[Ca^{2+}]_{in}$ of a superfused photoreceptor cell. Cells were continuously superfused with BSS (0-4 min; 24-31 min) or modified BSS containing 35 mM K^+ (4-24 min), as described in Methods. Fluorescence emission ratios were obtained at 10 s intervals throughout the experiment. Depolarization induced a large increase of $[Ca^{2+}]_{in}$ and this increase remained for as long as the elevated $[K^+]_{out}$ was applied. The $[Ca^{2+}]_{in}$ of the cell rapidly returned to basal levels when the high K^+ BSS was washed out of the superfusion well. This type of response is representative of that recorded from 3 cells.

always observed. Figure 1B shows an example of 2 photoreceptor cells that were recorded for > 2 h, in which $[Ca^{2+}]_{in}$ remained elevated during this extended period of depolarization. One of the cells (cell 1), which had an oval-shaped cell body, showed a moderate but sustained increase of $[Ca^{2+}]_{in}$ from basal levels of ~50 nM to ~200 nM in the presence of 35 mM K^+. The other photoreceptor (cell 2) had a very elongated cell body with a pronounced constriction between the inner segment and nuclear regions of the cell body. Intracellular $[Ca^{2+}]$ in this cell increased from ~50 nM to >1 µM in response to depolarization. A phase-contrast image of these cells, photographed under dim visible light and 380 nM illumination, is shown in Figure 1C along with the outline for integration of the fluorescence emission-ratio signal. The majority of the Ca^{2+}signals in these (Figure 1B) and most cells analyzed in this study was derived from the inner segment/cell soma regions.

In the experiments described above, fluorescence emission ratios were acquired at 1 min intervals. In most of the subsequent experiments the sampling frequency was increased to one ratio/10 s to improve temporal resolution. A typical response to depolarization under these conditions is shown in Figure 3. An initial peak of $[Ca^{2+}]_{in}$was frequently, but not always, observed. This peak was followed by a partial fall of $[Ca^{2+}]_{in}$ and a second rise to a plateau. It took a few minutes to get a stable $[Ca^{2+}]_{in}$, after which the levels remained high for as long as the elevated $[K^+]_{out}$ was applied. When the extracellular K^+ concentration was reduced, the $[Ca^{2+}]_{in}$ rapidly returned to the basal level.

The biphasic, initial response to depolarization was not observed in all cells. In some cells (e.g., Figure 6), a gradual increase taking several minutes was observed. It is unclear if this represents true differences in response or if transient peaks were missed in some cells because of the recording frequency of one ratio per 10 s. Even when the recording frequency was increased to one ratio per 1.5 s, both types of responses were observed (data not shown).

To determine if the increase of $[Ca^{2+}]_{in}$ observed during K^+ stimulation was caused by an influx of extracellular Ca^{2+}, 1 mM $CoCl_2$ was substituted for $CaCl_2$ in the superfusate during stimulation with 35 mM K^+. Co^{2+} blocks Ca^{2+} currents in a wide variety of cells [22]. Replacement of extracellular Ca^{2+} by Co^{2+} in the superfusion medium rapidly reduced the $[Ca^{2+}]_{in}$ of depolarized cells to basal levels (Figure 4 and Figure 5). When the superfusion medium was changed back to the normal, $CaCl_2$-containing high K^+ salt solution, the $[Ca^{2+}]_{in}$ increased again up to the original plateau level. This result suggests that the sustained increase of $[Ca^{2+}]_{in}$ is due to a continuous influx of calcium through voltage-gated membrane channels.

It is noteworthy that of the two cells recorded in Figure 5, only one showed a biphasic increase of $[Ca^{2+}]_{in}$ when initially depolarized (Figure 5, R7-R10). In this cell, the biphasic response was observed uniformly throughout the soma.

Cultured photoreceptor cells can respond to repeated 6 min periods of stimulation with 35 mM K^+ at least three times

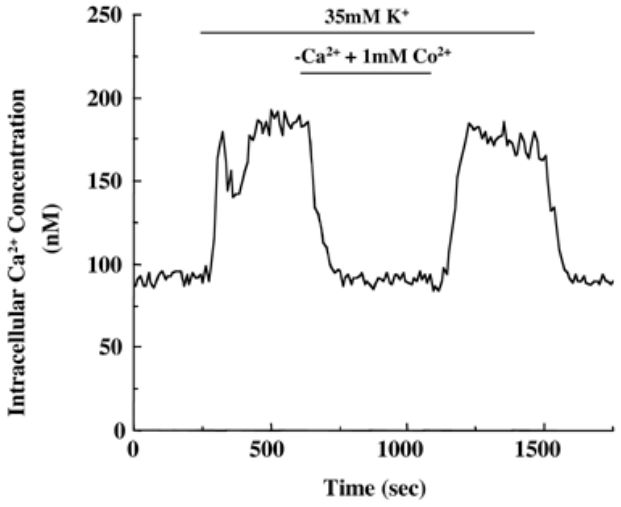

Figure 4. Application of 1 mM Co^{2+} in low Ca^{2+}superfusate during stimulation with 35 mM KCl. Cells were superfused as described in the legend to Figure 3 and recorded at a frequency of 1 ratio/10 s. Substitution of 1 mM $CoCl_2$ for $CaCl_2$ in the superfusion medium (in the continuous presence of 35 mM K^+) rapidly reduced the $[Ca^{2+}]_{in}$ of depolarized photoreceptor cells. The $[Ca^{2+}]_{in}$ increased again following the removal of Co^{2+} and the reintroduction of Ca^{2+}. A pseudocolor animation of 2 photoreceptor cells treated with Co^{2+} as described above but recorded with an emission-ratio frequency of 1/min can be viewed in Figure 5.

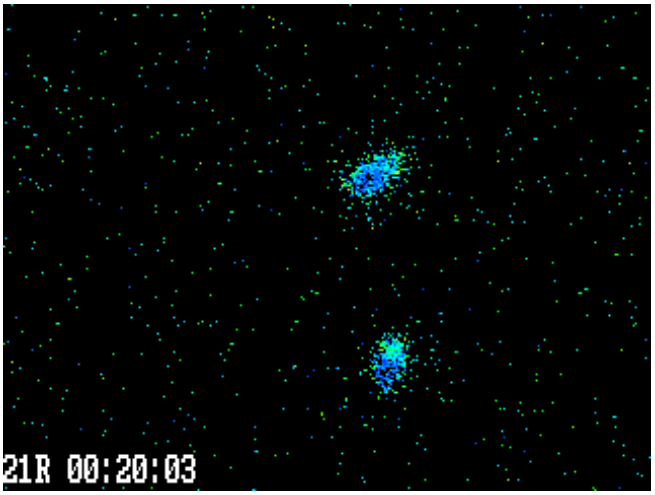

Figure 5. A pseudocolor animation of two photoreceptor cells treated with 35 mM K^+ and 1 mM Co^{2+}. A pseudocolor animation of two photoreceptor cells treated with Co^{2+} as described in the legend to Figure 4, recorded with an emission-ratio frequency of 1/min. In this experiment, cells were recorded under basal conditions for minutes 1-5. The following changes were made to the superfusion medium at the times indicated: 5 min, the extracellular K^+ concentration was increased to 35 mM; 10 min, the cells were exposed to 1 mM Co^{2+} in nominally Ca^{2+}-free salt solution containing 35 mM K^+; 21 min, Co^{2+} was removed and Ca^{2+}reintroduced in the continued presence of 35 mM K^+; 27 min, the extracellular K^+ concentration was lowered to basal levels (3.6 mM). Note that there is a quicktime movie of this figure in the online version at the following URL: http://www.molvis.org/molvis/v5/p1/uchida-fig3.html

with similar increases of $[Ca^{2+}]_{in}$ (Figure 6). The ratio of the $[Ca^{2+}]_{in}$ response to depolarization during the second stimulation to that of the first stimulation was 89.6 ± 8.8%; the ratio of the third to first stimulation periods was 95.4 ± 20.7% (8 cells).

Photoreceptor inner segments and synaptic terminals contain non-inactivating, voltage-gated Ca^{2+} channels with properties similar to those of L-type channels [3-6]. To investigate the role of these voltage-gated Ca^{2+} channels in the K^+-evoked $[Ca^{2+}]_{in}$ response, the effects of dihydropyridine modulators of L-type Ca^{2+} channels were examined. After establishing the response to the first 35 mM K^+ stimulation, the cells were treated with 3 μM nitrendipine or 10 μM nifedipine, Ca^{2+} channel antagonists. Neither drug affected $[Ca^{2+}]_{in}$ under basal conditions. However, they suppressed the 35 mM K^+-evoked increase of $[Ca^{2+}]_{in}$ by over 90% (Figure 7 and Figure 8). After washing out the drugs for 10 min, the cells stimulated by 35 mM K^+ showed partial recovery.

Bay K 8644 (1 μM), a dihydropyridine Ca^{2+} channel agonist, increased basal $[Ca^{2+}]_{in}$ and potentiated the effect of KCl (Figure 9, Table 1). The response to Bay K 8644 varied among individual photoreceptors. In some cells, Bay K 8644 alone, without K^+-stimulation, elicited large increases of $[Ca^{2+}]_{in}$(Figure 9 top panel, Table 1). Other cells showed little response to the agonist alone, but had K^+ responses that were greatly potentiated by Bay K 8644 (Figure 9, bottom panel).

Nitrendipine, nifedipine and Bay K 8644 were diluted from 10 mM, 50 mM or 10 mM stock solutions, respectively, in DMSO. DMSO as high as 0.1% has no effect on the $[Ca^{2+}]_{in}$ response to 35 mM KCl stimulation when it was applied alone to eight cells (data not shown).

DISCUSSION

The chicken photoreceptor cells recorded from in this study had estimated resting concentrations of intracellular Ca^{2+} of 50-100 nM. Upon depolarization, $[Ca^{2+}]_{in}$ generally increased to 300-400 nM, although smaller and larger responses were sometimes observed (see Figure 1B). The stimulation of $[Ca^{2+}]_{in}$ required Ca^{2+} influx through voltage-gated, dihydropyridine-sensitive channels. This response was consistently sustained, and appeared to require continuous Ca^{2+} influx.

The plasma membrane was partially depolarized by increasing the concentration of extracellular K^+ from 3.6 mM to 35 mM. The actual membrane potential was not measured in this study but can be estimated from the Goldman equation [23]. Assuming that the resting membrane potential under basal conditions (3.6 mM) is approximately -60 mV and chloride is at equilibrium, the Goldman equation predicts that 35 mM K^+ will depolarize the membrane to approximately -30 mV. Thus, the potential under basal conditions and in the presence of 35 mM K^+ approximates the potentials in bright light and darkness, respectively [1,24]. The basal $[Ca^{2+}]_{in}$ of chick photoreceptors in this study was similar to that reported previously for chick embryo photoreceptors [6] and for inner segments of salamander photoreceptor cells at a membrane potential of -50 mV [25].

The dynamics of the rise in $[Ca^{2+}]_{in}$ of chick photoreceptors stimulated with 35 mM K^+ showed several unique features. Firstly, the increase was very stable and persisted for as long as the depolarizing stimulus was applied. This required continuous calcium influx and suggests that the calcium channels do not inactivate. Secondly, the manner in which the calcium levels increased to that stable state suggests a multi-phasic mechanism. An initial peak of $[Ca^{2+}]_{in}$ was often followed

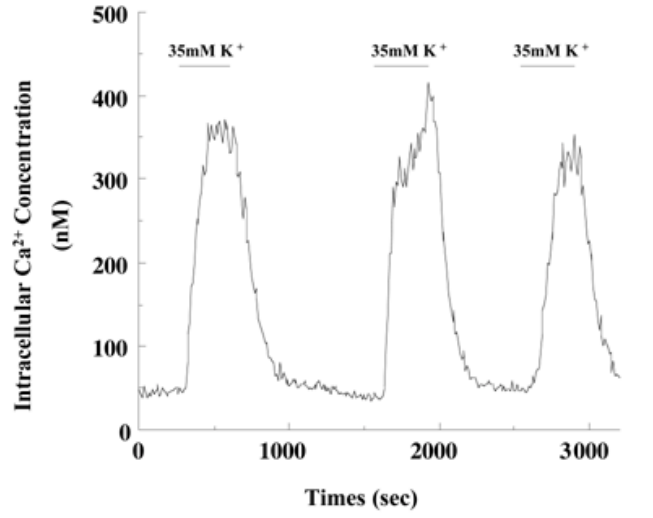

Figure 6. Photoreceptor cells responded to 35 mM KCl stimulation three times with similar increases of $[Ca^{2+}]_{in}$. Cells were continuously superfused using Protocol II, as described in Methods. Three 6 min pulses of 35 mM K^+ were delivered as indicated. The recording frequency was 1 ratio/10 s. This response is representative of those recorded from 8 cells.

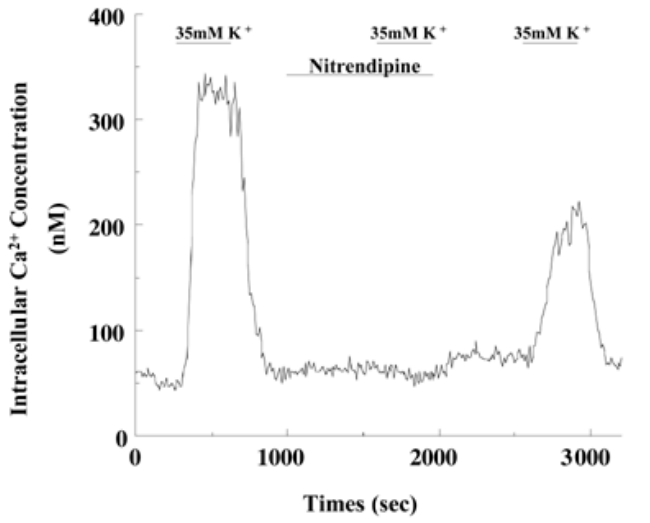

Figure 7. Effects of nitrendipine (3 μM) on basal- and 35 mM KCl-stimulated $[Ca^{2+}]_{in}$. Cells were treated as described in the legend to Figure 6. Nitrendipine (3 μM) was introduced 10 min prior to the second stimulation. The K^+-evoked increase of $[Ca^{2+}]_{in}$ was almost completely blocked by 3 μM nitrendipine while the basal level of the $[Ca^{2+}]_{in}$ was unaffected. This response is representative of those recorded from 8 cells.

by a partial decrease before a rise to the stable plateau. In other cells, a gradual increase of $[Ca^{2+}]_{in}$ taking several minutes to reach a maximum was observed. These patterns of change in intracellular Ca^{2+} may reflect positive feedback regulation of calcium channels and mobilization of intracellular stores.

Pre-depolarization of chicken photoreceptor cells increases subsequent depolarization-evoked $^{45}Ca^{2+}$ influx (Alonso-Gmez and Iuvone, unpublished observations). The effect of pre-depolarization is both time- and Ca^{2+}-dependent, and suggests that the initial Ca^{2+} influx results in activation or recruitment of channels. This may occur by phosphorylation, which alters the kinetics of gating and increases the permeability of L-type Ca^{2+}channels [26,27]. The involvement of phosphorylation in the regulation of photoreceptor calcium channels is supported by the observations that inhibitors of calmodulin and calmodulin-dependent protein kinase II decrease depolarization-evoked $^{45}Ca^{2+}$ influx in cultured photoreceptor cells [28].

The second rise or slow increase of $[Ca^{2+}]_{in}$ following application of 35 mM K^+ may reflect mobilization of intracellular Ca^{2+} stores. Depolarization and calcium influx through voltage-gated, dihydropyridine-sensitive channels lead to inositol tris-phosphate (IP3) formation in photoreceptor cell cultures [10]. IP3 binds to receptors, which in photoreceptor cells are on the endoplasmic reticulum of inner segments and synaptic terminals [29,30], to release intracellular calcium stores into the cytoplasm [31] increasing $[Ca^{2+}]_{in}$.

The dihyropyridine sensitivity of the $[Ca^{2+}]_{in}$ response to depolarization is consistent with reports that photoreceptor cells have Ca^{2+} channels with characteristics of L-type channels [3-6]. The dihydropyridine Ca^{2+} channel antagonists, nitrendipine and nifedipine, blocked the K^+-evoked increase of $[Ca^{2+}]_{in}$ by over 90%, indicating that the major voltage-gated Ca^{2+} channel subtype in the photoreceptor cells is dihydropyridine sensitive. However, our results do not preclude the existence of other, dihydropyridine-resistant Ca^{2+} currents [32].

Table 1. Effects of Bay K 8644 on basal and K+-stimulated $[Ca^{2+}]_{in}$

	Intracellular Ca2+ (nM)	
Condition	3.6 mM K+	35 mM K+
Control	65 ± 7	250 ± 41
Bay K 8644 (1 μM)	198 ± 41*	321 ± 47**
Washout	92 ± 7	203 ± 31

All measurements were made on photoreceptors exposed to the standard three KCl stimulation paradigm (Control, Bay K 8644, Washout) as described in Methods (Protocol II). Measurements under each condition represent the average $[Ca^{2+}]$in for 3 min before the control KCl stimulation (3.6 mM K^+) and the last 3 min of the 6 min stimulation period (35 mM K^+). Bay K 8644 was added 10 min prior to the second stimulation period. Data are expressed as mean ± SEM of 8 photoreceptors. Repeated measures ANOVA at 3.6 mM K^+ indicated a significant treatment effect (F= 8.966, df=7,2, p=0.003); asterisk (*) indicates p = 0.004 for comparison to Control and p = 0.017 for comparison to Washout. Repeated measures ANOVA at 35 mM K^+ indicated a significant treatment effect (F = 9.864, df = 7,2, p = 0.002); double asterisk (**) indicates p = 0.045 for comparison to Control and p = 0.002 for comparison to Washout.

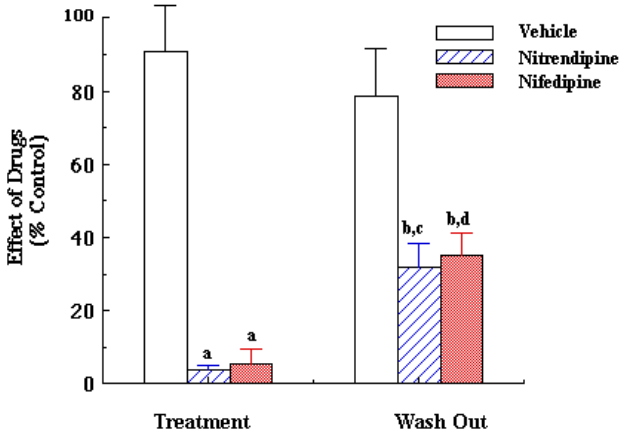

Figure 8. The K^+-evoked increase of $[Ca^{2+}]_{in}$ is blocked by 3 μM nitrendipine and 10 μM nifedipine. The bar graphs show the $[Ca^{2+}]_{in}$ response to K^+ of the second stimulation period (Treatment) and third stimulation period (Wash Out) expressed as a percentage of the first stimulation period. Vehicle, nifedipine, and nitrendipine were present from 10 min prior to the second stimulation until its end. The Ca^{2+} channel blockers reduced the response to K^+ by greater than 90%. The response partially recovered upon washout for 10 min. N = 8 per group. (a) $p < 0.01$ for comparison to Vehicle Treatment; (b) $p < 0.01$ for comparison to Vehicle Washout; (c) $p < 0.05$ for comparison to Nitrendipine Treatment; $p < 0.05$ for comparison to Nifedipine Treatment.

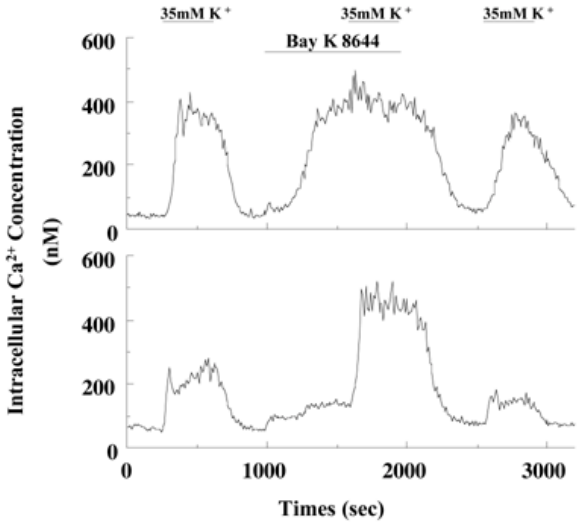

Figure 9. Effects of Bay K 8644 on basal and 35 mM KCl stimulated $[Ca^{2+}]_{in}$. Bay K 8644 (1 μM) was present from 10 min prior to the second stimulation period until its end and was then washed out with normal BSS. Bay K 8644 potentiated the response to 35 mM K^+ (bottom panel). In some photoreceptors, Bay K 8644 alone elicited a large response (top panel). Eight cells were recorded showing various stimulatory responses similar to these.

Bay K 8644 enhanced the $[Ca^{2+}]_{in}$ response to depolarization. A surprising result was that, in many cells examined, Bay K8644 increased $[Ca^{2+}]_{in}$ under basal conditions, without KCl stimulation. Bay K 8644 is thought to act by prolonging channel opening [33]. Thus, the open probability of the Ca^{2+} channels is apparently greater than zero under basal conditions. Calcium influx during channel opening would be potentiated by Bay K 8644 and the increase in Ca^{2+} conductance would tend to depolarize the cells, further increasing the open probability of the channels. The response to Bay K 8644 varied among individual photoreceptors. Patch clamp recordings of cultured chick photoreceptor cells indicate that the activation ranges of the voltage-gated Ca^{2+} channels also vary among cells, with thresholds observed between -70 and -25 mV [6]. Our results suggest that some cells have resting potentials near or above threshold, or that cells undergo spontaneous, transient depolarization under basal conditions. These possibilities are consistent with the observations that Bay K 8644 elicits small increases of inositol phosphate accumulation and serotonin *N*-acetyltransferase activity of photoreceptor cell cultures under basal conditions [10,34]. The variable channel activation thresholds [6] may also account for the cell-to-cell variability in the magnitude of the K^+-evoked increase of $[Ca^{2+}]_{in}$ observed in the present study.

It is noteworthy that the calcium channel antagonists have no detectable effect on basal $[Ca^{2+}]_{in}$. Thus, the spontaneous opening of channels under basal conditions, implied by the discussion above, has no significant effect on $[Ca^{2+}]_{in}$. This observation suggests that the Ca^{2+} buffering capacity of the cells under basal conditions meets or exceeds the Ca^{2+} influx. The existence of a large buffering capacity is supported by the observations that intracellular Ca^{2+} levels rapidly return to basal levels following removal of the depolarizing stimulus or blockade of Ca^{2+}influx. Intracellular free Ca^{2+} levels can be reduced by an ATP-dependent pump and a Na^+/Ca^{2+} exchanger in plasma membrane, by a Ca^{2+}- ATPase in the endoplasmic reticulum, and by a mitochondrial Ca^{2+} pump. The mechanisms operative in chick photoreceptor cells are still unknown.

Induction of serotonin *N*-acetyltransferase activity in retinal photoreceptor cells is mediated by a Ca^{2+}-dependent process that involves sustained Ca^{2+} influx through dihydropyridine-sensitive voltage-gated Ca^{2+} channels [14,16]. Ca^{2+} influx stimulates cyclic AMP formation [9], which in turn increases serotonin *N*-acetyltransferase activity [19,34]. Thus, the mechanisms responsible for the sustained increase of free intracellular Ca^{2+}concentration may play an important role in the regulation of serotonin *N*-acetyltransferase activity and melatonin synthesis.

In conclusion, we have examined the level of free intracellular Ca^{2+} in isolated cultured chick photoreceptor cells at rest and during depolarization. The sustained elevation of $[Ca^{2+}]_{in}$of depolarized photoreceptor cells requires continuous Ca^{2+} influx through the dihydropyridine-sensitive voltage-gated channels and may involve positive feedback regulation of channel activity and intracellular Ca^{2+} stores.

ACKNOWLEDGEMENTS

This study was supported in part by NIH grant EY04864. K. U. was partially supported by a fellowship from the Japanese Ministry of Education and Science. We are grateful to Jolanta Zawilska for helpful comments on the manuscript. A preliminary report of some of these data has been presented in abstract form [35].

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