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Insulin inhibits voltage-dependent calcium influx into rod photoreceptors

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Abstract

Insulin inhibits the ERG b-wave and modulates L-type calcium currents (I_{Ca}) in various preparations. We therefore examined insulin's effects on I_{Ca} and depolarization-evoked $[Ca^{2+}]_i$ increases in rod photoreceptors. Insulin inhibited I_{Ca} and caused a dose-dependent reduction in the depolarization-evoked Ca^{2+} influx with an EC_{50} of 2.1 nM. Tyrosine kinase inhibitors, lavendustin A (100 nM) and genistein (10 μ M), prevented insulin from reducing the depolarization-evoked Ca^{2+} increase in rods. Their less active analogues, lavendustin B and daidzein, had similar effects. An insulin receptor-specific tyrosine kinase inhibitor, HNMPA-(AM)₃ (50 μ M), prevented insulin (30 nM) from reducing the depolarization-evoked Ca^{2+} increase in rods. The results suggest that insulin inhibits Ca^{2+} influx through voltage-dependent I_{Ca} in rod photoreceptors via tyrosine kinase activity.

Keywords

Calcium channel; Calcium imaging; Fura-2; HNMPA-(AM)₃; Insulin; Lavendustin A and B; Retinal slice; Rod; Tyrosine kinase

INTRODUCTION

Insulin is generally thought of as an antidiabetogenic factor involved in glucose metabolism, but in the CNS and retina insulin appears to act primarily as a mitogen or growth factor [1,2]. The actions of insulin are initiated by tyrosine autophosphorylation of the insulin receptor (InsR) tail. Activated tyrosine kinase on the InsR cytoplasmic tail then phosphorylates tyrosine residues on an adapter protein known as insulin receptor substrate (IRS) which in turn triggers the intracellular signaling cascade [3]. Downstream actions of insulin include modification of ion channel activity. For example, insulin stimulates L-type Ca^{2+} channel activity in cardiac myocytes [4], skeletal myocytes [5] and *Aplysia* bag neurons [6], whereas insulin inhibits L-type I_{Ca} in pinealocytes [7].

InsRs are located on both the inner and outer segments of vertebrate photoreceptors [8] and insulin has been shown to produce a dose-dependent decrease in the a- and b-waves of the electroretinogram (ERG) [9]. The ERG b-wave reflects the activity of inputs from photoreceptors onto ON depolarizing bipolar cells [10]. Suppression of the b-wave therefore suggests that insulin may reduce transmitter release from photoreceptors. Transmission from photoreceptors is regulated by the activity of L-type Ca^{2+} channels [11,12]. Given the effects of insulin on the b-wave and the sensitivity of L-type Ca^{2+} channels in other preparations to insulin, we hypothesized that insulin might also influence the activity of L-type Ca^{2+} channels

in rod photoreceptors. The present results indicate that insulin inhibits depolarization-evoked Ca^{2+} , increases in rods as a consequence of inhibiting Ca^{2+} influx through voltage gated Ca^{2+} channels and that this inhibition is mediated by stimulation of insulin receptor tyrosine kinase activity.

Inhibition of rod I_{Ca} should alter neurotransmission at the first synapse in the visual pathway and, by minimizing increases in intracellular $[\text{Ca}^{2+}]$, might also provide neuroprotective benefits to rods.

MATERIALS AND METHODS

Retinal slices were prepared from larval tiger salamanders (*Ambystoma tigrinum*, Kons, Germantown, WI, 7–10 inches in length) as described by Thoreson *et al.* [12]. Briefly, salamanders were sacrificed by decapitation and pithed, an eye was enucleated, and the anterior segment of the eye was removed. The resulting eyecup was cut into 3–4 sections and one section was placed vitreal side down onto a piece of filter paper (2×5 mm, Millipore Type GS, 0.2 μm pores). The tissue and filter paper were cut into 100–150 μm slices using a razor blade tissue chopper (Stoelting). Slices were rotated 90° to allow viewing of the retinal layers when placed under a water immersion objective ($\times 40$, 0.7 NA) and viewed on an upright fixed stage microscope (Olympus BHWI).

Isolated retinal cells were prepared by finely mincing isolated retina with a double-edged razor blade and then gently triturating the retinal pieces. Isolated cells were plated on slides coated with a salamander-specific antibody, Sal-1 (kindly provided by Peter MacLeish).

Solutions were applied by a single-pass, gravity-feed perfusion system which delivered medium to the slice chamber at 1.0 ml/min. The normal amphibian superfusate that bathed the slices contained (in mM): 111 NaCl, 2.5 KCl, 1.8 CaCl_2 , 0.5 MgCl_2 , 10 HEPES and 5 glucose. For elevated KCl applications a solution was applied to the slices that contained (in mM): 63.5 NaCl, 50 KCl, 1.8 CaCl_2 , 10 HEPES and 5 glucose. For I_{Ca} measurements, the superfusate was switched to a Ba^{2+} solution to enhance Ca^{2+} currents. The Ba^{2+} solution contained (in mM): 99 NaCl, 2.5 KCl, 10 BaCl_2 , 0.5 MgCl_2 , 10 HEPES, 5 glucose, 0.1 picrotoxin and 0.1 niflumic acid. The pH of all solutions was adjusted to 7.8 with NaOH and the osmolality to 242 ± 5 mOsm. Solutions were continuously bubbled with 100% O_2 .

We used the perforated patch method of whole cell recording. Patch pipettes were pulled on a Narashige PB-7 vertical puller from borosilicate glass pipettes (1.2 mm O.D., 0.95 mm I.D., omega dot) and had tips of ~ 1 μm O.D. ($R=10$ – 15 M Ω). Pipettes were filled with a solution containing (in mM): 54 CsCl, 61.5 CsCH_3SO_3 , 3.5 NaCH_3SO_4 , 10 HEPES. The pH was adjusted to 7.2 with CsOH and the osmolality was adjusted, if necessary, to 242 ± 5 mOsm. Nystatin was mixed in dimethylsulfoxide (DMSO) at a concentration of 120 mg/ml, vortexed briefly, and then added to the pipette electrolyte solution to achieve a final concentration of 480 $\mu\text{g}/\text{ml}$. In successful recordings, seals > 1 G Ω were obtained in 30 s or less, and cells were usually fully perforated within 5 min of sealing. Cells were voltage clamped at -70 mV using an Axon 200B patch-clamp amplifier.

Rods were identified under infrared illumination by their large rod-shaped outer segments. After a recording was obtained, voltage ramps from -90 to $+60$ mV (0.5 mV/ms) were applied every 30 s to assess I_{Ca} . Drug solutions were applied after I_{Ca} and appeared stable for at least 2–3 min. Currents were acquired and analyzed using PClamp 7.0 software.

For measurement of intracellular Ca^{2+} changes, retinal slices were incubated in the dark with 10 μM Fura-2/AM $\pm 0.02\%$ pluronic F-127 (Molecular Probes, Eugene, OR) at 4°C for 45 min, followed by an additional incubation for 1.5 h in Fura-2/AM without pluronic. Isolated

retinal cells were incubated for 45 min in 10 μ M Fura-2 AM. Cells were perfused for 30 min before beginning data acquisition.

Slices were illuminated alternately through 340 nm and 380 nm interference filters mounted on a Sutter Lambda 10-2 filter wheel and the emission was collected through a 510 ± 20 nm bandpass emission filter. Digital fluorescent images were recorded with a cooled CCD camera (Sensi-Cam, Cooke Corp.) mounted on an upright fixed stage microscope (Nikon E600FN) equipped with a $\times 60$ water immersion objective (1.0 N.A.). Axon Imaging Workbench (AIW 2.2) was used to control the camera, filter wheel, and image acquisition. The 340 nm/380 nm fluorescence ratio, reflecting changes in $[\text{Ca}^{2+}]_i$, was collected from a region of interest centered over the rod inner segment. To activate voltage-dependent Ca^{2+} channels, cells were depolarized by increasing $[\text{K}^+]_o$ from 2.5 to 50 mM for 1 min. Images were acquired at 5–10 s intervals during elevated $[\text{K}^+]_o$ applications (acquisition time: 0.5–1 s). Elevated KCl applications were performed at 15 min intervals to minimize Ca^{2+} -dependent inactivation.

Insulin, genistein, daidzein, lavendustin A, and lavendustin B were obtained from Sigma Chemical Corp. (St. Louis, MO). HNMPA-(AM)₃ was obtained from Calbiochem (San Diego, CA). Insulin was prepared as a 5 mM stock solution in 0.1% BSA. All other stock solutions (1000 $\times$) were prepared in DMSO. Superfusion with 0.1% DMSO alone did not affect any of the I_{Ca} properties or elevated $[\text{K}^+]_o$ responses we studied.

Statistical significance was chosen as $p < 0.05$ and evaluated with Student's t -test using GraphPad Prism 3.0. Errors are reported as $\pm$ s.e.m.

RESULTS

Insulin suppresses rod photoreceptor I_{Ca}

In the presence of 10 mM Ba^{2+} , depolarizing voltage ramps from -90 to $+60$ mV produced inward currents in rods. Consistent with results indicating that rods possess predominately high voltage-activated, L-type Ca^{2+} channels [12,13], inward currents were first detected above -50 mV, peaked near -5 mV, and then diminished at more positive potentials. The average peak amplitude of I_{Ca} in rods was -183.7 ± 28.5 pA ($n=5$).

As illustrated in Fig. 1a, insulin (100 nM) inhibited rod I_{Ca} . Insulin decreased the peak amplitude of I_{Ca} in rods by $18.9 \pm 3.0\%$ ($n=5$, Fig. 1b) and did not appear to shift the peak inward current or the current-voltage relationship along the voltage axis (Fig. 1a). The onset of inhibition by insulin occurred within the first 1–3 min and maximal inhibition was seen within 5 min. The effect of insulin was not reversed after 15 min washout.

Insulin reduces Ca^{2+} influx

To examine the effect of insulin on Ca^{2+} influx through L-type channels in rods we loaded retinal slices with the Ca^{2+} -sensitive dye Fura-2. Figure 1c illustrates the inhibitory effect of insulin (100 nM) on Ca^{2+} responses in a rod inner segment evoked by application of 50 mM $[\text{K}^+]_o$ for 1 min. Insulin at concentrations of ≥ 1 nM produced significant inhibition of the K^+ -evoked $[\text{Ca}^{2+}]_i$ increase with an EC_{50} of 2.1 ± 0.11 nM (Fig. 1d). Maximal inhibition of K^+ -evoked $[\text{Ca}^{2+}]_i$ increase by insulin was obtained at concentrations of ≥ 30 nM ($14.0 \pm 2.0\%$, $n = 22$, $p = 0.0001$, Fig. 1d). Recovery of the K^+ -evoked $[\text{Ca}^{2+}]_i$ increase following washout of insulin was slow, requiring at least 45 min (data not shown). Due to this long recovery time and the possibility that insulin might produce desensitization, only one experimental trial with insulin was performed on each retinal slice.

To test for the possibility that an intermediary cell type might be involved in the insulin-dependent modulation of Ca^{2+} influx into rod photoreceptors, effects of insulin were tested on

mechanically isolated, solitary rods. As in retinal slices, insulin (100 nM) significantly inhibited the K^+ -evoked $[Ca^{2+}]_i$ increase in isolated rods by $31.4 \pm 11.2\%$ ($n = 6$, $p = 0.038$), indicating that insulin acts directly on rod photoreceptors.

Insulin reduces Ca^{2+} influx through a tyrosine kinase-mediated pathway

Upon stimulation by insulin, the InsR undergoes tyrosine autophosphorylation of the cytoplasmic tail which mediates recruitment of IRS proteins and is followed by downstream tyrosine phosphorylation and recruitment of various proteins and signaling molecules [3]. We used a selective inhibitor of InsR tyrosine kinase activity, HNMPA-(AM)₃, which inhibits InsR tyrosine phosphorylation but has no effect on protein kinase C or cyclic AMP-dependent protein kinase activities [14]. More-over, HNMPA-(AM)₃ has been shown to prevent insulin-stimulated enhancement of L-type I_{Ca} in human atrial myocytes [4]. Cells were pretreated for 1 h with HNMPA-(AM)₃ (50 μ M), which appeared to have no effect on Fura-2 loading or the magnitude of elevated $[K^+]_o$ responses in the absence of insulin. However, following pretreatment with HNMPA-(AM)₃, a maximally effective dose of insulin (30 nM) was unable to inhibit the K^+ -evoked $[Ca^{2+}]_i$ increase in rods (Fig. 2, $97.0 \pm 2.2\%$, $n = 14$, $p = 0.1357$). This suggests that disruption of InsR-mediated tyrosine kinase activity prevents insulin-stimulated reduction of Ca^{2+} in-flux through L-type channels in rods.

We tested two other tyrosine kinase blockers, genistein (10 μ M) and lavendustin A (100 nM). Genistein and lavendustin A compete with ATP for binding to tyrosine kinases [15]. Both genistein (Fig. 3a, $101.0 \pm 2.0\%$, $n = 16$, $p = 0.6$) and lavendustin A (Fig. 3c, $105.6 \pm 4.7\%$, $n = 13$, $p = 0.15$) prevented insulin (30 nM) from inhibiting the K^+ -evoked $[Ca^{2+}]_i$ increase in rods. Unexpectedly, we also found that their less active analogues, daidzein (10 μ M) (Fig. 3b, $110.0 \pm 10.0\%$, $n = 14$, $p = 0.3$) and lavendustin B (100 nM) (Fig. 3d, $95.5 \pm 2.9\%$, $n = 15$, $p = 0.1469$), also prevented insulin (30 nM)-mediated inhibition.

DISCUSSION

The results of this study show that insulin inhibits I_{Ca} and depolarization-induced Ca^{2+} influx in rod photoreceptors. Effects of insulin can arise from activation of InsRs but can also be due to insulin binding, albeit less potently, to IGF-1 receptors [16,17]. The potency of insulin's effects on depolarization-evoked Ca^{2+} responses ($EC_{50} = 2.1$ nM) suggests that the observed effects probably arise from actions at InsRs. This conclusion is also supported by the finding that HNMPA-(AM)₃, a selective blocker of InsR tyrosine kinase activity, blocks the effect of insulin (Fig. 3).

The effectiveness of pretreatment with 50 μ M HNMPA-(AM)₃ in blocking the inhibitory effects of insulin on rod Ca^{2+} responses (Fig. 2) suggests that InsR tyrosine kinase autophosphorylation is required for insulin action in rods [14]. This result is consistent with results from atrial myocytes in which pretreatment for 15 min with 1 mM HNMPA-(AM)₃ blocked insulin's effects on I_{Ca} [4]. In concert with the possibility that InsR activation stimulates tyrosine kinase activity, genistein and lavendustin A also blocked the inhibitory effects of insulin on Ca^{2+} influx (Fig. 3a,c). This is consistent with results from *Aplysia* neurons [6], human atrial myocytes [4] and pinealocytes [7], where the effects of insulin on L-type Ca^{2+} channels were also blocked by tyrosine kinase inhibitors. However, we also found that the less active analogues of genistein and lavendustin A, daidzein and lavendustin B, blocked insulin-mediated inhibition of the K^+ -evoked $[Ca^{2+}]_i$ increase in rods. One possible explanation for the effectiveness of both pairs of compounds is that rods may possess tyrosine kinase isoforms in which daidzein and lavendustin B are equally effective as their nominally more active counterparts. Another possibility is that these compounds may have actions at sites other than tyrosine kinase. This possibility is supported by results from pulmonary arterial cells

indicating that blockade of K^+ currents by genistein is not mediated by actions at tyrosine kinase [18].

What might be the physiological significance of insulin modulation of rod photoreceptor Ca^{2+} channels? Although it appears that insulin is unable to cross the blood–retinal barrier [19], the retina is capable of synthesizing insulin [20]; *in situ* hybridization and RT-PCR show that Müller cells contain mRNA for the prepro form of insulin [21,22]; and insulin and insulin-like immunoreactivity have been demonstrated throughout the retina, particularly in Müller cells [23]. Insulin levels in blood and various tissues are reported to range from 0.3 to 3.0 nM [24]. The EC_{50} of 2.1 nM observed in the present study therefore implies that physiological insulin levels have the capacity to produce inhibitory effects on rod I_{Ca} and Ca^{2+} influx.

Inhibitory effects of insulin on rod I_{Ca} might be neuroprotective. Blockade of L-type Ca^{2+} channels in rods of *rd* mice reduces the amount of photoreceptor degeneration, suggesting that photoreceptor Ca^{2+} channels play an important role in apoptosis [25]. Also consistent with a potential neuroprotective role is the finding that insulin can prevent retinal neurons from undergoing apoptosis during retinal development and in diabetes [1,2]. The decrease in tonic glutamate release from photoreceptors that would presumably accompany decreased activation of I_{Ca} might also minimize the potential for excitotoxic damage to post-receptoral neurons. In addition to possible neuroprotective benefits, the inhibitory effects of insulin on the ERG b-wave [9] and rod Ca^{2+} channel (described in this study) indicate that insulin could play a neuromodulatory role in regulating synaptic transmission from rods to second-order neurons. Finally, the present results suggest that alterations in retinal insulin levels or sensitivity to insulin, e.g., during diabetes, may affect photoreceptor cell survival and neurotransmission at the first synapse in the retina.

CONCLUSION

Insulin produces a dose-dependent inhibition in the influx of Ca^{2+} through high voltage-activated Ca^{2+} channels in rod photoreceptors. The physiologically relevant EC_{50} suggests that the actions of insulin are mediated by InsRs on rods. Actions of tyrosine kinase inhibitors suggest that the effects of insulin involve activation of InsR tyrosine kinase as well as possible activation of downstream tyrosine kinases. The ability of insulin to inhibit Ca^{2+} influx in rods is consistent with a role for insulin in modulating neurotransmission onto second-order neurons and raise the possibility that insulin might be neuroprotective for rods.

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Fig. 1.

(a) Current-voltage relationships of rod I_{Ca} illustrating inhibition by insulin (100 nM). Current-voltage relationship of I_{Ca} obtained in control superfusate (thin trace) is overlaid with one obtained after perfusion for 3 min with insulin (thick trace). The cell was held at -70 mV and the voltage was ramped from -90 to $+60$ mV at 0.5 mV/ms. (b) Bar graph showing the mean reduction in I_{Ca} amplitude by insulin (100 nM). (c) The effect of insulin (100 nM) on Fura-2 ratio changes in a rod inner segment from the retinal slice produced by 1 min applications of elevated (50 mM) $[K^+]_o$. (d) Concentration-dependent inhibition of the K^+ -evoked $[Ca^{2+}]_i$ increase in rods from retinal slice preparations by insulin. Fura-2-loaded slices were stimulated with elevated $[K^+]_o$, in the presence of different concentrations of insulin (0.001 – 1 μ M). Inhibition of K^+ -evoked $[Ca^{2+}]_i$ increase produced by insulin were normalized to prior control responses to high K^+ and expressed as percent inhibition. Each point represents the mean $\pm$ s.e.m. Number of experiments is shown in parentheses. * $p < 0.05$ compared with control response.

Fig. 2. Pretreating retinal slices for 1 h with 50 μ M HNMPA-(AM)₃, an inhibitor of insulin receptor tyrosine kinase, prevented insulin-mediated inhibition of depolarization-evoked $[Ca^{2+}]_i$ changes.

Fig. 3.

Effects of tyrosine kinase inhibitors on insulin-mediated inhibition of $[Ca^{2+}]_i$ changes in photoreceptor inner segments produced by 1 min applications of elevated (50 mM) $[K^+]_o$. **(a)** Genistein (10 μ M). **(b)** The less active analogue of genistein, daidzein (10 μ M). Genistein and daidzein were bath applied for 30 min prior to insulin application; shorter 3–5 min applications of genistein were ineffective at preventing the insulin-mediated reduction of K^+ -evoked $[Ca^{2+}]_i$ increase. **(c)** Lavendustin A (100 nM). **(d)** The less active analogue of lavendustin A, lavendustin B (100 nM). Lavendustin A and B were bath applied for 3 min prior to application of high K^+ solution or high K^+ solution plus insulin.