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Two Types of Voltage-activated Calcium Currents in Goldfish Horizontal Cells

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In horizontal cells (HCs) that were freshly dissociated from goldfish retina, two types of voltage-dependent calcium currents (I_{Ca}) were recorded using a patch-clamping configuration: a transient type current and a sustained type current. The cell was held at −40 mV, and the prepulse step of −90 mV was applied before command pulse between −65 and +55 mV. The transient Ca^{2+} current was activated by depolarization to around −50 mV from a prepulse voltage of −90 mV lasting at least 400 ms and reached a maximal value near −25 mV. On the other hand, the sustained Ca^{2+} current was induced by pre-inactivation for less than 10 ms duration. Its activation started near −10 mV and peaked at +20 mV. Co^{2+} (2 mM) suppressed both of these two components, but nifedipine (20 μM), L-type Ca^{2+} channel antagonist, blocked only the sustained current. Based on the activation voltage and the pharmacological specificity, the sustained current appears to be similar to L-type I_{Ca} and the transient type to T-type I_{Ca}. This study is the first to confirm that transient type I_{Ca} together with the sustained one is present in HCs dissociated from goldfish retina.

Key Words: Horizontal cell, Sustained calcium current, Transient calcium current, Goldfish, Whole-cell Patch-Clamp, Retina

INTRODUCTION

Horizontal cells (HCs) are one of the most extensively studied interneurons in the vertebrate retina. These cells integrate the signal input from photoreceptors (Naka, 1972; Stell & Lightfoot, 1975) and are responsible for surround inhibition of receptive field on bipolar cells (Dowling & Werblin, 1969). HCs are also known to send outputs to photoreceptors (Baylor & Fuortes, 1970; Burkhardt, 1977) or to bipolar cells (Dowling & Werblin, 1969; Naka, 1976). The HCs of teleost fish retina are particularly large in size, and they are consisted of several distinct layers along the distal margin of the inner nuclear layer, and their physiological properties have been studied to a great extent. Most HCs of teleost fish retina consist of 4 types: H1, H2, H3 and rod-HC (RH). Among these cells, Hl-type cells are the only ones known to contain and release GABA. This release of GABA from the Hl-type cells is believed to be largely independent of Ca^{2+} (Schwartz, 1982; Ayoub & Lam, 1985), therefore, the contribution of calcium current (I_{Ca}) on HCs remains controversial. The present study is a part of an effort to examine the role of Ca^{2+} channels in HCs. We have investigated the voltage-dependent Ca^{2+} channels in HCs dissociated from goldfish retina.

It is well established that Ca^{2+} plays a major role in the regulation of both metabolic and membrane mechanisms in all neurons. In general, voltage-activated Ca^{2+} channels have been classified into low-voltage-activated (LVA) and high-voltage-activated (HVA) Ca^{2+} channels. HVA Ca^{2+} channels include L-, N-, P/Q-, and R-types (Tsien et al, 1995). Each type of HVA Ca^{2+} channels can be identified by pharmacological specification. LVA Ca^{2+} channels are also generally referred to as T-type, which are characterized by activation at hyperpolarized voltage near rest and rapid inactivation (Huguenard, 1996). It has been reported that there is a wide variety of Ca^{2+} channels among various species and tissue types (Llinas & Yarom, 1981; Kaneko et al, 1989; Karschin & Lipton, 1989; Plummer et al, 1989). However, most studies on I_{Ca} in isolated HCs of teleost fish retina demonstrated sustained, high-threshold I_{Ca}: catfish (Shingai & Christensen, 1983), skate (Malchow et al, 1990), white perch (Lasater, 1986) and goldfish (Tachibana, 1983). The transient inward I_{Ca} was found only in cultured HCs isolated from white bass (Sullivan & Lasater, 1992). Since then, no other research has reported the presence of transient I_{Ca} in HCs of teleost fish retina. In the present study, two types of I_{Ca} were identified: one is a sustained I_{Ca} similar to one described previously in cultured HCs that were dissociated from teleost fish retina, and the other one is transient I_{Ca} that was first found in HCs that were dissociated from goldfish retina.

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ABBREVIATIONS: HC, horizontal cell; I_{Ca}, calcium currents; RH, rod-horizontal cell; LVA, low-voltage-activated; HVA, high-voltage-activated; RT, room temperature; NMDG, *N*-methyl-D-glucamine.

METHODS

Preparation

HCs were enzymatically and mechanically isolated from the retina of a goldfish (*Carassius auratus*). A goldfish (about 15 cm in total body length) was purchased from a local fish store and kept at room temperature (RT) with a light/dark cycle of 12/12 hrs. The details of dissociation procedures for goldfish have earlier been described (Tachibana, 1981; Yagi & Kaneko, 1988). Briefly, eyes were enucleated from pithed goldfish, and the retina was detached from pigment epithelium. The retina was treated with papain solution (12 U/ml) that was activated by 5 mM L-cysteine at RT. After washing of the enzyme with normal Ringer solution, it was mechanically triturated using a large-tip pipette for dissociation, and isolated cells were placed on the coverslip and kept at 4°C.

After dissociation, these horizontal cells could be classified into 4 types (H1, H2, H3 and RH) as described in previous studies (Stell, 1975; Paik et al, 2003). However, recordings were mainly made from H2 types in the present experiment. H2 and RH types were regarded as one, since they are sometimes too similar to distinguish one from the other. The experiments were carried out within a few hours of dissociation to use freshly dissociated HCs.

Solutions and pharmacological agents

The normal Ringer solution used for the dissociation contained the following (in mM): NaCl, 125; KCl, 2.6; $CaCl_2$, 2.5; $MgCl_2$, 1; HEPES, 10; and glucose, 10 (pH adjusted to 7.4 with HCl). In order to enhance the amplitude of I_{Ca} and prevent the contamination of other currents, the control Ringer solution was modified by increasing $[Ca]_o$ up to 10 mM, and completely replacing Na^+ and K^+ with *N*-methyl-*D*-glucamine (NMDG). The control Ringer solution contained the following (in mM): NMDG-Cl, 120; $CaCl_2$, 10; HEPES, 10; and glucose, 10. In order to suppress outward I_K (potassium current), NMDG was also introduced to the cells through a patch pipette. The patch pipettes used for the experiment were filled with a solution containing the following (in mM): NMDG-Cl, 125; $MgCl_2$, 2; $CaCl_2$ 0.5; EGTA, 2; and HEPES, 10 (pH adjusted to 7.4 with HCl). The pharmacological agents were added directly into the Ringer solution. Nipedipine was added into the Ringer solution after it was dissolved in dimethyl sulfoxide (DMSO). All pharmacological agents were purchased from Sigma (St. Louis, MO, USA).

Recordings

The cells attached to the bottom of the coverslip were placed inside a recording chamber, which was set on an inverted microscope stage. The recording chamber was continuously superfused with the Ringer solution. Membrane currents were measured by using a conventional patch-clamp technique in the whole-cell recording configuration (Hamill et al, 1981). Patch-clamp recordings were obtained using an Axopatch 1D amplifier (Axon instruments, Foster, CA) with pCLAMP acquisition software. Data were analyzed by Origin software (Microcal Software, Inc., Northampton, MA). Patch-clamp electrodes were made from borosilicate glass (B100-50-10; Sutter Instruments, Novato, CA) with a horizontal puller (P-87; Sutter Instruments). The holding potential was set at −40 mV, and the prepulse step of −90 mV was applied before the depolarizing test-pulse. The holding potential of −40 mV was needed to help the cells persist longer during the recording since the cells are likely to be damaged at times if holding voltage lingers lower than −40 mV.

RESULTS

Voltage-dependent I_{Ca} recorded from HCs of the goldfish retina

In order to enhance the amplitude of I_{Ca}, the horizontal cells were superfused with the control Ringer solution containing 10 mM $[Ca]_o$. When measured in 10 mM $[Ca]_o$, maximal amplitude of I_{Ca} increased about 2 folds, compared to 2 mM $[Ca]_o$. Na^+ and K^+ were replaced with NMDG in the external solution to block other cation-induced currents except for Ca^{2+}.

A series of current traces represented voltage-dependent inward currents evoked during depolarizing pulses that ranged from −65 mV to +55 mV (in 10-mV steps), with a prepulse voltage of −90 mV (Fig. 1A). These inward currents consist of two peaks. Both components were completely blocked by 2 mM Co^{+2} (data not shown), further supporting the idea that the transient currents along with sustained currents are carried by Ca^{2+}. The transient component of inward I_{Ca} was activated at around −45 mV and reached the maximal value near −25 mV. On the other hand, the sustained component showed a slowly initiating inward current without time-dependent inactivation. Its

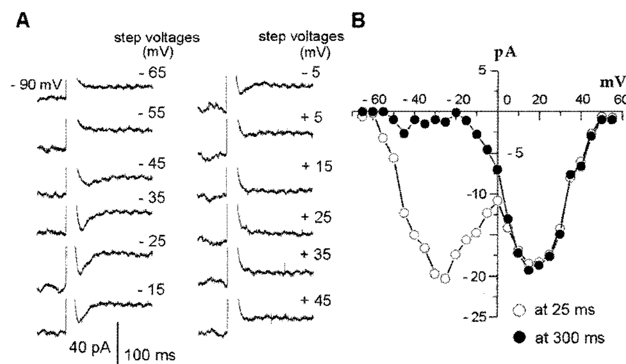

Fig. 1. (A) Voltage-dependent calcium current recorded from horizontal cells of the goldfish retina. A series of current traces were consisted of two types of inward currents evoked during depolarizing pulses that ranged from −65 mV to +55 mV in 10-mV steps with a prepulse voltage of −90 mV. In order to isolate I_{Ca}, Na^+ and K^+ were replaced with *N*-mehtyl-*D*-glucamine in pipette solution and external solution. The external calcium concentration was increased up to 10 mM. Transient component (upper trace) was activated at the lower voltage and reached maximal value around −25 mV, while sustained component peaked at around +15 mV. The cells were held at −40 mV. (B) Current-voltage relationship of I_{Ca}. All data were measured at the maximal value of membrane currents recorded from −65 mV to +55 mV in 5-mV steps. Data marked as open circles correspond to the responses measured at 25 ms after the onset of the test pulse; Data marked as filled circles correspond to the responses obtained at 300 ms after the onset of the test pulse.

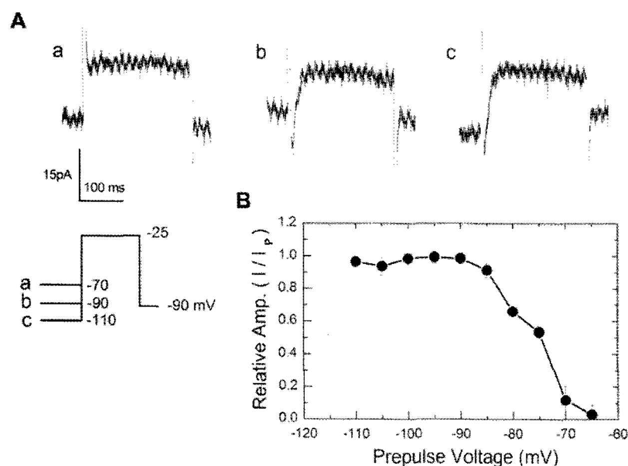

Fig. 2. The dependence of transient calcium current on prepulse voltage. (A) No inward current was evoked at prepulse voltage higher than −70 mV (a). Peak amplitude of inward current gradually increased as prepulse voltage became hyperpolarized more than −70 mV, and peak reached the maximal response around −90 mV (b). Response recorded at voltage of −110 mV was almost similar to maximal level (c). (B) Data marked as circles show the normalized peak amplitudes of transient I_{Ca} measured at various prepulse voltages from −110 mV to −70 mV, in 5-mV steps (mean± S.D., n=5).

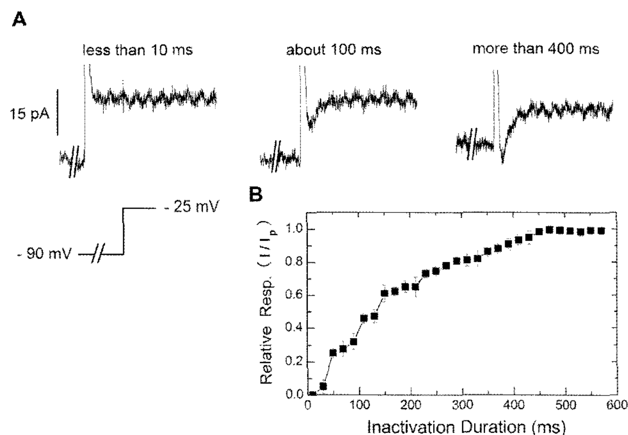

Fig. 3. The dependence of the transient calcium current on pre-inactivation duration. (A) Transient inward current was activated to the maximal value, when sufficient pre-inactivation duration (over 400 ms) was applied. The value of individual current trace was obtained when pre-inactivation duration was applied (a) less than 10 ms, (b) about 100 ms, (c) more than 400 ms. (B) Data marked as squares illustrate that peak amplitudes of transient I_{Ca} varied with changes in duration of pre-inactivation. Relative amplitude was given as individual peak amplitude divided by the greatest peak value (mean±S.D., n=5).

activation started near −10 mV, and the peak amplitude occurred at +15 mV. Fig. 1B shows the current-voltage plot measured from the current traces that were activated by depolarizing pulses (from −65 mV to +55 mV, in 5-mV step) with a prepulse voltage of −90 mV. Data corresponded to the amplitude of inward current measured at the 25 ms after the onset of the pulse (Fig. 1B, open circles) and at the 300 ms after the onset of the pulse (Fig. 1B, filled circles). The results revealed that the present voltage-dependent inward I_{Ca} was completely separated into two components. This clear separation induced by different activation ranges between the two components was a rare event, compared to that of other similar studies on retinal cells (Sullivan & Lasater, 1992; de la Villa et al, 1998).

Dependence of the transient I_{Ca} on prepulse voltage and duration of pre-inactivation

First, in order to find the threshold for activation of transient component, step prepulse was applied, as shown in Fig. 2. When the prepulse voltage was higher than −70 mV, no transient inward current was evoked at all (Fig. 2Aa). As the prepulse voltage fell down below −70 mV and approached about −90 mV, the transient component gradually became evident and its amplitude finally neared saturation value. Fig. 2Ab indicates that the response recorded at around −90 mV corresponds to the greatest amplitude. When the prepulse voltage was applied at a voltage more hyperpolarized than −90 mV, the amplitude was almost the same or rather diminished than the value measured at −90 mV (Fig. 2Ac). These results are summarized in Fig. 2B. The data marked as circles show the normalized peak amplitudes of transient calcium currents (measured at various prepulse voltages from −110 mV to −70 mV in increments of 5 mV). Each data point represents mean±

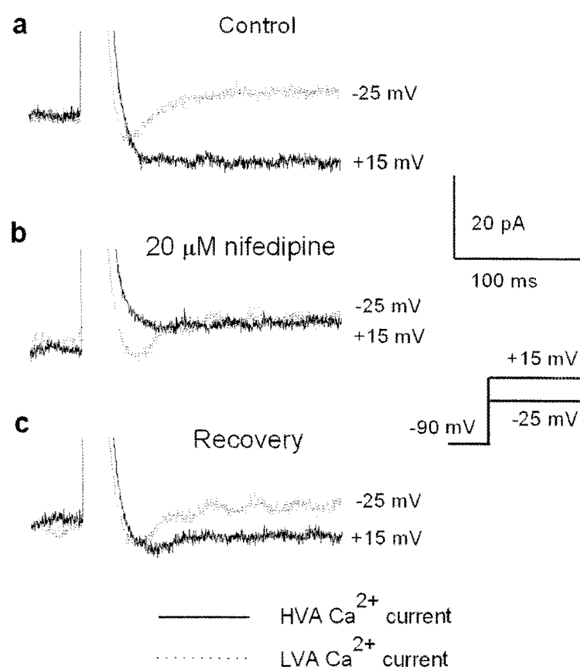

Fig. 4. Effects of nifedipine on two types of calcium currents. Superimposed current traces represented two types of inward currents evoked by depolarizing pulse to −25 mV and +15 mV, from a pre-voltage of −90 mV. The traces shown with a dotted line correspond to representative transient currents in peak amplitude, and the traces shown with a straight line correspond to representative sustained currents in peak amplitudes. The transient component and sustained component (a) before and (b) during the superfusion of control Ringer solution with 20 μM nifedipine, (c) after return to control Ringer solution.

S.D. of H2-type cells (n=5).

Secondly, the dependence of the transient I_{Ca} on pre-inactivation duration was studied and shown in Fig. 3. The transient inward current was not activated in pre-inactivation under 10 ms of duration. The transient component started to activate, when pre-inactivation duration was extended to about 100 ms. When sufficient pre-inactivation duration (over 400 ms) was applied, the transient component approached the maximal value. Fig. 3B demonstrates that the peak amplitude of transient I_{Ca} varied with changes in duration of pre-inactivation. Each data point represents mean±S.D. of H2-type cells (n=5).

Effects of nifedipine on two types of I_{Ca}

In an another trial, we examined the effect of nifedipine (an L-type Ca^{2+} channel blocker) to further investigate the two types of I_{Ca}. Superimposed current traces were selected as representatives for two types of inward currents. These currents were evoked by depolarizing pulses at −25 mV and +15 mV, respectively, from a pre-voltage of −90 mV (Fig. 4a). Through the superfusion of the control Ringer solution that contained 20 μM nifedipine, effects of selective blocking of the sustained component became apparent. Nifedipine blocked only the sustained component, but did not suppress the transient component (Fig. 4b). This pharmacological profile provides some evidence that this sustained I_{Ca} is mediated through the L-type calcium channel. However, it remains controversial whether the transient current corresponds to T-type, because there is a lack of pharmacological identification for the T-type current.

DISCUSSION

This research provides an evidence that two types of voltage-activated I_{Ca} are present in HCs which were freshly dissociated from goldfish retina. One is the sustained type that has occasionally been observed in most of HCs isolated from catfish (Shingai & Christensen, 1983), skate (Malchow et al, 1990), white perch (Lasater, 1986) and goldfish (Tachibana, 1983). The other is the transient I_{Ca}, which has never been found in HCs of any teleost fish retina, except only in cultured HCs of white bass retina (Sullivan & Lasater, 1992). Thus, our present study is the second to report on such transient current of inward I_{Ca} in HCs of teleost fish retina.

Our preparation used in the present study is unique, compared to earlier studies, on HCs from teleost fish retina (Tachibana, 1983; Lasater, 1986; Sullivan & Lasater, 1992), because the specimen (HCs) was freshly dissociated from goldfish retina. Our previous study was the first to be conducted on HCs that were freshly dissociated from goldfish retina using a patch-clamping (Paik et al, 2003). Although goldfish retina has been well studied more than any other fish retina, there are only very few studies with patch-clamping on freshly dissociated HCs. On the other hand, many studies have been conducted on freshly dissociated bipolar cells, because the bipolar cells dissociate easily into its original morphology. In the present study, the use of freshly dissociated HCs enabled us to discover the transient current that has not previously been seen in cultured HCs that were dissociated from goldfish retina.

Interestingly, in our current study, a variety of events were observed in each type of HCs, which were slightly different from the events reported in white bass HCs. The majority of H2 types (or RH types) displayed two types of I_{Ca} in large amplitude, while both H1 and H3 types showed only one type in relatively small amplitude (data not shown). Judging from the pharmacological profile and the threshold voltage for activation, it is highly possible that these I_{Ca} are almost similar to L-type and T-type currents. Notably, as shown in Fig. 1B, each component completely distinguished one from the other: such clear separation of two types of I_{Ca} has rarely been observed in similar studies of retinal cells (Sullivan & Lasater, 1992; de la Villa et al, 1998).

The presence of different I_{Ca} in HCs of goldfish retina, strongly confirms the notion that there is a wide variety of Ca^{2+} channels among various species and tissue types (Llinas & Yarom, 1981; Kaneko et al, 1989; Karschin & Lipton, 1989; Plummer et al, 1989). It is highly likely that individual differences are involved in different roles of I_{Ca} in various cell types (Sullivan & Lasater, 1992). However, in order to clearly elucidate the exact role of I_{Ca} in retinal HCs, further study should be conducted.

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