

Sustained and Transient Calcium Currents in Horizontal Cells of the White Bass Retina

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ABSTRACT Calcium currents were recorded from cultured horizontal cells (HCs) isolated from adult white bass retinas, using the whole-cell patch-clamp technique. Ca^{2+} currents were enhanced using 10 mM extracellular Ca^{2+} , while Na^+ and K^+ currents were pharmacologically suppressed. Two components of the Ca^{2+} current, one transient, the other sustained, were found. The large transient component of the Ca^{2+} current, which has not been seen before in HCs, is similar, but not identical, to the T-type Ca^{2+} current described previously in a variety of preparations. The sustained component of the Ca^{2+} current is similar, but not identical, to the L-type current described in other preparations. FTX, a factor isolated from the venom of the funnel-web spider, *Agelenopsis aperta*, preferentially and irreversibly blocks the sustained component of the Ca^{2+} current at very dilute concentrations. The sustained component of the Ca^{2+} current inactivates slowly, over the course of 15–60 s, in some HCs. This inactivation of the sustained Ca^{2+} current, when present, is primarily voltage dependent rather than Ca^{2+} dependent.

INTRODUCTION

Calcium ions are believed to be involved in the regulation of a wide variety of processes in nervous tissue, including neuronal plasticity, cell death, and neurotransmitter release. The role of Ca^{2+} currents in horizontal cells (HCs) of the retina is unclear, however, because transmitter release from H1-type HCs has been shown to be largely independent of Ca^{2+} (Schwartz, 1982, 1987; Ayoub and Lam, 1985). We have investigated voltage-dependent Ca^{2+} currents in HCs of white bass retina in an effort to draw some conclusions about the functional role of these currents from their characteristic properties.

Horizontal cells are second-order neurons in the retina that respond to levels of background illumination with graded changes in their membrane potential. They integrate information over large spatial regions (Naka, 1972) based on input from photoreceptors (Dowling and Werblin, 1969; Stell and Lightfoot, 1975), and in turn

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they are responsible for the inhibitory surround of the receptive field of bipolar cells (Dowling and Werblin, 1969; Werblin and Dowling, 1969). They also feed information back to photoreceptors (Baylor, Fuortes, and O'Bryan, 1971). In most teleost retinas there are four subtypes of HCs: H1s, H2s, H3s, and H4s. In vivo, HCs of the same subtype are extensively coupled through gap junctions (Naka, 1972). This coupling between like subtypes has made the study of ionic currents in HCs difficult in vivo. The use of single, isolated HCs in culture has overcome this obstacle.

We found two Ca^{2+} currents to be present in isolated HCs of white bass retina, using the whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981): one was a sustained Ca^{2+} current similar to that described previously in HCs of other species, and the other was a large transient current that has not been seen in other HC preparations. The sustained, high-threshold Ca^{2+} current has been found in cultured HCs isolated from the retinas of white perch (Lasater, 1986), goldfish (Tachibana, 1983), catfish (Shingai and Christensen, 1983, 1986), and skate (Malchow, Qian, Ripps, and Dowling, 1990); however, a study of the pharmacology of this current in these cells and a detailed characterization of its properties have not been carried out. Although Shingai and Christensen (1983) report a "fast inward current" in an extracellular solution containing 30 mM calcium and 0 mM sodium, the voltage dependence of this current is almost identical to that of the "slow sustained inward current," and it is much smaller, except at its peak at +30 mV, than the transient Ca^{2+} current described here. The "fast inward current" of Shingai and Christensen (1983) may represent an inactivating component of the sustained Ca^{2+} current.

MATERIALS AND METHODS

Isolation Procedure

Experiments were performed on HCs isolated from the retinas of white bass (*Roccus chrysops*). Details of the isolation procedure have been described for the white perch (Dowling, Pak, and Lasater, 1985; Lasater, 1986). After isolation, the cells were maintained in cell culture for periods up to 30 d. Most experiments were performed on cells that had been maintained from 1 to 7 d in culture.

Cells in culture maintained the characteristic morphology of their type (H1, H2, or H4) (Dowling et al., 1985; Lasater, 1986) and could be easily classified. For unknown reasons, H3-type cells were rarely encountered (see Sullivan and Lasater, 1990).

Experimental Solutions

Until the time of recording, cells were maintained in modified Leibowitz's tissue culture medium (L-15; Gibco Laboratories, Grand Island, NY). Before each experiment the culture medium was replaced with a salt solution consisting of 10 mM CaCl_2 , 130 mM NaCl, 2.8 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 8.4 mM HEPES, 10 μM tetrodotoxin (ITX; Sigma Chemical Co., St. Louis, MO) to block sodium currents, and 10 mM 4-aminopyridine (4-AP; Sigma Chemical Co.) to block the transient outward potassium current, and adjusted to pH 7.6 with HCl. The salt solution was bubbled with O_2 before use. In some experiments, where indicated, the sodium in the salt solution was replaced by an equimolar concentration of choline. For the experiments that generated the concentration-response curve for funnel-web spider toxin (see below), the calcium concentration was only 3 mM.

Test agents barium chloride, cadmium chloride, cobalt chloride, nickel chloride (all from Sigma Chemical Co.), and a purified factor (FTX) derived from the venom of the funnel-web spider, *Agelenopsis aperta* (the kind gift of Dr. R. Llinas, New York University Medical Center, New York, NY) were added directly to the salt solution. For those experiments investigating the effects of barium (Ba^{2+}), Ca^{2+} was removed from the salt solution. Bay K 8644 (Calbiochem Corp., La Jolla, CA) and nifedipine (Sigma Chemical Co.) were first dissolved in DMSO and then added to the salt solution (the DMSO was diluted 1,000–10,000 times). Purified ω -conotoxin fraction GVIA (the kind gift of Dr. B. M. Olivera, University of Utah, Salt Lake City, UT), a peptide from the venom of the snail *Conus geographus*, was dissolved in a salt solution containing 0.2 mg/ml of lysozyme to improve solubility of the toxin. Test agent-containing salt solutions were applied by superfusion of the culture dish, except for the experiments investigating the qualitative effects of FTX (see below). In some, but not all, cases sufficient time (usually 5–10 min) was allowed for full, or near full, recovery from drug effects during superfusion with test agent-free salt solution.

For the experiments investigating the qualitative effects of FTX, 10–30 μl of a 1:1,000 dilution of the column fraction that contained FTX (see Llinas, Sugimori, Lin, and Cherskey, 1989) was added directly to the culture dish during recording to give a final concentration of 1:100,000–1:300,000. For those experiments generating the concentration–response curve for FTX, the recording solution consisted of modified salt solution that contained only 3 mM Ca^{2+} to avoid displacement of the toxin by high Ca^{2+} . The concentration–response curve was generated by superfusion with FTX-containing, low Ca^{2+} -modified salt solution and subsequent superfusion with an FTX-free solution containing 2 mM Co^{2+} and 1 mM Ca^{2+} that was expected to block all Ca^{2+} currents (see below).

The patch pipettes used for recording in these experiments were filled with a solution consisting of 130 mM K-gluconate, 4 mM NaCl, 11 mM EGTA, 1 mM CaCl_2 , 1 mM MgCl_2 , and 8.4 mM HEPES, buffered to pH 7.4 with KOH. The free Ca^{2+} concentration of this solution was calculated to be 8.3 nM.

Recording Procedures

Voltage-clamp experiments were carried out using the whole-cell patch-clamp technique (Hamill et al., 1981) as described previously (Sullivan and Lasater, 1990). The series resistance of the patch electrodes was calculated from the peak capacitive transient current induced by a 10-mV voltage step recorded in the whole-cell patch-clamp configuration. The series resistance of the electrodes was $15 \pm 3.2 \text{ M}\Omega$ (mean \pm SD; $n = 7$) and was not compensated, since the series resistance error at the peak of the largest transient Ca^{2+} current was $< 5 \text{ mV}$ and would typically have been much smaller.

The leakage conductance was assumed to be voltage independent and to reverse around -70 mV (Tachibana, 1983; Lasater, 1986). The conductance of the leakage channels was estimated from the slope of the flattest portion of the I - V curve generated by a ramp stimulus that brought the membrane from -80 to -50 mV , the range of potentials over which the fewest voltage-dependent ionic currents are flowing in HCs. Over this range of potentials, the only current flowing, other than the leakage current, is a small potassium current that is moving outward through the anomalous rectifier channels (Tachibana, 1983; Perlman, Knapp, and Dowling, 1988). As the HC membrane is depolarized from -70 mV , the conductance of the anomalous rectifier channels decreases (Tachibana, 1983; Perlman et al., 1988). This will tend to flatten the slope of the I - V curve at potentials positive to -70 mV , and will lead to an underestimate of the leakage current. After the conductance of the leakage channels was estimated, the magnitude of the leakage current at any membrane potential could be calculated and subtracted from the total current. In this way, linear leakage currents were corrected off-line where indicated. Because of the unavoidable underestimate of leakage current, the

magnitude of the sustained inward Ca^{2+} current will also tend to be underestimated. This underestimate is likely to be small between -70 and -10 mV, the range of membrane potentials normally experienced by HCs in vivo (Dowling and Ripps, 1971; Wu, 1988).

Some voltage error may have occurred in some cells due to an inadequate space clamp. However, there was little evidence for this (i.e., a relaxation of the capacitive transient that could be fit only with more than two time constants, or sudden jumps in the current traces) even in recordings from the largest H4s, and no compensation for this error was attempted here.

Capacitive compensation, stimulus application, data collection, and experimental procedure were the same as described in Sullivan and Lasater (1990). Only cells with resting potentials between -50 and -80 mV were used, except for experiments investigating the effects of Ba^{2+} . Ba^{2+} tended to depolarize the cells, presumably by blocking the anomalous rectifier current (see below). The holding potential was set at -70 mV.

RESULTS

Sustained and Transient Ca^{2+} Currents

A high extracellular concentration of Ca^{2+} was used to enhance Ca^{2+} currents. Sodium currents were blocked by TTX, and the transient outward potassium current was blocked by 4-AP. The sustained outward potassium current (I_K) in bass HCs is not effectively blocked by extracellular tetraethylammonium (TEA; Sullivan and Lasater, 1990), nor by intracellular TEA (Sullivan, J. M., and E. M. Lasater, unpublished observations); therefore, TEA was not added to the recording or patch pipette solutions. Although intracellular Cs^{2+} was found to reduce both transient and sustained potassium currents (Sullivan, J. M., and E. M. Lasater, unpublished observations), the effects of Cs^{2+} took up to 15 min to reach a steady state in these large cells. This time course prohibited the use of Cs^{2+} in these experiments. I_K may have had a minor contaminating effect on current records at membrane potentials more positive than -10 mV, but this potassium current does not activate in these cells until the membrane potential rises above -10 mV, and does not become large until the membrane potential reaches $+50$ mV (Sullivan and Lasater, 1990). Therefore, it would not have reduced the apparent size of the Ca^{2+} currents at membrane potentials below -10 mV, the voltage region of most interest to us because -10 mV is the upper limit of the range of membrane potentials experienced by these cells in vivo (Dowling and Ripps, 1971; Wu, 1988).

Two components of the Ca^{2+} current isolated in this manner were seen: a transient component ($I_{\text{Ca}^{2+}\text{TR}}$) and a sustained component ($I_{\text{Ca}^{2+}\text{S}}$). The size of each component varied considerably from cell to cell, with no correlation between the relative sizes of the two components in individual cells. Generally, however, both components tended to be larger in H4-type HCs than in H2s, especially the transient component (Fig. 1). Both components were too small to be measured reliably in H1s, although Ca^{2+} currents were clearly present in this type of HC in the presence of Ca^{2+} current agonists (see Fig. 12). No run-down of $I_{\text{Ca}^{2+}\text{S}}$ was ever seen, even when cells were held for >20 min ($n = 10$). A small decline in $I_{\text{Ca}^{2+}\text{TR}}$ over 5–15 min was sometimes seen (6 of 10 HCs), but this current was never eliminated, even after holding the cell for >20 min. Experiments on individual cells rarely lasted longer than 10–15 min, and the occasional small decline in $I_{\text{Ca}^{2+}\text{TR}}$ did not interfere with our interpretation of the results.

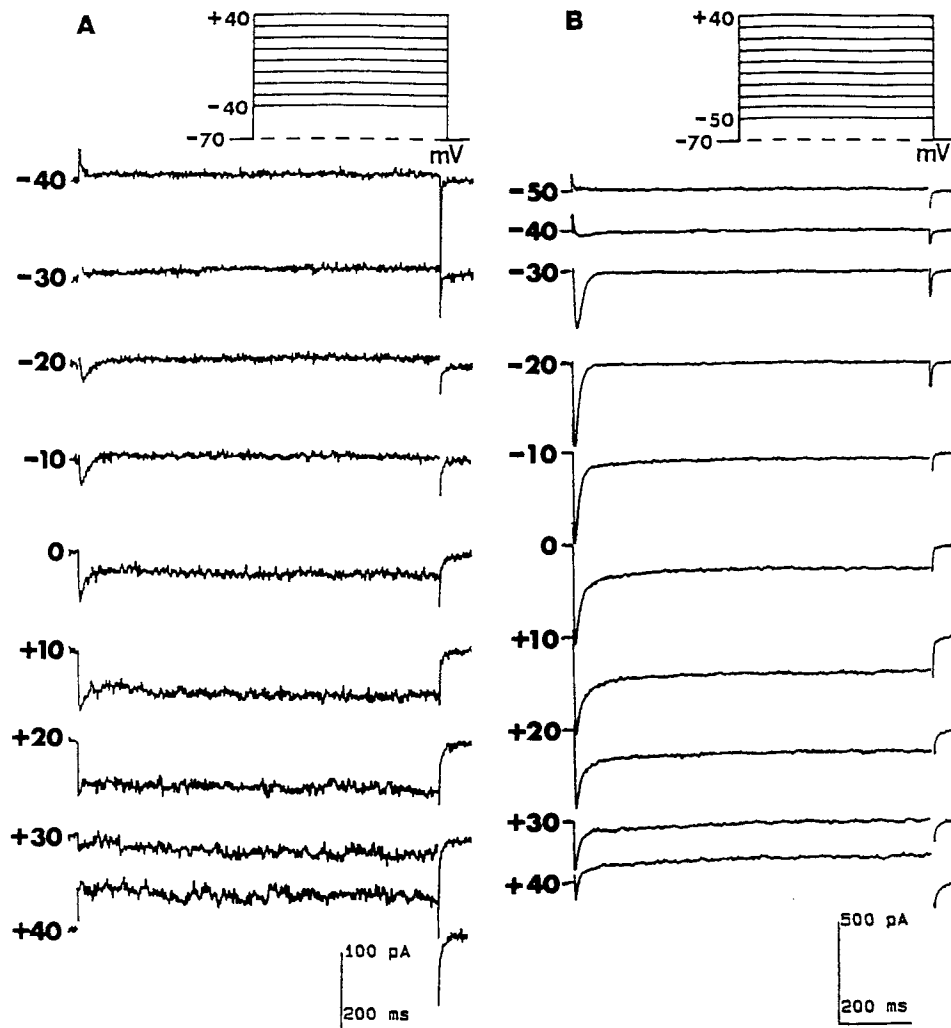


FIGURE 1. Examples of the sustained and transient components of the Ca^{2+} current. (A) Ca^{2+} currents in an H2-type retinal horizontal cell. (B) Ca^{2+} currents in an H4-type retinal horizontal cell. Although the magnitude of each of the components varied from cell to cell, these currents tended to be smaller in H2s than in H4s, and were very small in H1-type horizontal cells. Whole-cell Ca^{2+} currents were obtained from single, isolated horizontal cells in response to a series of voltage step commands from a holding potential of -70 mV to the levels indicated to the left of the corresponding current trace. Leakage currents were very small in both of these cells, and were not subtracted here. Capacitive transients have been removed for clarity.

Although the kinetics of $I_{\text{Ca}^{2+}\text{TR}}$ are similar to those of the sodium current in the closely related white perch (Lasater, 1986), the transient inward current seen here in HCs is not a TTX-resistant sodium current. Fig. 2 shows an example of a prominent $I_{\text{Ca}^{2+}\text{TR}}$ recorded in a modified salt solution in which sodium was replaced by an equimolar concentration of choline. (The leakage current was large in this modified salt solution and included a component with a reversal potential more positive than

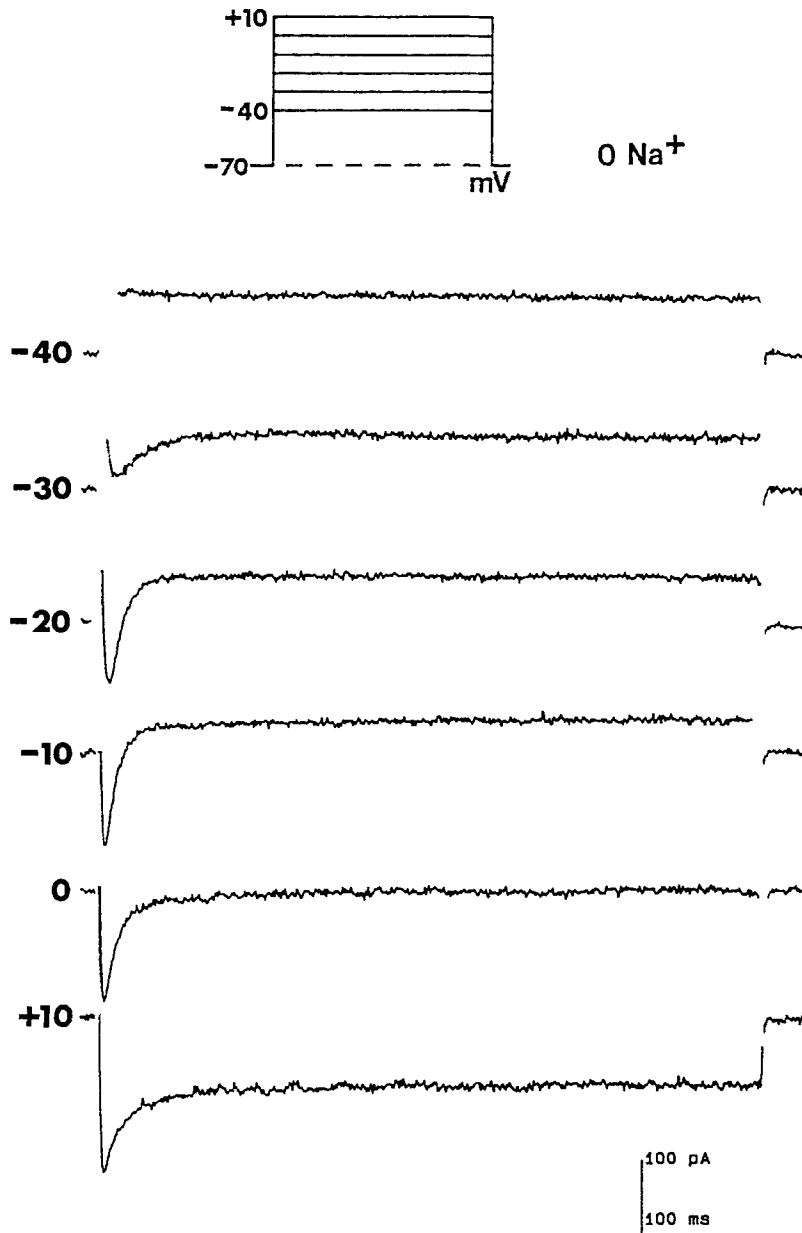


FIGURE 2. The transient component of the Ca^{2+} current was present in a sodium-free bathing solution. A large transient component of the Ca^{2+} current was clearly visible in this H4-type HC when equimolar choline replaced the sodium in the control salt solution. Current traces in this figure were only partially corrected for linear leakage current. The leakage currents in the choline bathing solution were larger than those in the sodium solution, and included a component that had a reversal potential more positive than -70 mV, which caused the cells to depolarize and made it difficult to calculate the leakage current component of the total current accurately. Capacitive transients have been removed for clarity. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV.

-70 mV which caused the HCs to depolarize and decline rapidly, so the choline bathing solution was not used regularly). In addition, $I_{Ca^{2+}TR}$ and $I_{Ca^{2+}S}$ were both completely and reversibly blocked by cobalt (Fig. 3), further supporting the notion that $I_{Ca^{2+}TR}$ is carried by Ca^{2+} ($n = 16$).

Figs. 4 and 5 show the I - V relationship for $I_{Ca^{2+}TR}$ and $I_{Ca^{2+}S}$, respectively, in H2s and H4s. $I_{Ca^{2+}TR}$ activated at membrane potentials above -60 to -50 mV, and exhibited a broad peak between 0 and +10 mV in H2s ($n = 24$) and between -20

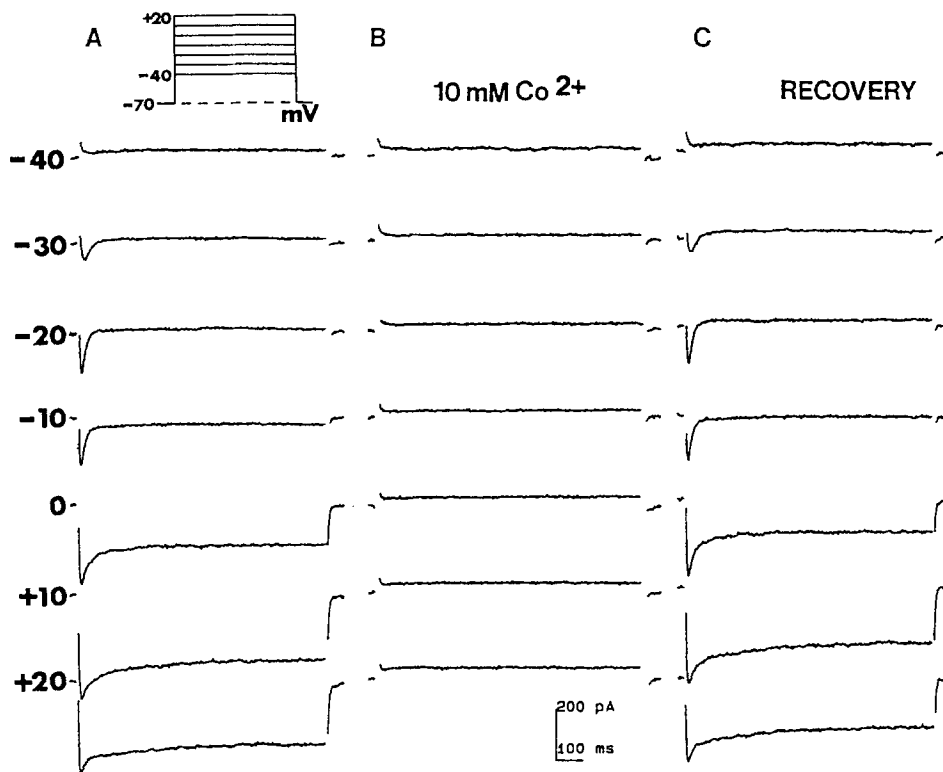


FIGURE 3. The transient and sustained components of the Ca^{2+} current were both rapidly and reversibly blocked by cobalt. Transient and sustained components of the Ca^{2+} current before (A), during (B), and after (C) superfusion of this H4-type HC with the control salt solution with 10 mM cobalt added. Linear leakage currents were corrected for in these traces. Capacitive transients have been removed for clarity. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV.

and 0 mV in H4s ($n = 24$). The inactivation of $I_{Ca^{2+}TR}$ could be fit by a single exponential in both H2s and H4s, with a time constant at -10 mV of 32.3 ± 6.9 ms (mean \pm SD; $n = 8$) in H2s, and 16.2 ± 1.53 ms ($n = 8$) in H4s. These values for the threshold of activation, the inactivation time constants, and the range of membrane potentials at which $I_{Ca^{2+}TR}$ peaks suggest that the transient component of the Ca^{2+} current is similar to the T current of other CNS neurons (Carbone and Lux, 1984,

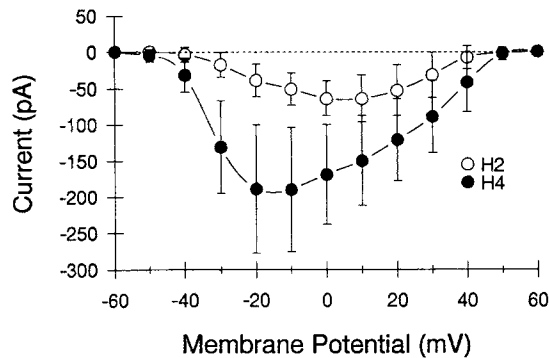


FIGURE 4. I - V relationship of the transient component of the Ca^{2+} current in H2-type (open circles) and H4-type (filled circles) HCs. The magnitude of the transient component was measured at each potential as the difference between the peak inward current and the sustained current at the end of the 1-s voltage step command pulse. Because the sustained current may not be fully ex-

pressed by the time the transient current reaches its peak (see Fig. 6), the magnitude of the transient component may be slightly underestimated. Each data point represents the mean \pm SD of 24 cells. Recording conditions and data collection were as described in Fig. 1.

1987; Nowycky, Fox, and Tsien, 1985; Fox, Nowycky, and Tsien, 1987; Karschin and Lipton, 1989; Ozawa, Tsuzuki, Iino, Ogura, and Kudo, 1989).

$I_{\text{Ca}^{2+}\text{S}}$ was not significantly activated until the membrane was stepped to potentials above -30 to -20 mV, and peaked at around $+10$ mV in both H2s ($n = 24$) and H4s ($n = 24$). The membrane potentials at which $I_{\text{Ca}^{2+}\text{S}}$ activates and peaks are very similar to the sustained Ca^{2+} currents recorded previously in HCs isolated from other species of fish (Tachibana, 1983; Lasater, 1986; Malchow et al., 1990). Although the Ca^{2+} current appears to reverse between $+40$ and $+50$ mV, this outward current probably reflects unblocked I_{K} and uncompensated leakage current. The presence of these outward currents will lead to an underestimate of the magnitude of $I_{\text{Ca}^{2+}\text{S}}$. This underestimate is likely to be quite small at potentials within the normal range of HC membrane potentials in vivo, where I_{K} is not activated and the driving force for the leakage current is not so great, but it could be larger at more positive potentials. The I - V characteristics of the sustained component of the Ca^{2+} current suggest that this

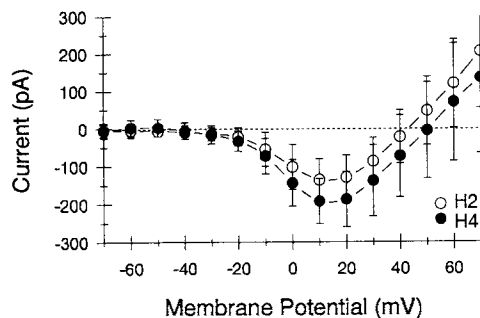


FIGURE 5. I - V relationship of the sustained component of the Ca^{2+} current in H2-type (open circles) and H4-type (filled circles) HCs. The magnitude of the sustained component was measured at the end of a 1-s voltage step command after correction for linear leakage currents. Because the leakage currents were slightly underestimated, $I_{\text{Ca}^{2+}\text{S}}$ was probably underestimated as well. The underestimate of $I_{\text{Ca}^{2+}\text{S}}$ is likely to be quite small at

potentials within the in vivo range of HC membrane potentials (see text). Each data point represents the mean \pm SD of 24 cells. Recording conditions and data collection were as described in Fig. 1.

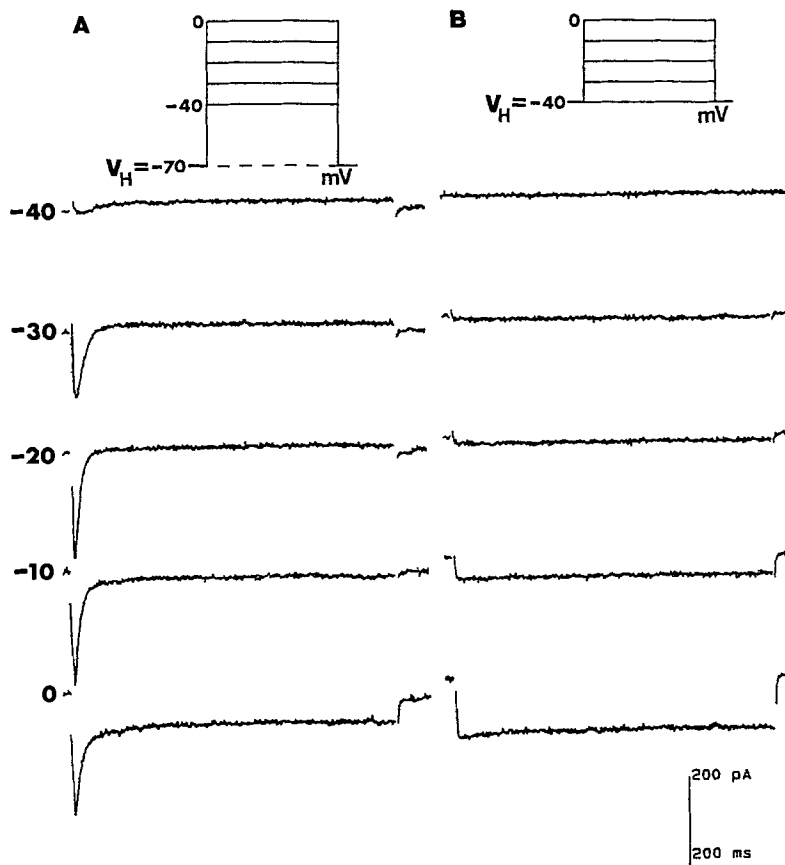


FIGURE 6. Differentiation between the transient and sustained components of the Ca^{2+} current by holding potential. (A) From a holding potential of -70 mV, voltage step-commands (as indicated to the left of the corresponding traces) elicited transient and sustained components of the Ca^{2+} current in this H4-type HC. (B) From a holding potential of -40 mV, the transient component of the Ca^{2+} current was eliminated, and voltage step commands elicited only the sustained component. The sustained component of the Ca^{2+} current appeared to be slightly larger when the cell was held at -40 mV before depolarizing step commands; however, this was probably due to steady-state inactivation of the small component of the transient outward potassium current that is insensitive to block by 4-AP (Sullivan and Lasater, 1990). Capacitive transients have been removed for clarity.

current is analogous to the L current of other CNS neurons (Carbone and Lux, 1984; Nowycky et al., 1985; Carbone and Lux, 1987; Fox et al., 1987).

Effect of Holding Potential

Fig. 6, A and B, shows that $I_{\text{Ca}^{2+}\text{-TR}}$ was inactivated when the holding potential of the membrane was shifted from -70 to -40 mV ($n = 8$). The sustained component of the Ca^{2+} current actually appeared to increase slightly with this change in holding potential. This increase was probably due to steady-state inactivation of the small

4-AP-resistant component of the transient outward potassium current in these cells (Sullivan and Lasater, 1990).

A double-pulse paradigm was used to investigate the voltage dependence of the inactivation of $I_{Ca^{2+}TR}$ (Fig. 7). The membrane potential was held at -70 mV and then stepped to a conditioning potential before being stepped to the test potential of -10 mV. The conditioning steps ranged from -90 to $+20$ mV in 10 -mV increments, and lasted for 100 ms. The peak of the transient inward current evoked by the test step was reduced as the conditioning step depolarized the membrane to potentials above -80 mV. $I_{Ca^{2+}TR}$ was half-inactivated after the membrane was stepped to -45 mV, and was fully inactivated after stepping the membrane to 0 mV ($n = 7$). It is interesting that over the in vivo range of depolarized potentials at which these HC

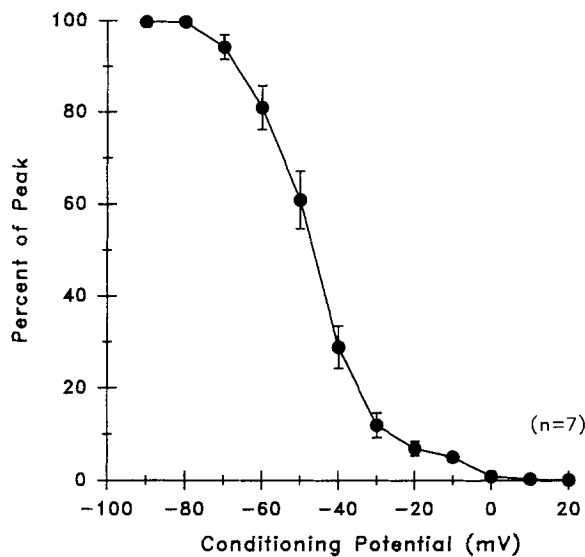


FIGURE 7. Voltage dependence of inactivation of the transient component of the Ca^{2+} current. The membrane potential was held at -70 mV before stepping to a conditioning potential for 100 ms. The conditioning potential ranged from -90 to $+20$ mV, in 10 -mV increments. After the conditioning potential, the membrane was stepped to a test potential of -10 mV. The peak of $I_{Ca^{2+}TR}$ was measured and plotted as a percentage of the largest current generated (the current generated by the test potential after a conditioning potential of -90 mV) versus

the level of the conditioning potential. Each data point represents the mean \pm SD of seven cells. $I_{Ca^{2+}TR}$ began to inactivate at a membrane potential between -80 and -70 mV, and was fully inactivated by 0 mV.

membranes sit under conditions of little or no illumination (~ -40 to -20 mV), the transient Ca^{2+} current is $>70\%$ inactivated.

The time dependence of the recovery from inactivation of $I_{Ca^{2+}TR}$ was studied by holding the membrane at -10 mV to inactivate the transient current and then stepping to -80 mV before stepping back to -10 mV (Fig. 8). As the time spent at -80 mV increased, the magnitude of the current evoked by the test step to -10 mV also increased. The current recovered to half of its control value (the size of the current evoked by the test step from a holding potential of -80 mV) when the membrane was held at -80 mV for 100 ms, and was fully recovered after 340 ms at -80 mV ($n = 3$).

These results suggest that the two components of the Ca^{2+} current are carried through two different types of channels, and are not the result of partial inactivation of a single channel type. The finding that $I_{Ca^{2+}TR}$ is inactivated at a holding potential

of -40 mV—a potential at which $I_{Ca^{2+}TR}$ is barely activated from a holding potential of -70 mV, and $I_{Ca^{2+}S}$ is not activated at all—is consistent with the interpretation that inactivation of $I_{Ca^{2+}TR}$, like the T current (Fox et al., 1987), is voltage dependent, not Ca^{2+} dependent.

Pharmacology

As seen in Fig. 9, when 3–5 mM Ba^{2+} replaced 10 mM Ca^{2+} in the bathing solution, $I_{Ca^{2+}S}$ was enhanced, while $I_{Ca^{2+}TR}$ was reduced or no longer visible ($n = 7$). It is not clear whether the channels carrying the transient current are less permeable to Ba^{2+} than they are to Ca^{2+} , or if the apparent reduction in $I_{Ca^{2+}TR}$ is the result of this current being obscured by the very large current flowing through the sustained current channels when Ba^{2+} replaces Ca^{2+} in the bathing solution. At any rate, the

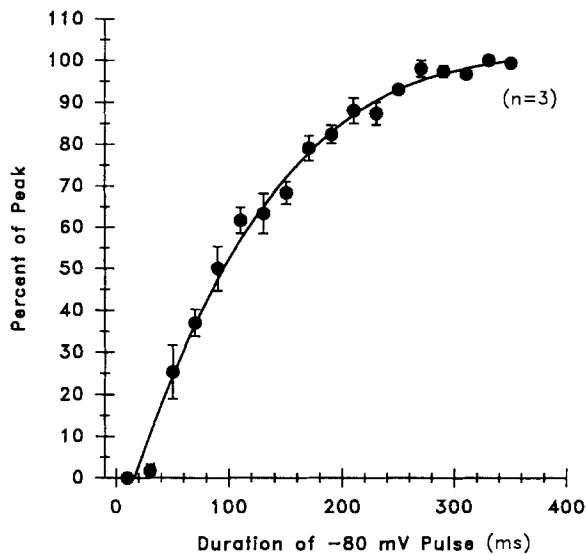


FIGURE 8. Time dependence of the recovery from inactivation of the transient component of the Ca^{2+} current. The membrane potential was held at -10 mV to inactivate $I_{Ca^{2+}TR}$, and then stepped to -80 mV for varying lengths of time before returning to -10 mV. The peak of $I_{Ca^{2+}TR}$ was measured and plotted as a percentage of the largest current generated versus the duration of the -80 -mV pulse. Each data point represents the mean \pm SD of one H2-type and two H4-type HCs. The curve is the best fit through the points. $I_{Ca^{2+}TR}$ took ~ 340 ms to recover fully from inactivation.

differential effect of Ba^{2+} and Ca^{2+} on the magnitude of the two components of the Ca^{2+} current further supports the conclusion that they are carried through two different types of channels (Nilius, Hess, Lansman, and Tsien, 1985; Nowycky et al., 1985; Carbone and Lux, 1987; Fox et al., 1987). Fig. 10 shows that when 10 mM Ba^{2+} replaced the Ca^{2+} in the salt solution, the magnitude of $I_{Ca^{2+}S}$ was enhanced almost fourfold and the voltage dependence of activation was shifted ~ 10 mV in the hyperpolarizing direction ($n = 8$). This enhancement of $I_{Ca^{2+}S}$ by Ba^{2+} , and the shift in voltage dependence, is characteristic of the sustained Ca^{2+} current described in other systems (Tachibana, 1983; Carbone and Lux, 1987; Fox et al., 1987; Karschin and Lipton, 1989).

In Figs. 9 and 10, an increase in the amount of current required to hold the membrane at -70 mV can be seen in the presence of Ba^{2+} . This is presumably due to blockade of the anomalous rectifier current (Tachibana, 1983; Shingai and Chris-

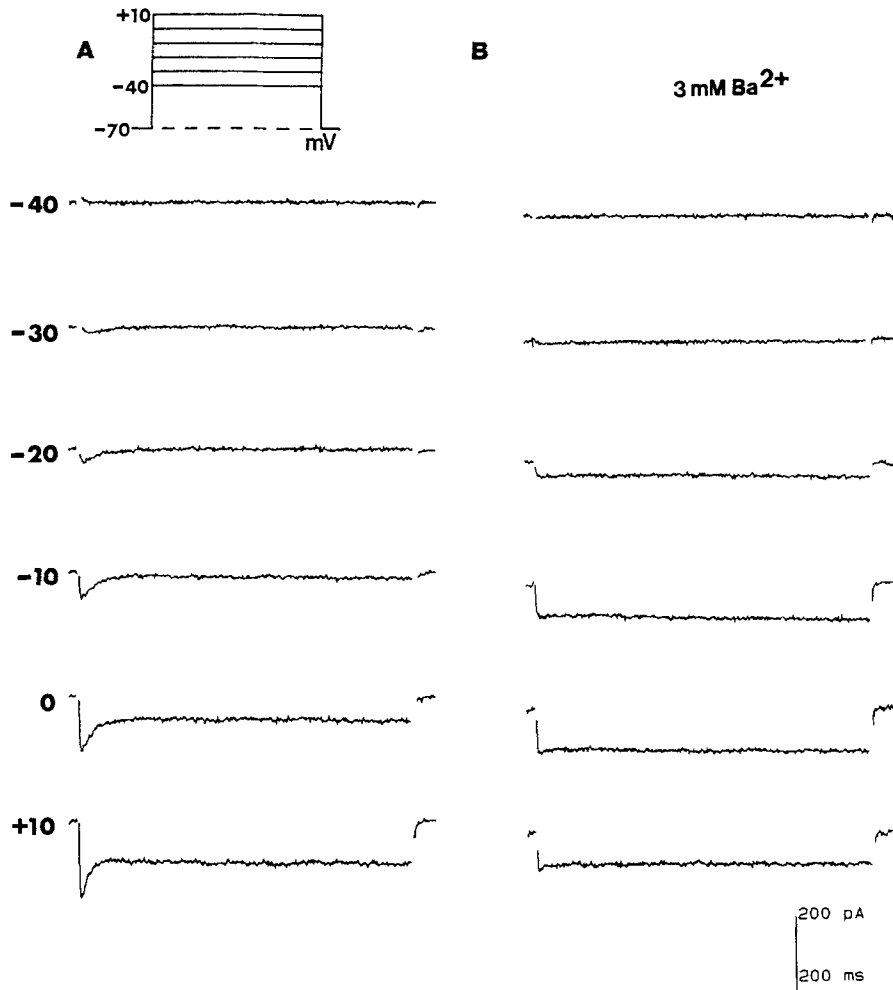


FIGURE 9. Enhancement of the sustained, but not the transient, component of the Ca^{2+} current by substitution of 3 mM Ba^{2+} for 10 mM Ca^{2+} in the bathing solution. When 3 mM Ba^{2+} replaced 10 mM Ca^{2+} in the bathing solution (B), the transient component of the Ca^{2+} current, present in the control salt solution (A), was eliminated in this H2-type HC, while the sustained component was enhanced. The apparent lack of alignment of the current traces in this figure is the result of the increase in current required to hold the membrane at -70 mV after blockade by Ba^{2+} of the anomalous rectifier current, which is believed to be responsible for maintaining the normal resting potential (see text). Linear leakage currents were corrected for in these traces. Capacitive transients have been removed for clarity. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV.

tensen, 1986; Sullivan, J. M., and E. M. Lasater, unpublished observations), which is believed to be responsible for maintaining the resting membrane potential of isolated HCs close to the equilibrium potential for potassium (Tachibana, 1981; Lasater, 1986; Shingai and Christensen, 1986). After blocking the small amount of current

that flows outward through anomalous rectifier channels at membrane potentials more positive than E_K , the remaining currents carried by permeant cations with equilibrium potentials more positive than E_K are opposed only by leakage current, and a larger holding current is required to maintain the membrane potential at -70 mV (Newman, 1989).

At submicromolar concentrations (0.05 – 0.1 μM), Bay K 8644, the dihydropyridine agonist, preferentially enhanced $I_{Ca^{2+}S}$ with little or no change in $I_{Ca^{2+}TR}$ ($n = 7$), as shown in Fig. 11. This differential effect of Bay K on $I_{Ca^{2+}TR}$ and $I_{Ca^{2+}S}$ suggests, again, that these are two different currents, analogous to the T- and L-type currents of other preparations (Nilius et al., 1985; Nowycky et al., 1985; Hirning, Fox, McCleskey, Olivera, Thayer, Miller, and Tsien, 1988; Karschin and Lipton, 1989). At concentrations of 1 – 10 μM , Bay K 8644 produced a large increase in $I_{Ca^{2+}S}$ that was sometimes accompanied by a small increase in $I_{Ca^{2+}TR}$ ($n = 12$). In Fig. 12, an

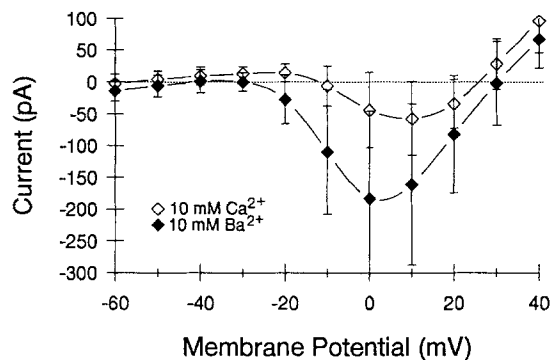


FIGURE 10. I - V plot of the sustained Ca^{2+} current in retinal horizontal cells showing the enhancement of amplitude and shift in voltage dependence when 10 mM Ba^{2+} (filled diamonds) replaced 10 mM Ca^{2+} (open diamonds) in the control salt solution. The magnitude of the sustained component of the current was measured at the end of a 1 -s voltage step command. Each data point represents the mean \pm SD of eight cells (four H2-type HCs, four H4-type HCs). Note the increase in current required to hold the membrane at -70 mV in 10 mM Ba^{2+} , due to blockade of the anomalous rectifier current, which is believed to be responsible for maintaining the normal resting potential (see text). These data were not corrected for leakage current. Holding potential, -70 mV.

example of the effects of 5 μM Bay K on an H1-type HC can be seen. The Ca^{2+} current in H1s was usually too small to be measured, but in four of four H1s tested, a prominent Ca^{2+} current could be seen after superfusion with Bay K-containing salt solution.

Surprisingly, the dihydropyridine antagonist nifedipine, which characteristically reduces the L-type current (Fox et al., 1987; Hirning et al., 1988; Karschin and Lipton, 1989), had no consistent effect on the HC Ca^{2+} currents at concentrations ranging from 10 to 50 μM ($n = 8$). The membrane potential at which the cell was held did not influence the inability of nifedipine to block $I_{Ca^{2+}S}$. Although high (30 mM) extracellular Ca^{2+} has been shown to reduce the effectiveness of blockade of Ca^{2+} currents in heart cells by the dihydropyridine antagonist nitrendipine (Lee and Tsien, 1983), the 10 mM extracellular Ca^{2+} used in our experiments is not likely to be responsible for the complete lack of response to nifedipine seen in most of the HCs tested.

Two sets of experiments were carried out to investigate the effects of FTX on the Ca^{2+} current components: one qualitative and one quantitative. The qualitative studies were carried out by adding directly to the culture dish 10–30 μl of a stock solution consisting of a 1:1,000 dilution of the column fraction containing FTX. The final concentration of toxin in these experiments ranged from 1:100,000 to 1:300,000. In all cases ($n = 8$), the addition of FTX eliminated 60–70% of the

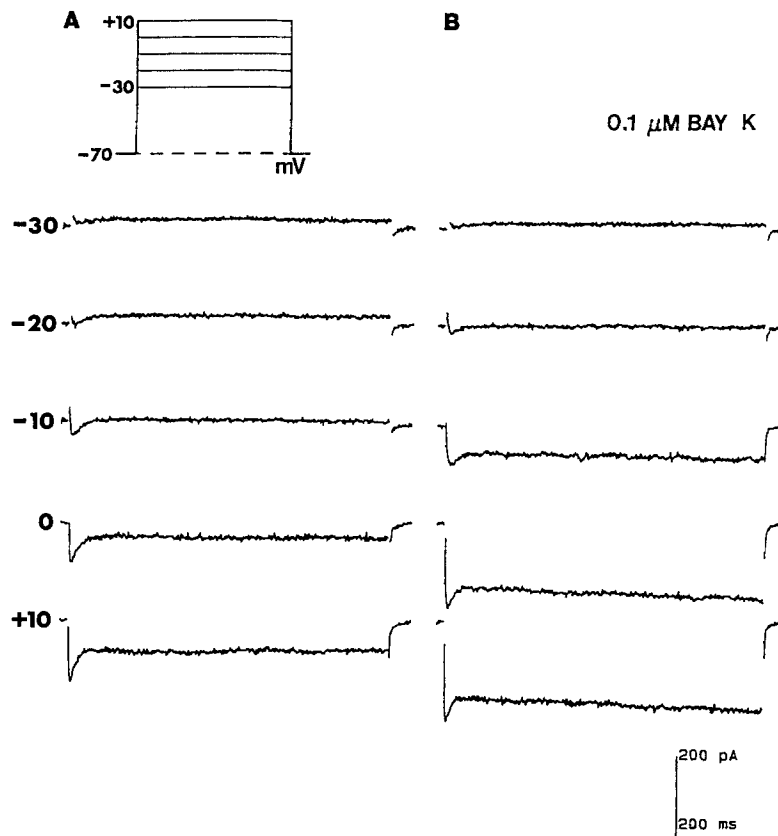


FIGURE 11. Enhancement of the sustained, but not the transient, component of the Ca^{2+} current by submicromolar concentrations of the dihydropyridine agonist Bay K 8644 (*B*), relative to currents in the control salt solution (*A*). Ca^{2+} currents in this example were recorded from an H2-type HC. The bathing solution in *B* was a salt solution containing 0.1 μM Bay K 8644. Capacitive transients have been removed for clarity. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV.

sustained Ca^{2+} current, while the peak transient component was reduced by 30–40% or less (Fig. 13, *A* and *B*). The block of $I_{\text{Ca}^{2+}\text{S}}$ was essentially irreversible, even after washing for up to 20 min ($n = 5$).

Because of the novelty of FTX, a concentration–response curve for the toxin was generated. To reduce the possibility of displacement of FTX by calcium ions, the concentration of Ca^{2+} in the recording solution was reduced from 10 to 3 mM for

these experiments. This reduction in Ca^{2+} concentration did not change the threshold for activation of $I_{\text{Ca}^{2+}\text{S}}$ or the membrane potential at which this component peaked, although the mean amplitude of the sustained current was reduced due to the reduction in driving force for Ca^{2+} . To generate the dose-response curve, four

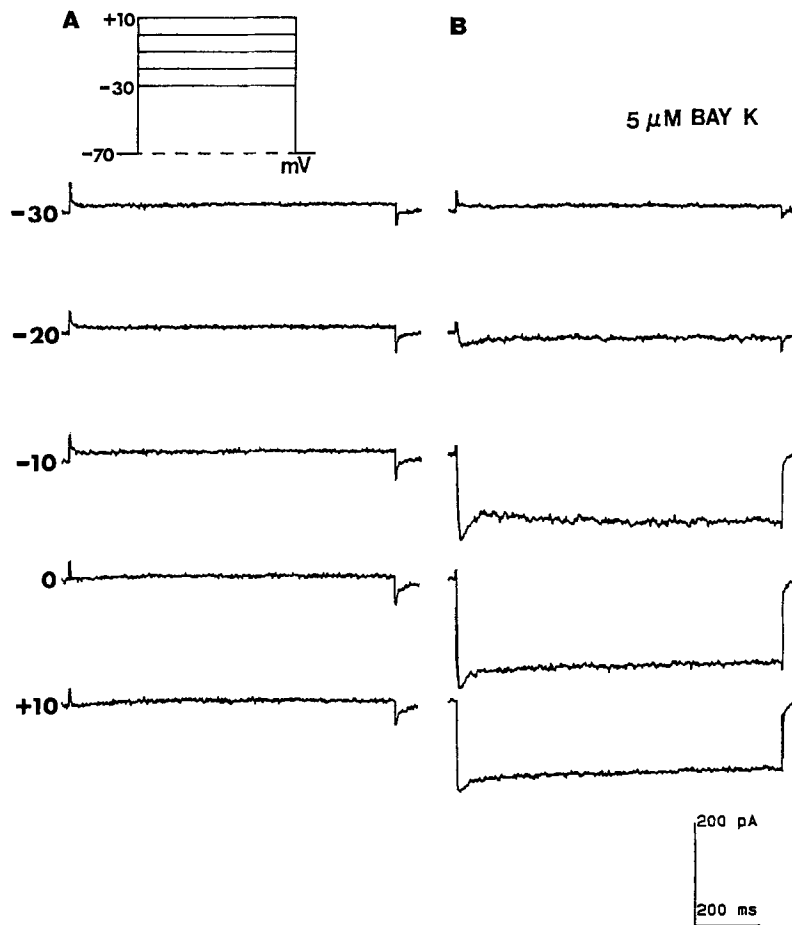


FIGURE 12. At micromolar concentrations, the dihydropyridine agonist Bay K 8644 produced an enhancement of both transient and sustained components of the Ca^{2+} current in this H1-type retinal horizontal cell (*B*). The transient and sustained components of the Ca^{2+} current were characteristically small for this HC cell type in control salt solution (*A*). The bathing solution in *B* was a salt solution containing 5 μM Bay K 8644. Capacitive transients have been removed for clarity. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV.

dilutions of the column fraction containing FTX were tested (Fig. 13 *C*); a maximum block was achieved with a 1:300,000 dilution.

Neither cadmium nor nickel, in concentrations ranging from 10 to 50 μM , selectively blocked either of the two Ca^{2+} current components. Even at these low micromolar concentrations, Cd^{2+} ($n = 16$) and Ni^{2+} ($n = 10$) reduced both $I_{\text{Ca}^{2+}\text{TR}}$

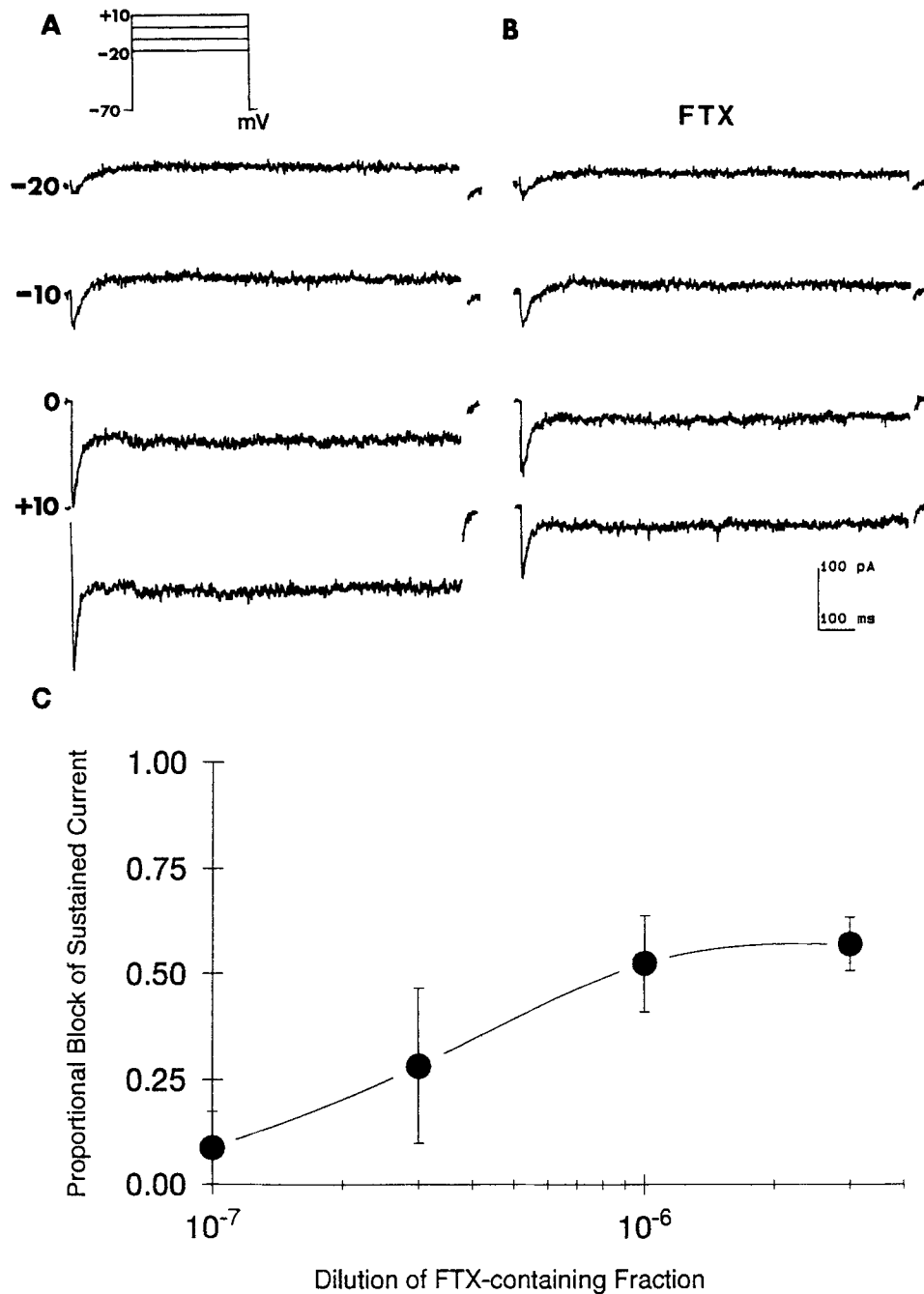


FIGURE 13. FTX, a factor derived from the venom of the funnel-web spider, irreversibly blocks the sustained component of the Ca^{2+} current at very low concentrations, but has a much smaller effect on the transient component of the Ca^{2+} current. Transient and sustained components of the Ca^{2+} current in an H2-type HC before (*A*) and after (*B*) direct addition to the

and $I_{Ca^{2+}S}$ comparably. These findings are in contrast to the results of others, who have found that 10–200 μM Cd^{2+} preferentially reduces the L and N currents (Nilius et al., 1985; Nowycky et al., 1985; Fox et al., 1987; Ozawa et al., 1989; Suzuki and Rogawski, 1989), while 25–500 μM Ni^{2+} selectively reduces the T current (Fox et al., 1987; Ozawa et al., 1989; Suzuki and Rogawski, 1989). In addition, the peptide ω -conotoxin had no effect on either component at concentrations up to 5 μM ($n = 5$). Previously, ω -conotoxin has been found to block the L and N currents irreversibly (McCleskey, Fox, Feldman, Olivera, Tsien, and Yoshikami, 1986; Fox et al., 1987; McCleskey, Fox, Feldman, Cruz, Olivera, Tsien, and Yoshikami, 1987). However, there have been recent reports that ω -conotoxin is ineffective in producing an irreversible blockade of high-threshold Ca^{2+} currents in some preparations (Suzuki and Rogawski, 1989), including retinal ganglion cells (Karschin and Lipton, 1989; Lasater and Witkovsky, 1990).

Inactivation of the Sustained Ca^{2+} Current

When the HC membrane was depolarized to around 0 mV from a holding potential of -70 mV, $I_{Ca^{2+}S}$ was sometimes seen to decline over 15–60 s (Fig. 14 B). However, in other cells, little or no inactivation was seen (Fig. 14 A). Inactivation, when present, was most likely to be voltage dependent rather than Ca^{2+} -dependent: when 2.5–10 mM Ba^{2+} replaced the Ca^{2+} in the salt solution, there was little or no change in the rate of inactivation seen over 15 s ($n = 14$). In five cells, two recorded in high Ca^{2+} and three recorded in 5 mM Ba^{2+} , the inactivation of $I_{Ca^{2+}S}$ at +10 mV was fit by a single exponential with a time constant of 13.5 ± 2.3 s (mean \pm SD). Evidence of inactivation was also seen in several experiments carried out with a low intracellular EGTA concentration suggesting that the EGTA was not masking a Ca^{2+} -dependent inactivation.

Low concentrations of Bay K (0.05–5 μM) also produced little or no change in the inactivation rate over 15 s ($n = 8$), in spite of large increases in $I_{Ca^{2+}S}$. Inactivation of $I_{Ca^{2+}S}$ was observed even after extensive washing of the culture dish with 10 mM Ba^{2+} -containing salt solution, suggesting that the decline in inward current, when seen, is not due to the activation of a Ca^{2+} -dependent potassium current. (In addition, HCs, including those of white bass, characteristically lack a Ca^{2+} -dependent

culture dish of 10 μl of a 1:1,000 dilution of the column fraction containing FTX, to give a final concentration in the dish of 1:300,000. Linear leakage currents were corrected for in these traces. Capacitive transients have been removed for clarity. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV. (C) Dose-response curve for four dilutions of the column fraction containing FTX. Dilutions tested were 1:300,000, 1:1,000,000, 1:3,000,000, and 1:10,000,000. Each point represents the mean \pm SD of four cells. The magnitude of block was calculated by comparing the peak sustained current after superfusion with the appropriate dilution of FTX (drug currents) to the peak current obtained in 3 mM Ca^{2+} salt solution (control currents). The values for drug and control currents were generated by subtracting the current traces obtained during block of the Ca^{2+} current by superfusion with cobalt from the traces obtained in FTX-containing 3 mM Ca^{2+} -salt solution, and in FTX-free 3 mM Ca^{2+} -salt solution, respectively. Holding potential was -40 mV, to inactivate the transient component of the Ca^{2+} current.

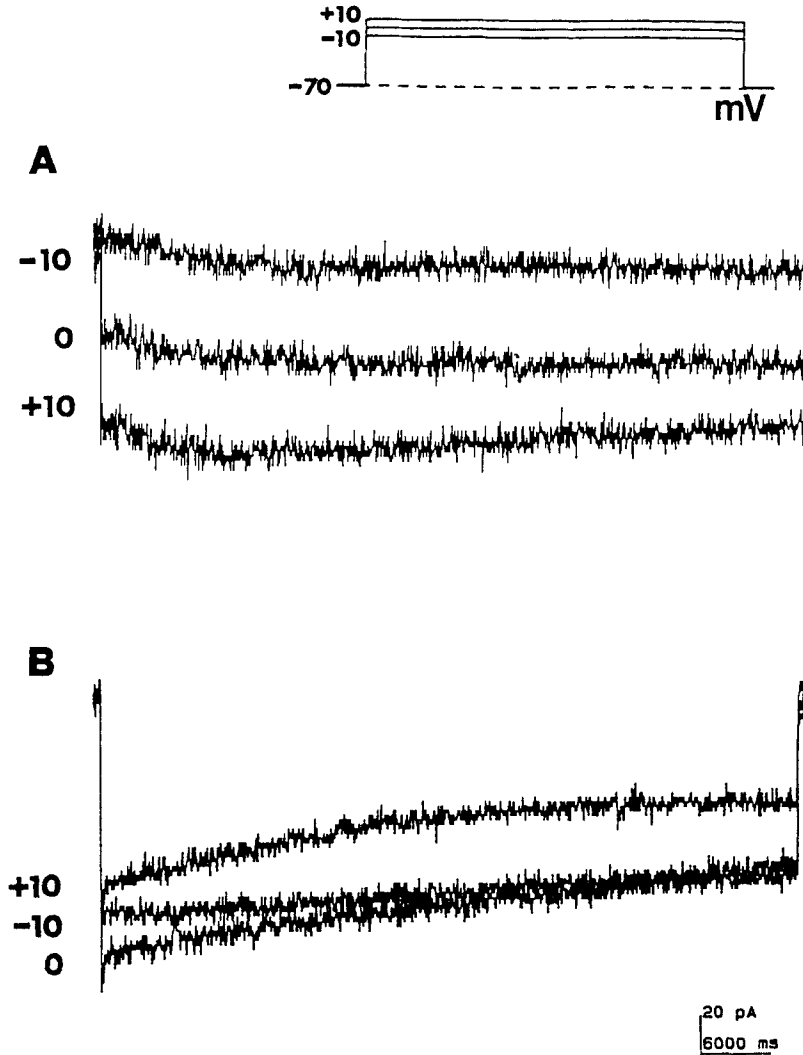


FIGURE 14. Inactivation of the sustained component of the Ca^{2+} current was sometimes, but not always, seen when the membrane was held at a depolarized potential for 1 min. In *A*, an example of a cell (in this case, an H2-type HC) in which the sustained component of the Ca^{2+} current did not inactivate with time when the membrane potential was stepped to -10 or 0 mV. Some inactivation of $I_{\text{Ca}^{2+}\text{S}}$ was seen in this cell when the membrane was stepped to $+10$ mV. The apparent increase in the inward current over the first 10 s was probably due to the inactivation of the small component of the transient outward potassium current that is insensitive to block by 4-AP (Sullivan and Lasater, 1990). In *B*, an example of a cell (in this case, an H4-type HC) in which the sustained component of the Ca^{2+} current did inactivate with time during a 1-min depolarizing step command. Linear leakage currents were corrected for in these traces. The voltage step command potential is indicated to the left of the corresponding current trace. Holding potential, -70 mV.

potassium current [Tachibana, 1983; Lasater, 1986; Shingai and Christensen, 1986; Sullivan and Lasater, 1990]). Finally, the decline in inward current is not due to a time-dependent increase in a voltage-dependent potassium current, because in three cells in which inactivation was seen in control salt solution, there was no time-dependent change in the magnitude of the current evoked by stepping the membrane to -10 , 0 , and $+10$ mV in the presence of 10 mM cobalt. We conclude that the decline in inward current reflects a voltage-dependent inactivation of $I_{Ca^{2+}S}$.

DISCUSSION

This study demonstrates the existence of two Ca^{2+} currents, one sustained and one transient, in horizontal cells isolated from white bass retina. Interestingly, previous studies in goldfish (Tachibana, 1983), catfish (Shingai and Christensen, 1983, 1986) and white perch (Lasater, 1986) found only a single sustained $I_{Ca^{2+}}$ in horizontal cells. We have characterized $I_{Ca^{2+}TR}$ and $I_{Ca^{2+}S}$ with regard to their voltage dependence as well as their pharmacology in an effort to better understand the role of Ca^{2+} currents in HCs.

Sustained and transient Ca^{2+} currents have been identified previously in a wide variety of preparations. The sustained, high-threshold Ca^{2+} current, referred to as the L current (Nowycky et al., 1985) or high voltage-activated (HVA) current (Carbone and Lux, 1984), is believed to be responsible for the "after-depolarization" seen in some preparations following the sodium spike (Llinas and Yarom, 1981a, b; Wong and Prince, 1981). A transient, low-threshold Ca^{2+} current, referred to as the T current (Nilius et al., 1985; Nowycky et al., 1985) or low voltage-activated (LVA) current (Carbone and Lux, 1984), has also been reported in some preparations. Interestingly, a transient, low-threshold Ca^{2+} current has been found in tiger salamander retinal bipolar cells (Maguire, Maple, Lukasiewicz, and Werblin, 1989), in mouse retinal bipolar cells (Kaneko, Pinto, and Tachibana, 1989), and in rat retinal ganglion cells (Karschin and Lipton, 1989). This transient current is believed to play a role in rhythmic membrane oscillation and to enhance low-threshold depolarizations (Llinas and Yarom, 1981b; Jahnsen and Llinas, 1984). A third Ca^{2+} current, referred to as the N current, which inactivates with time and has a high threshold for activation, has also been described in some preparations (Nowycky et al., 1985; Fox et al., 1987; Ozawa et al., 1989). The N current has been hypothesized to play a role in Ca^{2+} transmitter release coupling (Hirning et al., 1988). There was no indication of an N current in cultured white bass HCs.

This represents the first time that a large transient component of the Ca^{2+} current has been seen in HCs. In addition, this study provides further proof that the transient component of the Ca^{2+} current is present in adult vertebrate nervous tissue (Kaneko et al., 1989; Karschin and Lipton, 1989; Maguire et al., 1989). The transient component seen in white bass HCs has a number of features in common with the T current described in other preparations: the threshold for activation is above -60 mV, the time constant for inactivation is 15–35 ms, and inactivation is voltage dependent. In addition, the transient current peaks over a range of membrane potentials from -20 to $+10$ mV, inactivates at a holding potential of -40 mV, and is carried very poorly by Ba^{2+} (Nowycky et al., 1985; Carbone and Lux, 1987; Fox et al., 1987; Ozawa et al., 1989). However, unlike the T current, the transient component of

the Ca^{2+} current in white bass HCs is not preferentially blocked by micromolar concentrations of nickel. This sets it apart from other T currents and distinguishes it pharmacologically.

The sustained component has several features in common with the L current described in other preparations. It has a threshold for activation above -30 mV, is larger when Ba^{2+} replaces Ca^{2+} in the bathing solution, and is enhanced by Bay K 8644 (Nowycky et al., 1985; Fox et al., 1987; Hirning et al., 1988). However, FTX, a factor derived from the venom of the funnel web spider, preferentially blocks this component, suggesting that $I_{\text{Ca}^{2+}\text{S}}$ also has features in common with the P current, a sustained Ca^{2+} current with a low threshold that has been identified in Purkinje cells and the terminal of the squid giant axon (Llinas et al., 1989). In addition, unlike the L current, the sustained component of the Ca^{2+} current in white bass HCs is not preferentially blocked by micromolar concentrations of cadmium or ω -conotoxin, nor is it antagonized by nifedipine. Thus, this retinal current also has its distinguishing pharmacological features.

The finding that $I_{\text{Ca}^{2+}\text{TR}}$ and $I_{\text{Ca}^{2+}\text{S}}$ each has a constellation of pharmacological and kinetic properties that is unique to white bass HCs supports the notion that there is a wide variety of Ca^{2+} channels among different species and tissue types (Kaneko et al., 1989; Karschin and Lipton, 1989; Llinas et al., 1989; Plummer, Logothetis, and Hess, 1989). Only now are we beginning to appreciate the diversity of Ca^{2+} channel types that have been described (Bean, 1989). It is likely that these individual differences reflect differences in the functions of these currents in different cell types.

In teleost HCs, the release of GABA from the H1-type cell has been shown to have a large Ca^{2+} -independent component (Schwartz, 1982, 1987; Ayoub and Lam, 1985). Thus, the primary role of Ca^{2+} currents in HCs may not be Ca^{2+} transmitter release coupling. The transient current might contribute to that portion of transmitter release that is dependent on Ca^{2+} , or might simply serve to help shape the response of the HC membrane at the termination of a bright light stimulus, when the membrane potential rises rapidly from a strongly hyperpolarized potential to a more depolarized potential. For example, in many species including some teleosts, when the horizontal cell is hyperpolarized by light there is a transient "off" spike of the membrane potential at light offset. It has been proposed that the transient nature of the spike might be due to the activation of an outward potassium current (for example, see Stone and Witkovsky, 1987). But, because of its inactivating nature, $I_{\text{Ca}^{2+}}$ could significantly shape this spike. This latter role is similar to that of the transient low-threshold Ca^{2+} current in other preparations, which serves to enhance low-threshold depolarizations (Llinas and Yarom, 1981*b*; Jahnsen and Llinas, 1984). In any case, $I_{\text{Ca}^{2+}\text{TR}}$ would seem to be well suited to HCs, being activated over almost the entire range of membrane potentials normally experienced by these cells in vivo.

In contrast to the transient current, the sustained Ca^{2+} current will be activated only when the HC membrane is relatively depolarized; that is, in the presence of little or no background illumination. This sustained inward current could aid in maintaining the membrane potential of the cell at a depolarized level, much as the high-threshold Ca^{2+} current in other preparations is responsible for the after-depolarization seen following the sodium spike (Llinas and Yarom, 1981*a, b*; Wong and Prince, 1981). $I_{\text{Ca}^{2+}\text{S}}$ could thereby serve to reduce the amount of photoreceptor

neurotransmitter required to hold the HC membrane at chronically depolarized potentials.

It is expected that any effect of transient and sustained Ca²⁺ currents on the HC membrane potential will *secondarily* affect Ca²⁺-independent release of transmitter from HCs. However, it is important to keep in mind that the experiments investigating the Ca²⁺ independence of neurotransmitter release have only measured the Ca²⁺ independence of GABA release, and thus far have only used H1-type HCs. This correlates with our finding that the Ca²⁺ currents in H1s are almost immeasurably small. It may be that other HC types that possess larger Ca²⁺ currents exhibit a strong coupling between Ca²⁺ entry and transmitter release. It is therefore possible that while GABA release from H1s is primarily Ca²⁺ independent, other (as yet unidentified) neurotransmitters undergo Ca²⁺-dependent release from some or all HC types. Further insight into the role of Ca²⁺ currents in HCs must await studies identifying the transmitters of the other HC types, and the subsequent demonstration of the Ca²⁺ dependence or independence of their release.

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