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Separation of Ionic Currents in the Somatic Membrane of Frog Sensory Neurons

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Summary. Electrical properties of isolated frog primary afferent neurons were examined by suction pipette technique, which combines internal perfusion with current or voltage clamp using a switching circuit with a single electrode. When K^+ in the external and internal solutions was totally replaced with Cs^+, extremely prolonged Ca spikes, lasting for 5 to 10 sec, and Na spikes, having a short plateau phase of 10 to 15 msec, were observed in Na^+-free and Ca^{2+}-free solutions, respectively. Under voltage clamp, Ca^{2+} current (I_{Ca}) appeared at around -30 mV and maximum peak current was elicited at about 0 mV. With increasing test pulses to the positive side, I_{Ca} became smaller and flattened but did not reverse. Increases of $[Ca]_o$ induced a hyperbolic increase of I_{Ca} and also shifted its I–V curve along the voltage axis to the more positive direction. Internal perfusion of F^- blocked I_{Ca} time-dependently. The Ca channel was permeable to foreign divalent cations in the sequence of $I_{Ca} > I_{Ba} > I_{Sr} \gg I_{Mn} > I_{Zn}$. Organic Ca-blockers equally depressed the divalent cation currents dose- and time-dependently without shifting the I–V relationships, while inorganic blockers suppressed these currents dose-dependently and the inhibition appeared much stronger in the order of $I_{Ba} = I_{Sr} > I_{Ca} > I_{Mn} = I_{Zn}$.

Key Words frog sensory neuron · internal perfusion · Na spike · Ca spike · ionic currents · Ca current

Introduction

Frog spinal ganglion cells are capable of producing action potentials in Ca^{2+}-free solution containing EDTA, while membrane excitability is lost in Na^+-free solution (Koketsu & Koyama, 1962). However, in the absence of Na^+, the ganglion cells produce extremely prolonged action potentials if TEA^+ is present (Koketsu, Cerf & Nishi, 1959). The results suggest that both Na^+ and Ca^{2+} carry inward currents during depolarization of the ganglion cells. These conductances have not been characterized as completely as those in axons (Hodgkin & Huxley, 1952) and large snail neurons (Akaike, Lee & Brown, 1978*b*; Brown, Morimoto, Tsuda & Wilson, 1981; Akaike, Ito, Nishi & Oyama, 1982; Oyama, Nishi, Yatani & Akaike, 1982), because of the following difficulties: separation of conductance in the axon from that in the nerve cell body, separation of the ionic currents, and adequate space clamp of the ionic current by a single electrode. The combination of both the suction pipette method of intracellular perfusion and the voltage-clamp system with switching circuit modified and developed in our laboratory makes it possible to investigate ionic mechanisms of the excitable membrane in this ganglion cell under controlled external and internal ionic conditions.

We report here the electrical and pharmacological properties of currents in these perfused cells determined by voltage clamp.

Materials and Methods

The experiments were carried out on isolated dorsal root ganglion cells of the American bullfrog (*Rana catesbeiana*). Isolation of neurons and adequacy of internal perfusion by the suction pipette method have been described (Hattori et al., 1983).

A schematic diagram of the experimental arrangement and the voltage-clamp circuit is shown in Fig. 1. Voltage-clamp circuit for a single electrode was used (Wilson & Goldner, 1975). Before the neuron was aspirated by a suction electrode, the system was tested to ascertain both the maximum sampling rate and current injection that the suction electrode would allow. The electrode could carry average current of more than 100 nA at a switching frequency of 10 kHz without showing signs of polarization or other artifacts. Switching frequency of 10 kHz was used. A phase compensation feed-back loop allows stability of the clamp circuit. The voltage clamp was tuned to give the fastest possible response, as follows: the holding potential was set to the resting potential, the gain was gradually increased, and the compensation circuit was adjusted. As the gain reached its maximum level, the circuit was delicately adjusted until the voltage trace was critically clamped. In this system the series resistance (R_s) does not affect performance.

Na^+ current (I_{Na}), and Ca^{2+} current (I_{Ca}) were monitored on a storage oscilloscope (Tektronix 5113), simultaneously recorded with a photosensitive paper recorder system (Medelec, MS 6), and stored on a digital tape recorder (Kennedy 9700C). The linear components of the transient capacitative and leakage currents associated with the ionic currents were subtracted during the experiments by adding the current responses to equal

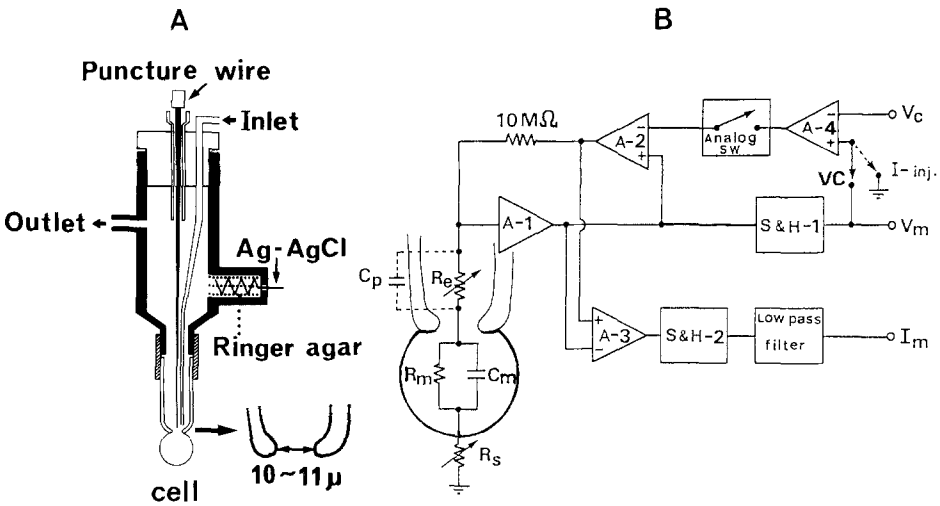

Fig. 1. Schematic illustration of a suction electrode and current or voltage-clamp circuits. (*A*): A newly modified suction electrode. Membrane potential and current injection were performed through a 1 mm diameter Ag-AgCl wire mounted into Ringer-agar. (*B*): Switching circuit for a single electrode and the equivalent electrical circuit of neuron aspirated by a suction electrode. R_m and C_m, membrane resistance and capacitance; R_s, series resistance; R_e and C_p, electrode resistance and capacitance; V_c, command signal; V_m, membrane potential; and I_m, average membrane current. In the voltage-clamp mode, output from *S & H-1* is feed-back to the high-gain differential amplifier *A-4* while the *A-4* input is grounded in the current injection mode. *A-1* is a high input impedance and low input capacity preamplifier. Low input capacitance (capacity compensation) is obtained by the *A-1* power supply with the potential of the input signal plus the flowing power supply voltage. Membrane potential is sampled by the sample- and hold-amplifier *S & H-1* when the analog switch is opened. *A-2* is the voltage-to-constant-current converter. When the analog switch is open, the potential across 10 MΩ is zero and no current flows through it. Current is measured across 10 MΩ by the differential amplifier *A-3* and *S & H-2* when the analog switch is closed. This pulsed output is filtered by a low-pass filter to obtain average current. The changes of electrode resistance (R_e) and series resistance (R_s) do not affect performance

Table 1. Ionic composition of external and internal test solutions (mM)

External solution	NaCl	Tris-Cl	KCl	CsCl	$CaCl_2$	$MgCl_2$	Glucose	TEA-Cl	4-AP
Normal	112		2		2		5		
Na-free		112	2		2		5		
Ca-free	112		2			5	5		
Na-, Ca-free		112	2			5	5		
Na-, Ca-, K-free		81		2		5	5	25	3
I_{Ca} ES[a]		87		2	2		5	25	3

Internal solution	KCl	K-aspartate	Cs-aspartate	TEA-Cl	EGTA	CsF
Normal	30	100			5	
I_{Ca} IS[b]			105	25	5	
CsF				25	5	105

Various K^+-free external test solutions (ES) were made by substituting Cs^+ for K^+ in normal, Na^+-free, Ca^{2+}-free, and Na^+- and Ca^{2+}-free external solutions. Various divalent cation solutions were made by the replacement of Ca^{2+} in I_{Ca} ES with other divalent cations.

[a] External solution. [b] Internal solution.

but opposite voltage steps using a signal averager (Nihon Kohden, ATAC-150).

Isolated neurons were perfused with the external and internal solutions throughout the experiments. The composition of the main test solution is shown in Table 1. The Ca^{2+} current (I_{Ca}) was separated from I_{Na} and K^+ current (I_K). I_{Na} was removed by the replacement of Na^+ in the external solution with $Tris^+$. I_K was suppressed by substituting Cs^+ for K^+ in both external and internal solutions and by adding TEA^+ to both solutions (Akaike et al., 1978*b*; Akaike, Nishi & Oyama, 1981*b, c*; Akaike et al., 1982). 4-aminopyridine (4-AP) was also added to the external solution to block a transient fast K^+ current (I_A) (Connor & Stevens, 1971; Thompson, 1977; Akaike et al., 1983*a*). For study of the Ba^{2+}, Sr^{2+}, Mn^{2+} and Zn^{2+} currents, Ca^{2+} in the external solution was replaced by equimolar Ba^{2+}, Sr^{2+}, Mn^{2+} or Zn^{2+}, respectively. The pH of all external and internal solutions, except that for recording I_{Zn}, was adjusted to pH 7.4 with Tris. The solution for I_{Zn} was adjusted to pH 6.8 (Oyama et al., 1982). Experiments were carried out at room temperature of 18 to 20 °C.

A modified suction pipette method (Akaike, Nishi & Oyama, 1981*b*; Oyama et al., 1982) was used for voltage clamp

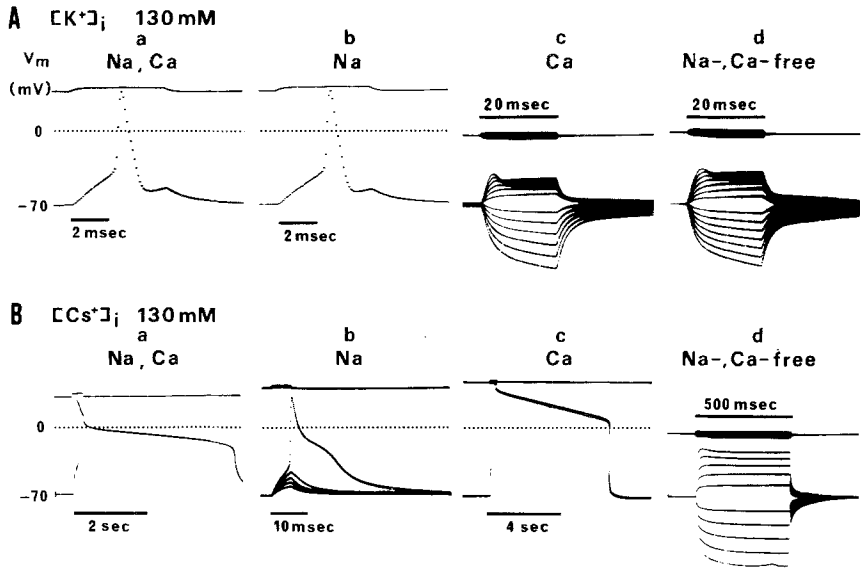

Fig. 2. Membrane potentials in various external and internal test solutions. (*A*): Membrane potentials in normal (*a*), Ca^{2+}-free (*b*), Na^+-free (*c*), and Na^+- and Ca^{2+}-free (*d*) external solutions. The neuron was internally perfused with an internal solution containing 130 mM K^+. In normal and Ca^{2+}-free solutions, the action potentials were elicited by currents between 0.5 to 0.8 nA. The electrotonic potentials in both Na^+-free and Na^+- and Ca^{2+}-free test solutions were evoked by currents of 0.2, 0.5, 0.8, 1.1, 1.5, 1.8 and 0.2, 0.5, 0.8, 1.1, 1.5, 1.8, 2.1 nA, respectively. Note no spikes but the anomalous potassium rectification in the record *c*. The potassium rectification decreased in the Na^+- and Ca^{2+}-free solution (*d*). All records were obtained from the same neuron. Membrane potentials were fixed at −70 mV by DC current injection. (*B*): Membrane potentials of neurons perfused with K^+-free Cs^+ external and internal solutions. Na^+ and Ca^{2+} spike (*a*), Na^+ spike (*b*), Ca^{2+} spike (*c*), and electrotonic potentials evoked by currents of 0.2, 0.5, 0.8, 1.1 and 1.5 nA (*d*). Note the marked prolongation of action potentials in records *a* and *c*. Different time scales are given for each record

and internal perfusion. 3-mm OD Pyrex® glass was pulled to a shank length of 2.5 to 3 mm. The tip of the pipette was cut at about 40 μm OD and then fire polsihed to 10 to 11 μm ID. The same suction pipette was used for the enzymatically treated ganglion cells throughout all present experiments for a few months.

Drugs employed in the present experiments are: tetrodotoxin (Sankyo), diltiazem (Tanabe), verapamil (Knoll), D-600 (Knoll), trypsin (Sankyo), and collagenase Type I (Sigma). They were dissolved in solution just before use and the pH was adjusted if necessary.

Results

Membrane Potentials of the Dorsal Root Ganglion Cells

When a nerve cell body immersed in normal external solution was aspirated with suction pipette containing normal internal solution, a huge negative potential shift of more than −100 mV occurred. Successive electrical ruptures, of the aspirated membrane by application of 10 to 50 msec oscillatatory square wave pulses of 5 to 20 nA depolarizing constant current gave resting membrane potential of −70 to −90 mV. Resting potentials were greater than values measured with the glass microelectrode technique; i.e., −65 to −85 mV in the isolated frog, bullfrog, and toad dorsal ganglion cells (Ito, 1957; Koketsu et al., 1959; Nishi, Minota & Karczmar, 1974). Thereafter, neurons perfused with normal external solution with 2 mM K^+ and internal solution with 130 mM K^+ were hyperpolarized to −95 to −100 mV within 10 min. The measured potentials were close to the K^+ equilibrium potential (E_K) of −105.1 mV. At this stage, membrane resistance estimated from the current-voltage relationships was 31 ± 3 MΩ ($n=8$, mean ± SE). Also, the suprathreshold depolarizing current pulses evoked all-or-none action potentials with overshoots of +30 to +40 mV. The results suggest that resting and action potentials of dorsal root ganglion cells are unaffected by enzymatic digestion for about 15 min.

Membrane Potentials in the Presence or Absence of K^+ Conductance

Bathing the ganglion in a Ca^{2+}-free medium hyperpolarized neurons maintained at −70 mV by DC current injection by another 10 to 15 mV. However, the configuration of action potential was unchanged as long as the resting potential was maintained at −70 mV (Fig. 2*A* (*a, b*)). Replacement of Na^+ by $Tris^+$ also hyperpolarized the resting potential maintained at −70 mV by 10 to

15 mV, but in this condition the neurons no longer produced all-or-none action potentials and just showed a marked K^+ rectification (Fig. 2*A* (*c*)). The K^+ rectification was considerably reduced in the Na^+- and Ca^{2+}-free external solution (Fig. 2*A* (*d*)).

In contrast, when K^+ in the external and internal solutions was successively replaced with Cs^+ in the presence of both TEA and 4-AP, a prolonged action potential with a slower rising phase appeared (Fig. 2*B* (*a*)). The degree of prolongation of action potential was variable among individual cells. The configuration of the action potential was not affected by the removal of Na^+ only in the external solution (Fig. 2*B* (*c*)). These graded voltage-dependent action potentials were sensitive to external Ca^{2+} concentration and were blocked by organic and inorganic Ca^{2+}-antagonists such as diltiazem (3×10^{-5} M), verapamil (10^{-5} M), D-600 (10^{-5} M), Co^{2+}, Ni^{2+}, and Cd^{2+}, as has been found in other excitable tissues (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Bayer, Kaufman & Mannhold, 1975; Kostyuk, Krishtal & Shakhovalov, 1977; Akaike et al., 1978*b*; Akaike, Brown, Nishi & Tsuda, 1981; Akaike et al., 1982). Unexpectedly, there was a "pure" Na-spike in the K^+- and Ca^{2+}-free external solution, which was followed by a plateau phase of 10 to 15 msec (Fig. 2*B* (*b*)). Both the peak and plateau phases in the action potential were sensitive to tetrodotoxin (10^{-6} g/ml) but were not affected by external application of Co^{2+} and Cd^{2+}. On internal and external perfusion of the neurons with Na^+-, K^+-, and Ca^{2+}-free solutions, the electrotonic potentials were symmetrical at hyperpolarizing and depolarizing current pulses as compared with those in the presence of K^+ ion (Fig. 2*A* (*d*) and *B* (*d*)).

Elimination of Outward Current

Under voltage-clamp condition, it was difficult to analyze the inward currents in dorsal root ganglion cells because of contamination by the outward K^+ current (I_K), as has been found in other preparations (Kostyuk et al., 1977; Akaike et al., 1978*b*; Lee et al., 1979). The usual I_K blockers, TEA and 4-AP (Thompson, 1977; Akaike et al., 1978*b*; Gustafsson, Galvan, Grafe & Wigström, 1982; Akaike et al., 1983*a*), did not completely suppress I_K in this neuron. Therefore, the dorsal root ganglion cells were perfused externally and internally with solutions containing K^+ blockers and Cs^+. Perfusion with this solution finally abolished the outward currents so that the inward currents, which consisted of two components, fast and slow, became large and distinguishable.

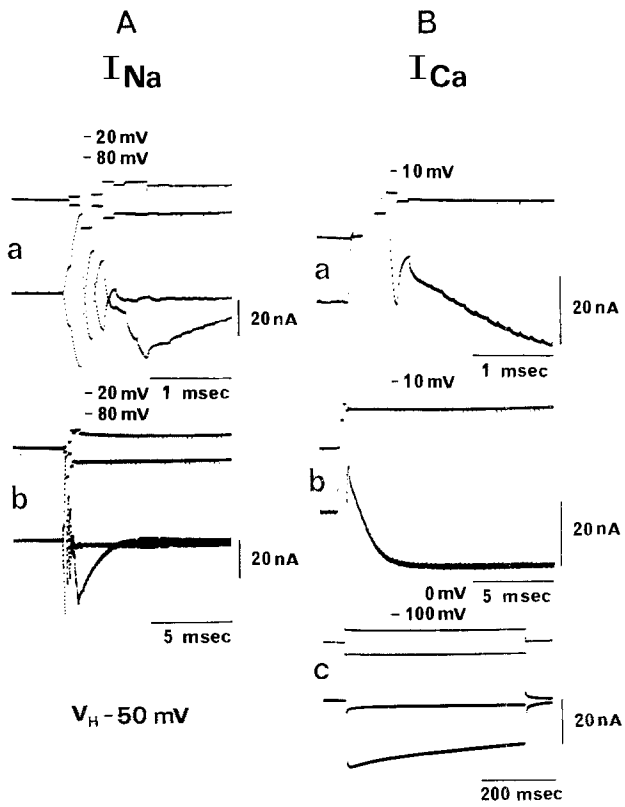

Fig. 3. Sodium and calcium currents (I_{Na} and I_{Ca}) in frog dorsal root ganglion cells. (*A* (*a* and *b*)): The voltage steps were to −30 and +30 mV and were applied from holding potential (V_H) of −50 mV. The I_{Na} arose rapidly and reached its peak level within 1 msec. In this case the switching frequency was 10 kHz. Membrane potential almost changed to the new value within 0.5 msec. The capacitive current samples are off scale. (*B* (*a–c*)): I_{Ca} arose slowly as compared with I_{Na}. Clamp currents elicited by voltage steps are indicated by the numbers on each voltage trace. Note a very slow inactivation process of I_{Ca} (*c*)

Separation of Inward Currents

The fast component of the inward current disappeared following equimolar substitution of $Tris^+$ for Na^+ or application of TTX, indicating that the fast inward current was carried by Na^+. Moreover, when the Na^+ inward current (I_{Na}) was blocked with TTX or substitution with $Tris^+$ for Na^+, the amplitude and time course of the remaining slow inward current did not change, suggesting that Na^+ did not play a role in the appearance of the slow inward current. The transient peak of I_{Na} appeared at about 1 msec after a depolarizing step from a holding potential (V_H) of −50 to −20 mV (Fig. 3*A* (*a, b*)). However, at larger depolarizing voltages the peak of the current was often obscured by the capacitative current transient, and also the initial 500 μsec was not resolved in the voltage-clamp circuit used in the present experiments. Therefore, analysis of I_{Na} was not carried out in this study.

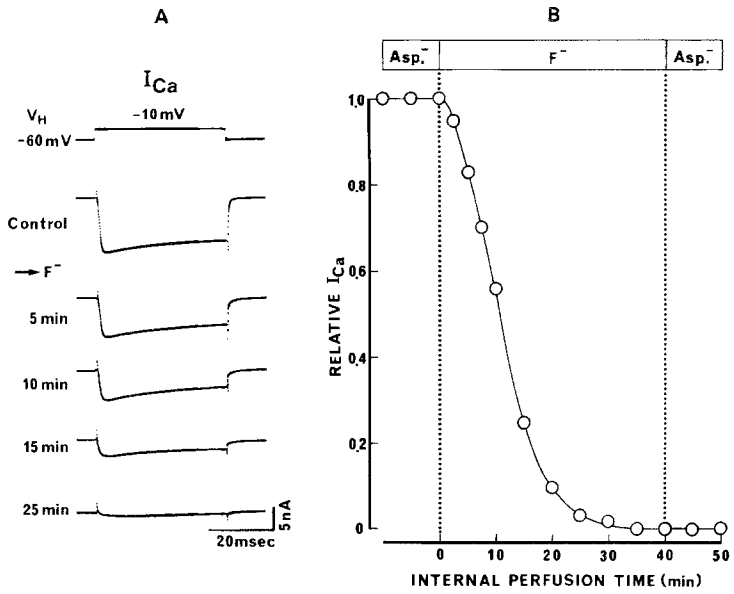

Fig. 4. Blockage of I_{Ca} by the internal application of F^-. (*A*): Effect of the internally perfused F^- on the I_{Ca} by voltage step of −10 mV. V_H, −60 mV. Sample records of I_{Ca} were obtained after subtraction of leakage and capacitative currents. From top to bottom: control in 105 mM Cs-aspartate internal solution, 5, 10, 15, and 25 min, respectively; after the perfusion of 105 mM CsF internal solution. (*B*): Time course of changes of I_{Ca} after the internal perfusion of F^-. Data were obtained from the same neuron. Upper sign on graph indicates changes of the internal perfusate

The slow inward current was carried by Ca^{2+}, since perfusion of the cell interior by a solution containing F^- abolished the slow inward current, as was shown in snail neurons (Kostyuk et al., 1977; Akaike et al., 1981*c*; Oyama et al., 1982) (Fig. 4). In all neurons I_{Ca} inactivated very slowly. The peak inward Ca^{2+} current (I_{Ca}) appeared much later than that of I_{Na} (Fig. 3*B* (*a–c*)). In the following sections, the properties of I_{Ca} are described.

Current-Voltage Relationship for Calcium Current

A slowly rising inward current appeared at depolarizing voltage steps of 20 to 25 mV from a holding potential of −60 mV. I_{Ca} rose smoothly, reached its peak within a few msec, and persisted for more than 10 sec. The time to peak was shorter at large depolarizing voltages, and the maximum peak current occurrred at a membrane potential level of 0 to +5 mV when the external Ca^{2+} concentration ($[Ca]_o$) was 2 mM. With successive increases of the depolarizing voltage steps, the peak current became smaller and finally reversed at potential levels beyond +80 to +90 mV (Fig. 5*A*). However, the outward current at high voltage steps is likely a nonspecific outward current (I_{NS}). This outward current, which appears after the complete suppression of I_{Ca} by substitution of Co^{2+} for Ca^{2+}, appears at voltages of around +20 to +25 mV and becomes nonlinearly larger at greater depolarizing voltages (Akaike et al., 1978*b*, 1981*a*, *b*; Byerly & Hagiwara, 1979; Brown et al., 1981; Oyama et al., 1982). This means that I_{Ca} is contaminated with I_{NS} at voltages greater than about

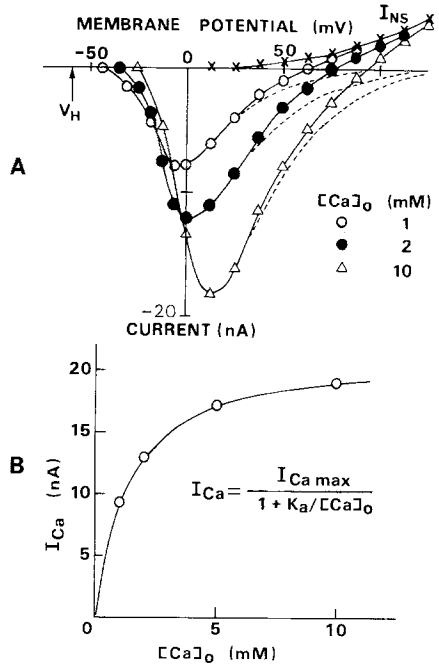

Fig. 5. I_{Ca} at various external Ca^{2+} concentrations ($[Ca]_o$). (*A*): Effect of varying $[Ca]_o$ on the current-voltage (*I–V*) curves. Each *I–V* curve was obtained between 3 to 5 min after changing the external test solutions. Note shifts of current thresholds and peak current voltages in the *I–V* curves to the less negative potentials with increasing $[Ca]_o$. V_H, −60 mV. After subtraction of the nonspecific outward current (I_{NS}) for each I_{Ca} *I–V* relationship, the real I_{Ca} *I–V* curves (dashed lines) at various $[Ca]_o$ were obtained. Reversal current of I_{Ca} was not observed after the subtraction. These inward currents became closer to zero at higher voltage steps. (*B*): Relationship between peak I_{Ca} and $[Ca]_o$. V_H, −60 mV. I_{Ca} increased in a hyperbolic manner as $[Ca]_o$ was increased. The experimental points were well fitted by a theoretical solid line described by the equation shown in *B*. In this equation, I_{Ca} is a peak transient current, $I_{Ca\,max}$ (21.7 nA) is I_{Ca} when all the sites are occupied by Ca^{2+}, and K_a is an association constant of the calcium binding site. The value for K_a calculated using the data is 1.37 mM

+20 mV. Therefore, the real current-voltage (*I–V*) relationship for I_{Ca} must be corrected for I_{NS}. The *I–V* relationship for I_{Ca} thus obtained is shown in Fig. 5*A*, in which a null potential rather than a reversal potential for I_{Ca} is seen at around +120 mV, a value similar to that found in snail neurons (Akaike et al., 1978*b*, 1981*b*, *c*; Oyama et al., 1982).

The amplitude and configuration of I_{Ca} recorded from neurons perfused externally and internally with Na^+- and K^+-free solutions for at least 30 min, were constant for as long as 3 to 5 hr; the survival time was shorter than the 5 to 10 hr found for I_{Ca} in snail neurons (Akaike et al., 1982; Oyama et al., 1982; Nishi, Akaike, Oyama & Ito, 1983). However, depolarizing voltage steps of more than +50 mV and/or with duration longer than a few hundred msec at short intervals resulted in both a progressive increase of the holding current toward the inward direction and a rapid decrease of membrane resistance. Finally, the electrical activities of cell membrane were completely lost.

Changes in External Ca^{2+} Concentrations

Increases in external Ca^{2+} concentration ($[Ca]_o$) from normal $[Ca]_o$ of 2 mM to 5 or 10 mM increased the peak value of I_{Ca} and caused it to inactivate more rapidly. Increases in $[Ca]_o$ also shifted the maximum peak and threshold voltages of the *I–V* relationship in the positive direction, and the null potential became more positive at higher $[Ca]_o$. On the other hand, decreases in $[Ca]_o$ reduced I_{Ca}, slowed inactivation, and shifted the *I–V* relationship in a negative direction along the voltage axis (Fig. 5*A*). The relationship between the maximum peak I_{Ca} and $[Ca]_o$ was hyperbolic, as reported for barnacle muscle fibers (Hagiwara & Takahashi, 1967), tunicate eggs (Okamoto, Takahashi & Yoshii, 1976), snail neurons (Akaike et al., 1978*b*), and *Paramecium tetraurelia* (Satow & Kung, 1979) (Fig. 5*B*).

Selectivity of Ca^{2+}-Channels for Other Divalent Cations

Substitution of Ba^{2+} or Sr^{2+} for Ca^{2+} in Mg^{2+}-free external solution resulted in smaller currents, which activated more or less slowly and inactivated more slowly, as has been reported for *Helix* and *Aplysia* neurons (Eckert & Lux, 1976; Magura, 1977; Adams & Gage, 1979; Oyama et al., 1982). Mn^{2+} and Zn^{2+} also evoked substantial amounts of inward current, and the time courses of activation and inactivation processes were much slower than those of I_{Ba} and I_{Sr}. The *I–V* relationships

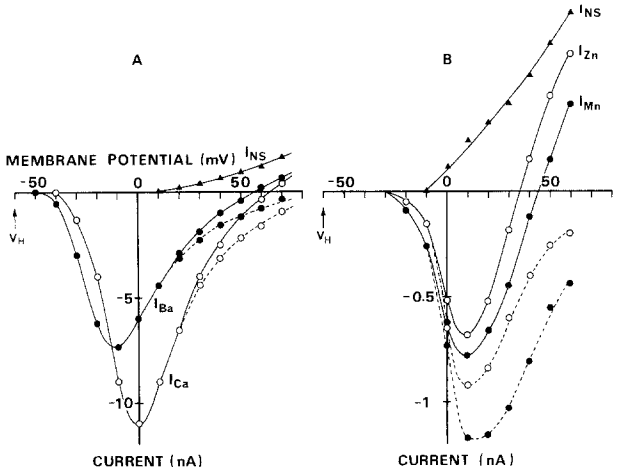

Fig. 6. The *I–V* relationships in the various divalent cation solutions. (*A*): I_{Ca} (○) and I_{Ba} (●). Each current was recorded in test solution containing 2 mM Ca^{2+} or Ba^{2+}. I_{NS} (▲). Real *I–V* relationships after the subtraction of I_{NS} are shown by the dashed line. (*B*): The *I–V* relationships for I_{Mn} and I_{Zn}. The currents were measured in test solution containing 2 mM Mn^{2+} or Zn^{2+}

for I_{Ca}, I_{Ba}, I_{Zn} and I_{Mn} are shown in Fig. 6*A* and *B*, in which the threshold and peak currents of I_{Zn} and I_{Mn} occur at potentials less negative as compared with those of I_{Ba}. The sequence estimated from the amount of the voltage shift in the positive direction of the maximum peak in each *I–V* relationship was $Mn^{2+} = Zn^{2+} > Ca^{2+} > Ba^{2+} = Sr^{2+}$ (Fig. 6*A* and *B*). In addition, the relative maximum peak inward currents carried by these cations to I_{Ca} were in the order of $I_{Ca} > I_{Ba} = I_{Sr} > I_{Mn} > I_{Zn}$ (Fig. 7*A* and *B*). The results indicate that some divalent cations can permeate the Ca^{2+}-channels of frog dorsal root ganglion cells as well as those of snail neurons (Kawa, 1979; Akaike et al., 1981*c*; Oyama et al., 1982). Figure 7*B* also suggests that the critical size, which limits the permeation of divalent cations through the Ca^{2+} channel, may be just below the size of the crystal ionic radius of Zn^{2+} (74 pm) (Shannon, 1976; Edwards, 1982).

Effects of Organic and Inorganic Ca^{2+}-Antagonists on I_{Ca}

The time-dependent inhibition of the organic Ca^{2+}-antagonist diltiazem (10^{-4} M) on I_{Ca} produced by a voltage step from V_H of −60 to 0 mV is shown in Fig. 8. Similar results were obtained by other organic Ca^{2+}-antagonists, verapamil and D-600. These agents also produced dose-dependent depression of I_{Ca} without shifting the peak voltage of the I_{Ca} *I–V* relationship. In contrast, inorganic Ca^{2+}-antagonists, such as Co^{2+} and

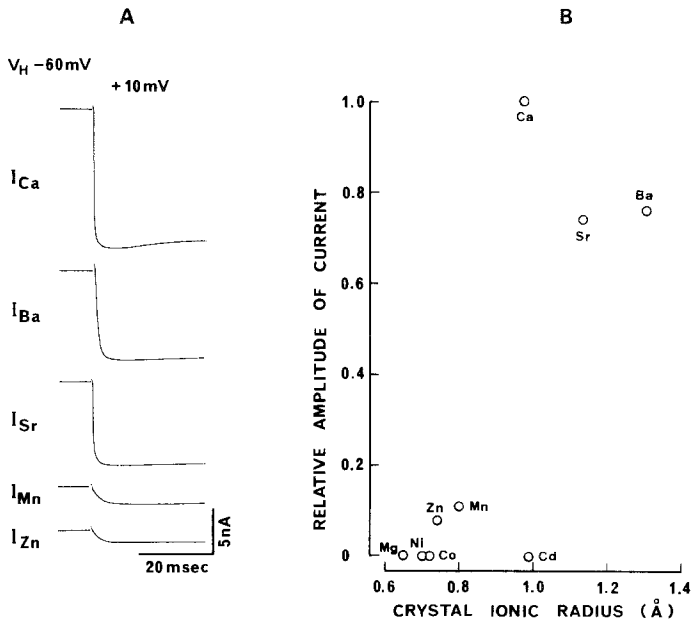

Fig. 7. Divalent cationic currents. (*A*): I_{Ca}, I_{Ba}, I_{Sr}, I_{Mn} and I_{Zn} measured in each test solution containing 2 mM Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+} or Zn^{2+}, respectively. The current records were obtained after the subtraction of leakage current, linear capacitative current, and I_{NS}. V_H, −60 mV. These subtractions might not be taken very seriously, especially for nonlinear outward current (I_{NS}), because the I_{NS} might change with the different divalent ions. In the present experiments, however, it should be pointed out that the I_{NS} hardly appeared at the voltage step from V_H of −50 to +10 mV, as seen in Figs. 5*A*, 6*A* and *B*, and Fig. 8*B*. (*B*): The relative amplitudes of peak currents of various divalent cation currents for I_{Ca} were plotted as a function of their crystal ionic radius. Each point is an average value of 5 to 6 measurements from different neurons

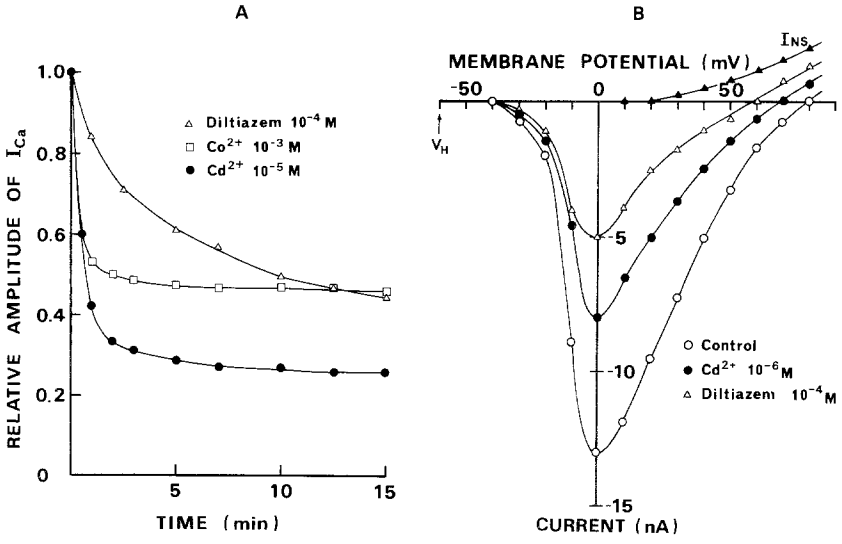

Fig. 8. Effects of organic and inorganic Ca^{2+} antagonists on the I_{Ca}. (*A*): Blockage of I_{Ca} by diltiazem, Co^{2+} and Cd^{2+}. Note the time-dependent blockage of I_{Ca} by an organic Ca^{2+} blocker, diltiazem. Drugs were added to the bathing medium at time zero. V_H, −60 mV. (*B*): Effects of external application of diltiazem or Cd^{2+} on the I_{Ca} *I–V* relationship. Control (○), the nonspecific outward current I_{NS} (▲), 5 min after adding Cd^{2+} (●), and 5 min after adding diltiazem (△). V_H, −60 mV

Cd^{2+}, depressed I_{Ca} in a dose-dependent manner but not in a time-dependent manner, since the inhibitory effects of Co^{2+} and Cd^{2+} were complete within a few minutes after application of these divalent cations (Fig. 8*A*). Adding 5 to 10 mM Co^{2+} to the bath shifted the threshold and peak currents in the *I–V* relationships to more positive direction, while a small amount of Cd^{2+} (10^{-5} to 10^{-6} M) inhibited I_{Ca} without shifting the threshold of the *I–V* relationship (Fig. 8*B*).

Internal application of diltiazem, verapamil, and D-600 at 6×10^{-6} to 3×10^{-4} M also depressed I_{Ca} in a dose- and time-dependent manner. The depression occurred over the entire voltage range. The threshold in the *I–V* relationship was not shifted. These inhibitory actions were partially but not completely reversible following a washout period of 30 min after removal of the drugs. The effects of drugs applied externally and internally on I_{Ca} were almost equi-potent.

When I_{Ca} was evoked by a voltage step from a V_H of −60 to 0 mV with a pulse duration of 4 sec, the inward current inactivated slowly. Adding high concentrations of Co^{2+} (5 to 10 mM) reduced I_{Ca} without changing the time course of the inactivation process, while the organic Ca^{2+} antagonists and 10^{-6} M Cd^{2+} (the concentration of Cd^{2+} that did not shift the threshold in the *I–V* relationship) reduced I_{Ca} and enhanced inactivation from the peak level. In addition, organic

Ca^{2+} antagonists suppressed equally the inward currents carried by Ca^{2+}, Ba^{2+}, Sr^{2+}, Mn^{2+} or Zn^{2+} while Co^{2+} (5 to 10 mM) inhibited selectively in the order of $I_{Ba}=I_{Sr}>I_{Ca}>I_{Mn}=I_{Zn}$. Co^{2+} at a concentration of 5 mM reduced the peak I_{Ba} by about 90%, the peak I_{Ca} by about 50%, and the peak I_{Mn} by about 20% of the control.

Discussion

In *Helix* neurons immersed in normal external solution, the Na spike is dominant regardless of the presence of Ca^{2+}. However, Ca spike can be elicited by removal of Na^+ from the external solution; the Ca spike shows no appreciable reduction in amplitude but decreases in the maximum rate of rise or fall as compared with those of Na spike in the presence of Ca^{2+}. Substitution of Cs^+ for K^+ in both the external and internal medium leads to full-sized Ca spikes of the all-or-none type lasting for 10 to 60 sec (Akaike et al., 1981 *b*, *c*; Oyama et al., 1982). In the present experiments, dorsal root ganglion cells of frog could not produce Ca spikes in the presence of K^+ in the external and internal medium. When K^+ in the medium was replaced with Cs^+, neurons could evoke a Ca spike lasting for more than 6 sec. The results suggest that absence of spikes in the presence of K^+ results from the fact that K^+ conductance is greater in frog sensory neurons than in snail neurons. There seems to be no essential difference in the properties of the Ca^{2+} channel between these vertebrate and invertebrate neurons, since ionic selectivity for permeable divalent cations and sensitivity to Ca^{2+} antagonists are quite similar for neurons of the vertebrate and invertebrate.

Recently our work on the large, identifiable F_1 neuron of *Helix aspersa* (*see* Kerkut et al., 1975) indicated that inactivation of I_{Ca} occurs in a bi-exponential manner, consisting of fast and slow time constants (τ_{h_1} 30 to 50 msec, τ_{h_2} 600 to 800 msec at a voltage step of +15 mV from holding potential of −50 mV) (Akaike et al., 1981 *b*, *c*, 1983; Oyama et al., 1982). The results are quite identical to the time course of I_{Ca} inactivation data obtained from molluscan neurons (Connor, 1979; Adams & Gage, 1980; Brown et al., 1981; Byerly & Hagiwara, 1982) and to the implication drawn from intracellular indication data (Gorman & Thomas, 1978; Ahmed & Connor, 1979; Smith & Zucker, 1980). The rates of inactivation of I_{Ca} observed in these recent works are considerably smaller than those reported in our earlier paper (Akaike et al., 1978*b*). In our study, very small cells having maximum inward I_{Ca} of about 20 nA or less were used to minimize series resistance effects of the single-suction pipette combined with the usual voltage-clamp circuit. Also in our study, the combination of TEA and 4-AP was not used. Therefore, the fast inactivation in our previous paper may have been due to the contamination of a small amount of potassium outward currents to the small I_{Ca}, though the differences in cell sizes could also be a contributing factor.

Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in production of inward currents in crustacean muscle fibers (Fatt & Ginsborg, 1958; Hagiwara, Fukuda & Eaton, 1974), egg cells (Okamoto et al., 1976), heart muscle fibers (Vereecke & Carmeliet, 1971; Kohlhardt, Haastert & Krause, 1973; Reuter, 1973), and snail neurons (Oyama et al., 1982). The apparent selectivity sequence is $Ca^{2+}>Sr^{2+}>Ba^{2+}$ for sea urchin egg cell, $Sr^{2+}>Ba^{2+}>Ca^{2+}$ for tunicate egg cell, $Sr^{2+}>Ca^{2+}>Ba^{2+}$ for mouse egg cell, and $Ba^{2+}>Sr^{2+}>Ca^{2+}$ for snail neuron (Okamoto, Takahashi & Yamashita, 1977; Oyama et al., 1982). In frog sensory neuron, ion selectivity was $Ca^{2+}>Ba^{2+}>Sr^{2+}$. These variations among various preparations may account for the difference in both "affinity" to the Ca^{2+}-binding site and membrane stabilizing action of the three cations (Hagiwara & Byerly, 1981; Akaike et al., 1982).

Until recent years, transition metal divalent cations such as Co^{2+}, Mn^{2+}, Zn^{2+} and Ni^{2+} have been thought to suppress I_{Ca} in molluscan neurons (Geduldig & Gruener, 1970; Kostyuk et al., 1977; Akaike et al., 1978*a*, *b*; 1981; 1982) by occupying the binding site for Ca^{2+} in Ca^{2+} channel in a dose-dependent manner. Kawa (1979), Oyama et al. (1982), and Akaike, Nishi and Oyama (1983*b*) found all-or-none-type action potentials and substantial inward current in snail neurons immersed in Ca^{2+}-free solutions containing Zn^{2+} or Mn^{2+}. Furthermore, Oyama et al. (1982) reported that there is a very strong interaction between Zn^{2+} and Ca^{2+} at the common site in Ca^{2+}-channel and that I_{Zn} is markedly reduced by adding Ca^{2+} in a concentration 1/200 times that of Zn^{2+}. In the present experiments, both Zn^{2+} and Mn^{2+} could pass the frog cell membrane in the absence of external Ca^{2+}. The results suggest that the cell membrane of various tissues may be essentially permeable to Zn^{2+} and Mn^{2+} as well as to Ca^{2+}, Ba^{2+} and Sr^{2+} but that in the presence of Ca^{2+}, I_{Mn} or I_{Zn} may be easily hidden by the strong negative interaction between Ca^{2+} and Mn^{2+} or Zn^{2+} at the common binding site of the Ca^{2+} channel.

According to Hagiwara and Byerly (1981), when *Limnea stagnalis* neurons, treated with 0.2% trypsin for 90 min, were well perfused internally by the suction pipette technique, I_{Ca} disappeared

regardless of the presence of I_{Na} and I_K. They concluded that the decay of I_{Ca} was due to "wash-out" effects of some soluble intracellular factor necessary to maintain the physiological function of the neuronal Ca^{2+} channel. Kostyuk, Krishtal and Pidoplichko (1975) and Fenwick, Marty and Neher (1981) also offered similar conclusions in the enzymatically well-treated snail neuron and bovine chromaffin cell. In contrast, using a suction pipette technique, we could record a large and stable I_{Ca} of more than 80 nA for more than 5 hr from snail neurons of 80 to 100 μm diameter without enzyme treatment (Akaike et al., 1982; Oyama et al., 1982) and also a stable I_{Ca} of 20 nA from frog sensory neurons of 30 to 40 μm diameter for more than 3 hr following the first 15 to 20 min after the beginning of internal perfusion, in which the neurons were treated for 10 to 15 min with a small amount of trypsin of 0.05% plus 0.3% collagenase. The initial perfusion time was no doubt adequate since in both preparations the action potentials were extremely prolonged, lasting for 6 to 60 sec, and since little or no inactivation of I_{Ca} was present. In snail neurons, complete exchange of internal K^+ or Cl^- was directly measured by K^+ or Cl^- selective microelectrode (Lee, Akaike & Brown, 1978). In addition, the reversal potential (E_{GABA}) for the GABA response in frog sensory neurons was equal to the Cl^- equilibrium potential (E_{Cl}), and the E_{GABA} behaved as a simple Cl^- electrode within the initial 20 min of perfusion following changes of internal Cl^- concentration (Hattori et al., 1983). However, it was difficult to make continuous measurements of I_{Ca} over periods of several hours in both *Helix* neurons and frog sensory neurons that were treated with high concentrations of trypsin (0.1% or more). A part of the cell membrane of such neurons was gradually sucked into the interior of the suction pipette with time, although it occurred very slowly. Finally, the shape of the whole cell was slightly transformed, and this reduced I_{Ca} with but a slight decrease of I_{Na}. GABA-induced Cl^- responses were also reduced in frog sensory neurons treated with high concentrations of trypsin. Therefore, we never used the enzyme-treated *Helix* neurons and we took care to use trypsin-treated preparations at concentrations of less than 0.05% for 10 to 15 min even for frog sensory neurons, which are enveloped by thick, tough connective tissues.

Hagiwara and Byerly (1981) also reported decay of the I_{Ca} without changing the membrane resistance and capacitance by applying a suction pipette with tip interior diameter of about 25 μm to cells of 90 to 100 μm in diameter. In our experiments, however, it was impossible to constantly maintain the neurons without changing the shape of cells with diameter of about 100 μm by a pipette with such a large interior tip. We were unable to use pipettes with tip interior diameters exceeding 15 to 18 μm for cells 80 to 100 μm in diameter. By using a suction pipette having a suitable tip interior diameter, the recordings of I_{Ca} are stable and the cell lasts for more than several hours. The results mentioned above suggest that application of suction pipette to the cells may require the presence of some membrane constituents such as connective tissue on the cell membrane which would prevent damage of plasma membrane from the direct touch of the suction pipette tip and maintain the original cell shape for negative pressure from suction pipette. Therefore, the "wash-out" interpretation offered by several investigators may account for the mechanical injury of cell plasma membrane from the suction pipette or glass electrode.

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