INTRACELLULAR DIVALENT CATIONS AND PLATEAU DURATION OF SQUID GIANT AXONS TREATED WITH TETRAETHYLAMMONIUM

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Abstract Squid giant axons were intracellularly perfused with a solution containing 100 mM K^+ and 5 mM tetraethylammonium (TEA) to obtain action potentials with a long-lasting plateau. Effects of intracellular divalent cations on the plateau of the action potentials were examined by introducing 0.1–10 mM Ca^{2+} , Sr^{2+} , Mg^{2+} , Ba^{2+} , or Mn^{2+} into the perfusate under these conditions. Ca^{2+} , Sr^{2+} and Mn^{2+} were found to shorten the plateau duration drastically. Upon removal of the divalent cations in the perfusate, there was a recovery in the plateau duration. The recovery was imperfect in the case of Ca^{2+} , nearly perfect in the case of Sr^{2+} , and perfect in the case of Mn^{2+} . Voltage-clamp experiments carried out on axons intracellularly perfused with the control (*i.e.*, divalent cation free) perfusate revealed the existence of a late inward current through the membrane. Intracellularly administered Mn^{2+} (1–3 mM) blocked this late inward current and decreased the membrane conductance during the late period of voltage clamping. Intracellular Mg^{2+} (1–10 mM) prolonged the action potential plateau produced by TEA reversibly in some axons; however, this effect was masked by an opposing (*i.e.*, irreversibly plateau-shortening) effect in most axons. Ba^{2+} applied intracellularly prolonged the action potential duration regardless of the presence or absence of TEA in the perfusate. We emphasize that the effects of divalent cations applied internally are very similar to those observed when these cations are applied externally, except that the effective concentration is far lower in the case of internal application. It is suggested that the primary site of action of divalent cations might be on or near the internal surface of the axonal membrane.

In 1968, Tasaki presented a theoretical argument that the excited state of the axonal membrane is terminated because of an increase in the concentration

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of interdiffusing ions which accumulate near the membrane to form stagnant layers. He regarded the intracellular accumulation of Ca^{2+} to be most crucial. However, at that time there was no experimental evidence to support this hypothesis. Intracellular injection of Ca^{2+} either did not produce any pronounced effect on the action potential of squid giant axons (BEGENISICH and LYNCH, 1974), or if any, a slight prolongation of the duration together with a decrease in amplitude and in rate of rise (TASAKI *et al.*, 1967; YAMAGISHI, 1977). However, a recent experiment by ISENBERG (1975) indicated that the intracellular Ca^{2+} shortens the plateau duration of the action potential of cardiac Purkinje fibers. The effect was ascribed to an increased potassium conductance which is observed in the resting membrane of several invertebrate and vertebrate neurons (MEECH, 1972; KRNEVIC and LISIEWICZ, 1972; MEECH, 1974a, b). It is possible, therefore, that the effect of Ca^{2+} on the plateau potential in squid axons is also large enough to draw conclusions without ambiguity, even though the effect on the natural brief action potential is not clear.

In the present paper, we report that intracellularly administered Ca^{2+} , Sr^{2+} and Mn^{2+} markedly shorten the duration of the action potential plateau in tetraethylammonium (TEA)-treated squid giant axons. A voltage clamp study revealed that a major effect of intracellular Mn^{2+} is to reduce or eliminate the late inward current observed when TEA-treated axons are clamped with a depolarizing voltage pulse.

METHODS

Freshly excised giant axons of squid, *Dorytheus bleekeri*, (400–625 μm in diameter) were used. All experiments were done from axons mounted horizontally in the Lucite chamber which contained artificial sea water. We used the double cannulation technique for the intracellular perfusion (WATANABE and TASAKI, 1972). The length of the perfused zone was usually 13–17 mm. At the initial stage of perfusion we used a solution containing protease (Protease Type VII, Sigma P5255, 0.1 mg/ml) for 2–4 min to remove the major portion of axoplasm.

The membrane potential was recorded with an axially inserted glass capillary electrode (80 μm in diameter) filled with 0.6 M or 3 M KCl-agar; the tip of the electrode was placed at approximately the midpoint of the perfused zone. Electrical stimuli were delivered either through an internal electrode made of platinized platinum wire (50 μm in diameter), or through a pair of platinum electrodes placed externally at one end of the axon. In voltage clamp experiments we employed the technique described by MOORE (1971).

Since intracellularly administered Ca^{2+} usually produces a detrimental effect of the excitability of squid giant axons, rapid replacement of the internal solution was found essential in order to obtain reversible results. For this purpose we modified the perfusion system to reduce the dead space and employed several solenoid valves to facilitate rapid exchange of solutions (see Fig. 1). The desired

Fig. 1. The experimental set-up for the intracellular perfusion which allowed a rapid exchange of internal perfusates. A, a schematic representation of the system. AX, giant axon. IN, inlet pipette for the perfusate. OUT, outlet pipette for the perfusate. BS, a compartment of about $10 \mu\text{l}$ volume at the base of the inlet pipette. H, the holder of the inlet pipette and its base compartment, mounted on a micromanipulator. A, B and C, reservoirs for perfusates; a, b, c and d, electric valves which usually close the tubings from the respective reservoirs and the waste to the base compartment. W, an Erlenmeyer flask to receive waste perfusate. M, a branch of tubing which opens at the top end. B, time sequence for valve operation and stimulation; a, b, c and d indicate timing of pulses which open the respective electric valves shown in A; e indicates periods during which one of the perfusates shown in A flows; f indicates timing of stimulation with the resultant action potentials; l, the basic repeating period; m, the period of opening of valves; n, the period between opening of a valve and delivery of a stimulus. C, upper traces show changes of transmitted light through the central portion of the perfused zone. Lower traces show the timing of application of electric pulses which drove the valves. The perfusing fluid was switched from a transparent solution to a dyed solution (left), and was switched back to the transparent solution again (right). Time: 10 s.

perfusate was selected by an opening of the proper valve. During perfusion with one solution, all four valves (a, b, c, and d) in Fig. 1 were closed and the hydrostatic pressure applied to the perfusate was maintained by the level of the surface of the solution in tubing M which was open at its upper end. When rapid exchange of solutions was required, two valves, one of the three reservoir valves (a, b or c) and the waste valve (d), were opened simultaneously. When these two valves were open, the perfusate flowed rapidly from one of the reservoirs (A, B or C) to the waste (W). The flow washed away the content of the small compartment (BS) at the base of the inlet pipette and replaced it with the new perfusate.

The volume of compartment BS was about 10 μ l. This compartment was usually rinsed with 200 μ l of new perfusate. The amount of the perfusate required for rinsing was controlled by the length of the valve opening time (usually 2 s) and by the hydrostatic pressure difference between reservoirs and the waste. The height of the reservoirs and of the waste were adjusted in such a way that the fluid level in the tubing M did not change appreciably during the valve operation. We could not measure pressure changes which might be generated inside the axon during the valve operation accurately; no change in the axon diameter was detected under a dissection microscope (see Fig. 2). The axon was never excited by the valve operation.

The rate of exchange of the fluid was estimated by the use of a dyed perfusate. When the optical density of the axon was measured through a photodiode in the middle portion of the perfused zone (see Fig. 1C), a change in transmitted light intensity could be detected within 2 s after opening the valves and a steady state was reached within about 20 s. The actual exchange process of the internal perfusate was further examined by the use of 16 mm movie (see Fig. 2). A new dyed perfusate is seen to emerge from the inlet pipette within a fraction of a second and to enter the outlet pipette within 2 or 3 s. The axon was filled with the new perfusate almost completely within 10 s. However, the density of the color at the edge of the axon continued to increase during another period of 10 s. In most experiments the valves were operated periodically at 30 s interval, in order to prevent contamination of the perfusate by the ingredients in other tubings which have openings to the compartment.

It is known that the excitability of a perfused axon can be maintained for a long period of time when the internal perfusate contains fluoride or phosphate as the major anion (TASAKI *et al.*, 1965). We therefore used a solution containing 90 mM KF, 10 mM K^+ (as phosphate salts) and 5 mM TEA-Cl as a standard perfusate to maintain the excitability. Divalent cations, Ca^{2+} , Sr^{2+} and Mg^{2+} are known to combine with fluoride and to cause precipitation. Before testing the effects of Ca^{2+} , Sr^{2+} and Mg^{2+} , therefore, we replaced the standard perfusate with a control perfusate, which usually contained 100 mM K-glutamate and 5 mM TEA-Cl. BaF_2 is soluble in water, its solubility product being $1.7 \times 10^{-6} M^3$. In experiments with Ba^{2+} , we therefore used, as the control perfusate, a solution containing 25 mM KF and 5 mM TEA-Cl. MnF_2 is also soluble in water; in experiments with Mn^{2+} , we used a solution containing 100 mM KF and 5 mM TEA-Cl as the control perfusate. We often added 0.1 mM EDTA to the control perfusates in order to ensure the maintenance of low internal divalent cation concentration. However we did not find any detectable effect of eliminating EDTA from the control solutions. The tonicity of the solution was adjusted with glycerol. The pH of the internal perfusate was kept at 7.3 ± 0.1 by addition of Tris-glutamate or potassium phosphate buffer.

In typical experiments we switched the intracellular solution successively

Fig. 2. Photomicrographic pictures of a perfused squid giant axon during the exchange of intracellular perfusates. The pictures were selected from a 16 mm movie film driven at a rate of 24 frames per second. The valves opened at 0s and closed at about 2s. The new perfusate was dyed deeply with chlorphenol red. The thick curved lines are edges of a window made at the bottom of the lucite chamber for optical measurements. The bar at the bottom right indicates 1 mm.

in the order of the standard, control, test, control, and standard perfusates. The period of application of the control or the test perfusate was within 2 min (see Figs. 4 and 6).

In most experiments artificial sea water was used as the external medium which contained the following concentrations (expressed in mM) of salts: 450 NaCl, 10 KCl, 50 CaCl₂, 10 MgCl₂; its pH was adjusted by 1 mM Tris-HCl buffer to a value of 8.0–8.2.

We kept the following parameters approximately constant during one experiment: the stimulus frequency usually at a rate of once in 30 s, the interval between valve operation and stimulation usually at about 20 s (see Fig. 1B), the room temperature at $20 \pm 1^\circ\text{C}$, and the hydrostatic pressure on the intracellular perfusate at 28 ± 3 cm H₂O.

RESULTS

Effects of intracellular Ca²⁺

Figure 3 shows the typical result of an experiment of intracellular perfusion with Ca²⁺. Replacement of the TEA-free perfusate with the standard TEA-containing perfusate converted the action potential from a brief spike to one followed by a plateau with a duration of several seconds. We often observed a burst of spikes during the plateau phase (Fig. 3-1). A subsequent switching of the perfusate from the standard to the control often prolonged the plateau duration transiently (Fig. 3-2), then shortened it gradually. It was usually difficult to

Fig. 3. Effects of 0.3 mM Ca²⁺ on the plateau of TEA-treated axon. Top traces show the whole action potentials on a slow time base. Bottom traces show only the initial part of the action potentials on a rapid time base. 1: records taken with the standard perfusate as the intracellular solution. 2: records taken 50 s after switching to the control perfusate which contained (in mM) 100 K-glutamate, 5 TEA-Cl, 0.1 K-EDTA and 2 Tris-glutamate. 3: records taken 50 s after switching to the test perfusate which contained 100 K-glutamate, 5 TEA-Cl, 0.3 CaCl₂ and 2 Tris-glutamate. 4: records taken 50 s after switching to the control perfusate. 5: records taken 50 s after switching to the standard perfusate. Axon 59, 550 μm in diameter.

maintain the action potential duration constant for more than 5 min with the control perfusate inside the axon. When the internal solution was further switched to the test perfusate containing CaCl_2 , shortening or disappearance of the plateau was invariably observed (Fig. 3-3). After a few minutes, when the internal solution was switched back to the control perfusate, the plateau reappeared and grew to some extent (Fig. 3-4). Such a recovery was observed in two-thirds of the axons examined. The recovery was promoted further by switching back to the standard perfusate; after several minutes of perfusion, the amplitude and the duration of the action potential usually approached to the initial level of those before application of the test solution (Fig. 3-5).

A Ca-buffered test solution (*i.e.*, a mixture of EDTA and Ca-glutamate) with a pCa of 7.6 did not show any detectable effect of the plateau duration. The lowest effective Ca^{2+} concentration in the range we could examine was 0.1 mM. In some axons, Ca^{2+} at this concentration was enough to suppress the plateau formation almost completely, but in others it simply shortened the plateau duration. The reversibility was very good at this concentration. When the axon was perfused with 0.3 mM Ca^{2+} , the plateau was abolished conspicuously as shown in Fig. 3. Perfusion with Ca^{2+} at concentrations higher than 0.3 mM caused a more rapid and complete suppression of the plateau. The reversibility was not always good at higher concentrations.

When the interior of the axon was exposed to Ca^{2+} -containing internal perfusates, the action potential amplitude, the rate of potential rise, and the magnitude of the resting potential were reduced as shown in Fig. 4. During this membrane depolarization, the membrane resistance decreased to about 2/3 of the control value (data are not shown). Spontaneous firings were sometimes observed at the initial stage of perfusion with the Ca^{2+} -containing medium.

Intracellular Ca^{2+} suppressed the plateau formation effectively, but it always left a vestige of the plateau. Even at a high concentration of intracellular Ca^{2+} , the action potential showed a hump at its falling phase (see Fig. 3-3). The duration of the action potential was always shortened by Ca^{2+} only to a value around 10 ms, which was still much longer than the action potential observed in axons without TEA treatment. Thus the suppressing effect of intracellular Ca^{2+} on the plateau was limited; it did not nullify the effect of TEA completely.

When the plateau in TEA-treated axons has a limited duration around 10 ms, intracellular Ca^{2+} did not shorten the plateau duration effectively. For example, when the internal K^+ concentration was 400 mM, the plateau duration was comparatively short in the presence of the same concentration of intracellular TEA. In such a case, application of Ca^{2+} at a concentration as high as 5 mM did not influence the duration. Another example is the case in which external Ca^{2+} concentration was raised to 150 mM. In this case the duration of the action potential under perfusion with the TEA solution of the usual concentration was about 10 ms; again intracellular application of Ca^{2+} changed the shape and

Fig. 4. Time course of electrophysiological parameters of an axon during a series of experiments to test the effect of Ca^{2+} . Internal media were the standard perfusate during the periods a, the control perfusate during the periods b, and the test perfusate with 0.3 mM Ca^{2+} during the period c. Top: the duration of the action potential. Second: the amplitude at the peak of the action potential. Third: the rate of rise of the action potential. Bottom: the resting membrane potential.

the amplitude of the action potential only slightly (Fig. 5). The latter experiment indicates that the effect of intracellular Ca^{2+} on the plateau duration is similar to the effect of extracellular Ca^{2+} , except that the intracellular Ca^{2+} was several hundred times more effective than the extracellular Ca^{2+} .

Effects of intracellular Mg^{2+}

Under the present experimental conditions, a high intracellular Mg^{2+} concentration was found to be detrimental to the maintenance of the excitability. After exposing the interior of the axon to a test solution containing 1–10 mM Mg^{2+} ,

Fig. 5. Suppression of the effect of intracellular Ca^{2+} with high extracellular Ca^{2+} . A and B are records of two series of experiments successively performed on the same axon. The external medium in A contained (in mM) 375 NaCl and 150 CaCl_2 , with a small amount of Tris-Cl buffer. The external medium in B contained 461 NaCl, 10 KCl, 52 CaCl_2 and 10 MgCl_2 , with a small amount of Tris-Cl buffer. Internal media in 1 and 5 were the standard perfusate; in 2 and 4 the control perfusate; and in 3 the test perfusate with 0.3 CaCl_2 (see METHODS for the recipe). Axon 61, 640 μm in diameter.

Fig. 6. Effects of 5 mM Mg^{2+} on the plateau duration of TEA-treated axons. Plateau durations of three different axons are plotted against time. The external medium was the standard artificial sea water. Internal media in periods a were the standard perfusate; in periods b the control perfusate; and in periods c the test perfusate containing 5 mM Mg^{2+} . A: axon 191, 465 μm in diameter. B: axon 184, 615 μm in diameter. C: axon 182, 510 μm in diameter.

9 axons out of 12 examined showed irreversible reduction in peak amplitude and plateau duration of the action potential. In these 9 axons either a simple reduction of plateau duration (Fig. 6C) or a transient prolongation followed by a reduction (Fig. 6B) was observed; in either case the reduced plateau duration did not recover after switching to the control perfusate. In the remaining 3 axons, Mg^{2+} definitely prolonged the plateau at this concentration range of Mg^{2+} (see Fig. 6A). When the test perfusate contained 5 mM Mg^{2+} the extent of prolongation was roughly 50% of the plateau duration in the control perfusate. This effect of Mg^{2+} was reversible. In summary, our experimental results can be explained as a summation of two opposing effects of Mg^{2+} : an irreversible shortening action and a reversible lengthening action. It is not clear whether or not Mg^{2+} exerts any reversible shortening effect on the plateau, as Ca^{2+} does.

Effects of intracellular Sr^{2+}

We examined the effects of Sr^{2+} on five axons. On intracellular application of Sr^{2+} at concentrations between 0.5–1.0 mM, the plateau duration was invariably reduced (Fig. 7A). The effects on other properties of the axon were also similar to those of Ca^{2+} : the resting membrane was depolarized, the membrane resistance was decreased and the peak amplitude was reduced by Sr^{2+} . The only significant difference was that the recovery on switching to the control perfusate was always better with Sr^{2+} than with Ca^{2+} .

Effects of intracellular Ba^{2+}

As described in METHODS, it was unnecessary to use glutamate in the control perfusate because BaF_2 is soluble in water. After exposing the interior of the axon to the test perfusate which contained Ba^{2+} (0.03–1 mM), the effect of Ba^{2+} was found to be highly reversible. Unlike Ca^{2+} and Sr^{2+} , Ba^{2+} always prolonged the plateau, as shown in Fig. 7B. The resting membrane was depolarized only slightly (2–3 mV with 1 mM Ba^{2+}). The membrane resistance has not been affected appreciably. The most conspicuous change, apart from prolongation of the plateau duration, was a marked decrease in the rate of rise of the action potential. With 0.3 mM Ba^{2+} it decreased to a value between one-third and one-tenth of the control. The recovery was excellent when the perfusate was switched back to the control.

In one axon we used TEA-free perfusing solution in order to test whether or not Ba^{2+} alone can produce an action potential with a long duration. Following intracellular application of 1 mM Ba^{2+} , the axon started to fire repetitively without electrical stimulation. The individual action potentials had a duration of about 15 s. This observation indicates that Ba^{2+} alone can prolong the duration of action potentials.

Effects of intracellular Mn^{2+}

Mn^{2+} is known to be a divalent cation with little toxic effect when applied

Fig. 7. Effects of intracellular Sr^{2+} , Ba^{2+} and Mn^{2+} on action potentials of TEA-treated axons. External medium was always the standard artificial sea water. Internal media in top (control) and bottom (recovery) records were the same in individual axons. The vertical lines indicate 100 mV. A: axon 198, 620 μm in diameter. The control perfusate contained (in mM) 90 K-glutamate, 10 K^+ as K-phosphate, 5 TEA-Cl and 0.1 K-EDTA. The test perfusate contained 1 SrCl_2 and all the solutes in the control perfusate except K-EDTA. B: axon 177, 400 μm in diameter. The control perfusate contained 0.33 BaCl_2 and all the solutes in the control perfusate except K-EDTA. The peaks of the initial spikes are not well reproduced in the figures for this axon; they were 97 mV in Control, 90 mV in Test and 100 mV in Recovery. C: axon 217, 510 μm in diameter. The control perfusate contained 100 KF, 5 TEA-Cl, and 2.5 Tris-Cl. The test perfusate contained 1 MnF_2 and all the solutes in the control perfusate.

internally (BEGENISICH and LYNCH, 1974). We found that intracellular application of Mn^{2+} (0.1–5 mM) for about 10 min did not have serious detrimental effect on the excitability. In these experiments the control perfusate contained 100 mM KF and 5 mM TEA-Cl (with Tris-HCl buffer), and the test perfusate was prepared by adding MnF_2 or MnCl_2 crystals to the perfusate. As expected reversibility was good in the presence of fluoride.

Mn^{2+} at a concentration of 1 mM or higher always shortened the plateau duration (Fig. 7C). The reversibility was very good. At 0.3 mM, the shortening effect was observed only in some axons.

With Mn^{2+} application, the resting potential was either unaffected or slightly (2–3 mV) increased; the membrane resistance was either unchanged or slightly decreased; the amplitude of action potentials did not change appreciably, and the rate of rise was decreased only slightly. These changes were also highly reversible.

Voltage clamp experiments with Mn^{2+}

Because Mn^{2+} experiments could be performed with a high degree of reversibility, it was possible to measure the membrane conductance during application of Mn^{2+} with the use of the voltage clamp technique.

When the axon was internally perfused with the control solution containing 100 mM K^+ and 5 mM TEA, the depolarizing clamping pulse produced an initial peak current followed by a late current which lasted up to the end of the clamping pulse of about 10 ms (Fig. 8). The initial peak current was similar to the one obtained in an unperfused axon, but the late current was inwardly directed through the membrane in the voltage range of 20–110 mV. This late inward current is due to the existence of intracellular TEA; if TEA was eliminated from the axon interior, it was replaced by a late outward current as was described by HODGKIN *et al.* (1952). The late inward current under the influence of TEA was similar to

Fig. 8. Current-voltage relationship obtained by the voltage clamp technique with the clamping pulse of 11.4 ms duration. A: initial peak currents. B: late currents measured at the end of the clamping pulse. Circles: membrane currents with the control perfusate. Triangles: membrane currents with the perfusate containing 1 mM MnF_2 . The holding potential was set at the initial resting potential. The arrow indicates the external potential level. The leak current measured by a 100 mV hyperpolarizing pulse was about 0.01 mA/cm². C: specimen records of the late inward current; initial currents were too large to be illustrated in these records. Top, control. Middle, with Mn^{2+} -containing perfusate. Bottom, control again. Clamping voltages were: top, 82 mV; middle, 87 mV; bottom, 79 mV. Downward deflections represent the inward current. Calibration, 0.1 mA/cm². Time, 5 ms. Axon 217, 510 μ m in diameter.

those appeared in the figures published by TASAKI and HAGIWARA (1957, Fig. 11) and by ARMSTRONG and BINSTOCK (1965, Fig. 2). We observed that the late inward current disappeared on extracellular application of tetrodotoxin (10^{-5} M); this finding is consistent with the view that the late inward current passes through the sodium channel (ADELMAN, 1971).

When 1 mM Mn^{2+} was added to the perfusate, the initial peak current was slightly decreased, but its reversal potential did not change appreciably (Fig. 8). This is consistent with the fact that the peak amplitude and rate of rise changed very little in an unclamped axon with this perfusate. A drastic change appeared at the later phase of the clamping current (Fig. 8C). The late inward current was always profoundly inhibited, and the V-I curve obtained from values at the end of the clamping pulse came closer to the V axis. The slope of the curve was always small (Table 1), and the V-I curve sometimes did not cross the V axis, showing that the membrane potential was not stable in the later stage of the clamping. The late inward current reappeared when Mn^{2+} was removed from the axon interior; the effects of Mn^{2+} were highly reversible.

Table 1. Effects of intracellular Mn^{2+} on the late membrane conductance.¹⁾

Axon No.	Concentration of Mn^{2+} (mM) ²⁾	Conductance (mS/cm ²)		Decrease (%)	Reversal potential(mV) ³⁾	
		Control	Test		Control	Test
213	1	3.33	1.43	57	98	NE ⁴⁾
215	3	4.55	2.11	54	110	NE
216	1	7.36	3.84	48	109	103
217	1	5.46	3.47	36	113	96
218	3	4.38	1.12	74	97	NE

1) Measured from the gradient of the V-I curve at the reversal potential of the control late current.

2) Intracellular concentration of Mn^{2+} of the test perfusate.

3) Measured from the resting level.

4) Non-existent because the curve did not cross the potential axis.

The results are clearly consistent with the observation that the plateau of an unclamped axon disappeared when Mn^{2+} was introduced intracellularly.

DISCUSSION

Since Ca^{2+} , Sr^{2+} and Mn^{2+} shortened the plateau whereas Ba^{2+} and Mg^{2+} lengthened it, the effects of divalent cations cannot be explained as due to a single characteristic action of these cations. It is necessary to regard the action of divalent cations as a sum of at least two opposing effects. The shortening effect of Ca^{2+} , Sr^{2+} and Mn^{2+} may be regarded as similar to the effect of acidic perfusates (TERAKAWA and WATANABE, 1976; WATANABE *et al.*, 1977). The similarity is strengthened by our recent finding that acidic perfusates also suppress the late

inward current (Terakawa *et al.*, data to be published). Probably Ca^{2+} , Sr^{2+} , Mn^{2+} and proton act on the negative fixed charges on or near the membrane; they neutralize the charges or shield their Coulomb force. This may lead to a decrease in the membrane conductance measured by voltage clamp, resulting in shortening of the plateau duration. The similarities between effects of divalent cations and the proton on polyelectrolytes have been pointed out by MICHAELI (1960). On the other hand, the lengthening effect of Ba^{2+} and Mg^{2+} is probably to be ascribed as due to their ion-specific, or 'lyotropic' effects which cause a salting-in of macromolecules. Unfortunately, however, the lyotropic series of cations vary according to the target macromolecules. A series which is consistent with the present results can be extracted from the literature (see, *e.g.*, HÖBER, 1945, p. 304; GUSTAVSON, 1956, p. 179). It is uncertain, however, if these results have any relevance to the phenomenon presented in this paper. A better approach seems to be to compare the protein-solubilizing power of the divalent cations on the squid axon axoplasm and to determine the sequence of these cations according to their power. This is, however, an experiment to be performed in future.

Our experimental results with Ca^{2+} are very similar to the shortening effect of intracellular Ca^{2+} on the action potential of cardiac muscle fiber (ISENBERG, 1975). In the cardiac muscle, it has been proposed that the shortening effect might be due to an activation of the potassium conductance mechanism (see MEECH, 1974a, b). It is possible that the same mechanism explains the shortening of the plateau by intracellular Ca^{2+} in TEA-treated squid axons. For the following reasons, however, we are inclined to think that here the mechanism is different. (1) Intracellular introduction of Ca^{2+} caused a membrane depolarization even though the calculated potassium equilibrium potential (approximately -58 mV) was larger than the resting membrane potential (around -40 mV). (2) The voltage clamp experiment indicates that the intracellular introduction of Mn^{2+} reduces the conductance at the later period of the clamping. The shortening action of Mn^{2+} on the plateau can therefore be hardly explained in terms of activation of any ionic channels. If the effect of Mn^{2+} is assumed to be similar to that of Ca^{2+} , it is improper to ascribe the Ca^{2+} effect to an activation of any ionic channels. The above reasonings are, however, still indirect.

Intracellularly applied Ca^{2+} shortened the duration of plateau potential of TEA-treated squid giant axons. The effect is similar to that of extracellularly applied Ca^{2+} (see Fig. 5), except that, with intracellular application, the necessary concentration of Ca^{2+} was several hundred times lower than that with extracellular application. The similarity between effects of externally and internally applied divalent cations is not confined to Ca^{2+} . Thus externally applied Ba^{2+} prolongs the duration of action potentials in many excitable tissues, *e.g.*, in the cockroach giant axon (NARAHASHI, 1961), in frog muscle fibers (SPERELAKIS *et al.*, 1967), in arthropod muscle fibers (WERMAN *et al.*, 1961; HAGIWARA and NAKA, 1964)

and molluscan nerve cells (ECKERT and LUX, 1976). The effect is qualitatively the same with that of intracellularly applied Ba^{2+} described in this paper. Further, extracellularly applied Mn^{2+} shortens the duration of action potentials of Ba^{2+} -treated barnacle muscle, and action potentials of the frog cardiac muscle (HAGIWARA and NAKAJIMA, 1966). Again the effect is qualitatively the same with that of intracellularly applied Mn^{2+} described in this paper. These results lead to a hypothesis that the primary site for some of the effects of the divalent cations is located on or near the internal surface of the membrane, and the extracellular and intracellular divalent cations produce the effects after binding the site of the plasma membrane. This hypothesis is not quite new (see, e.g., the discussion given by KASS and TSIEN, 1976), and is clearly consistent with the proposition made by TASAKI (1968) that the termination of the excited state may be a result of change in the ionic composition within and near the membrane.

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