Special Lecture*

THE Ca ION PERMEABILITY OF THE CELL MEMBRANE

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IT is a great honor to be here as an invited lecturer of this Society, and I am very grateful to Professor Matsuda for his kind introduction. The Ca ion permeability, which is the title of my talk, is a broad subject. Therefore, I would like to limit my talk to the membrane potential-dependent Ca ion permeability, in which we have been interested for the last several years.

The electrical potential of the interior of the excitable cell, such as a nerve or muscle cell, is negative by 50 ~ 80 mV against the solution surrounding the cell. When the cell undergoes excitation, this potential difference changes its sign, i.e., the potential at the interior becomes temporarily more positive against the external saline. This phenomenon is referred to as the action potential.

About 30 years ago, based upon a series of experiments with the giant axon of a squid, Hodgkin & Huxley¹ in England proposed the "Na theory" to explain the excitation phenomenon. According to this theory, the action potential is produced by the permeability increase of the membrane to Na ions. This brings the membrane potential towards the Na equilibrium potential V_{Na} . This permeability increase is then followed by the K permeability increase, and this brings the membrane potential rapidly back to V_K which is close to the resting potential (Fig. 1). During the last 2 decades, a considerable amount of experimental results have been accumulated to show this principle is applied to a number of different excitable tissues.

Shortly after the Na theory was proposed, Fatt and Katz² found that this general principle

has to be modified slightly in some excitable tissue, such as crustacean muscle fibers. In those tissues the initial permeability increase of the action potential occurs to Ca ions instead of Na ions, and this is followed by the K permeability increase. Those action potentials are referred to as Ca spikes, and the mechanism of the membrane responsible to the Ca permeability increases as the Ca channel. The classical action potential should then be called the Na spike. I have been

Fig.1. Diagram of Na and K conductances during the action potential. (Hodgkin & Huxley¹).

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Fig.2. Ca spikes of barnacle muscle fibers. (Hagiwara & Naka³).

interested in Ca spikes for over ten years. Our earlier studies of the Ca spike were performed with barnacle muscle fibers. The species of barnacle we used is *Balanus nubilus* first described by Charles Darwin and distributed along the Pacific coast of the American continent. They are particularly abundant in the north of Oregon, U.S.A. and south of Valparaiso, Chile. The size of the barnacle is quite large and the size is sometimes comparable to that of a football. The diameter of the muscle fiber becomes up to 2 mm and, therefore, every technique applied to the squid axon is equally applicable to this preparation.

The top two records of Fig. 2 show Ca spikes of the same barnacle muscle fiber obtained before and after replacing the external Na ions with impermeant Tris ions. Any Na spike should disappear under this condition. In this preparation, however, the action potential is virtually unchanged. (A slight increase of the resting potential suggests that the resting Na permeability is not negligible.) In contrast, the change in the external Ca concentration results in a change in the action potential. This is shown by the middle 5 records. The Ca concentration was

Fig.3. The Na current (C) and the K current (B) sum up to the net membrane current (A). (Hodgkin & Huxley⁵).

MEMBRANE CURRENTS OF A BARNACLE MUSCLE FIBER DURING VOLTAGE CLAMP

Fig.4. Membrane currents carried by Ca⁺⁺ in the barnacle muscle fiber under voltage clamp⁶

gradually increased from 20 mM to 338 mM. The membrane potential at the peak of the spike is plotted against the logarithm of the Ca concentration at the bottom of the figure. When the Ca concentration is close to its normal value, which is 20 mM in the barnacle saline, the rate of increase is 29 mV for a tenfold increase in the

concentration. The slope is expected from the Nernst equation in a Ca electrode and is illustrated by a broken line. As the concentration increases, the rate becomes smaller; in other words, the relation tends to saturate. A similar saturation phenomenon, which is not usually seen for the Na spike, is often found for Ca spikes in a variety of preparations.^{3,4}

The Na spike is produced by the opening of the Na channel, and this is followed by the opening of the K channel. As I have mentioned, the opening occurs first at the Ca channel for the Ca spike. Properties of the Na, Ca and K channels differ in several aspects. In fact, those differences provide the basis for us to believe that they correspond to different physical entities of the membrane. The term "channel" does not necessarily mean that its molecular structure in the membrane is actually channel-like. Of course, many of us believe that this is the case. The Na, Ca and K channels are distinguished in three major areas. They are (a) the kinetics of the membrane current as a function of the membrane potential and the time, (b) the ion selectivity, and (c) pharmacological properties.

Figure 3 shows the membrane current obtained during the voltage clamp of the squid giant axon (Hodgkin & Huxley⁵). The total membrane current is an initial rapid inward current followed by a gradual development of the outward current. This is the sum of the inward Na current and the outward K current. The Na conductance increases rapidly so that, at its peak, the K conductance is still negligible. The Na conductance increase is always followed by an inactivation. In other words, the Na conductance follows m^3h kinetics; in contrast, the K conductance shows no inactivation, and it follows n^4 kinetics.

Figure 4⁶ shows membrane currents obtained during the voltage clamp of a giant muscle fiber of a barnacle. In this preparation, the inward current is carried by Ca ions. The K current was reduced by applying TEA to the external solution. The number to each record indicates the membrane potential during the voltage clamp. Two traces in each record were obtained before and after replacing the external Ca with impermeant Mg. In other words, the difference between the paired traces roughly represents the inward Ca current. These records show that the Ca permeability increase is not very fast compared with the increase in the K permeability. Therefore, at relatively positive membrane potential, the K current has already become sig-

Fig.5. Ion selectivities of the Na and K channels.

nificant at the time when the Ca current reaches its peak value. The peak amplitude of the net inward current is plotted against the membrane potential with filled circles, while the peak amplitude of the actual inward Ca current obtained as a difference between the two paired traces is plotted with open circles. They deviate significantly as the membrane potential becomes more positive. The activation of the Ca channel is always faster than that of the K channel, but it is slower compared with that of the Na channel. This is the first point. The second point of the Ca current is that the inactivation is not very clear. Some scientists believe that the Ca channel shows no inactivation; others believe that the inactivation is real enough, albeit slow. There is also the belief that the apparent decline of the Ca current is due to the blocking effect of internal Ca ions which enter during the Ca current.⁷ In short, the kinetics of the Ca channel represent an intermediate between those of the Na and the K channels.

The most important difference is the one in their ion selectivity. It is obvious that the Na channel is permeable to Na ions, and the K channel is permeable to K ions. The permeability of either channel to other alkali cations is not zero. Figure 5 shows the selectivity of the Na

Ca PERMEABILITY

	IONIC RADII Å	PERMEABILITY
Mg	0.65	-
Ni	0.70	-
Co	0.72	-
Fe	0.75	-
Mn	0.80	+
Ca	0.99	++
Sr	1.13	++
Ba	1.35	++

Fig.6. The permeability of the Ca channel.

and K channels in terms of permeability ratios. The X axis is the crystal radius of the ion. For the Na channel, P_{Na} was taken as unity; for the K channel, P_K was taken as unity. Continuous lines show the data obtained with frog Ranvier node by Hille^{8,9}. Broken lines show the data obtained with a squid giant axon. The Na channel data are from Chandler & Meves¹⁰ and the K channel data are from this laboratory¹¹. The Na channel is permeable to Li, but it is not very permeable to K, Rb, or Cs. Tl and NH_4 are not alkali cations, but their ionic diameters are similar to those of alkali cations and are moderately permeant to the Na channel. For the K channel, Tl is more permeant than K. An important conclusion which can be drawn from the data is that the selectivity of either the Na or K channel is common between frog axon and squid axon. Assuredly, frog and squid are very far apart phylogenetically. Therefore, this indicates that when we work on the Na or K channels, we are dealing with unique molecular structures regardless of the species of animal.

The Ca permeability of the Na channel of the squid axon is not zero, but P_{Ca}/P_{Na} as estimated by Baker, Hodgkin and Ridgeway¹² is less than 1/100. In other words, the permeability to divalent cations is very small. In contrast, the Ca channel is permeable to divalent cations, and permeabilities to monovalent cations are negligible. Figure 6 lists divalent cations together with their crystal radii. The Ca channel is permeable to large cations such as Ba, Sr and Ca, and it is impermeable to small cations such as Mg, Co, Ni, and Fe. One of the intermediate-

sized cations, Mn, always shows small but significant permeability in the Ca channel. The Cd ion has a radius of 0.97 Å; therefore, it is larger than Mn. We have examined the permeability of Cd without success, since Cd is toxic and the cell membrane becomes very leaky in a very short time. However, Cd is found to be a permeant ion for the Ca spike of an insect larval muscle.¹³

As I mentioned already, the Ca spike was first found in crustacean muscle fibers. During the last decade, it has become clear that a variety of tissues other than crustacean muscles show Ca spikes. Action potentials of smooth muscles are all Ca spikes. At least no evidence is known for the Na spike. Vertebrate striated muscle fibers all produce Na spikes, but always include a small component of Ca spike. In contrast, striated muscle fibers of invertebrates show pure Ca spikes. Action potentials of vertebrate heart muscle have both Na and Ca components. These Ca spikes are obviously important for the excitation-contraction coupling. The membrane of presynaptic terminal has the potential-dependent Ca permeability. Endocrine secretory cells, such as the Langerhan's β cells of pancreas, anterior pituitary cells, and the chromaffin cell of the adrenal medulla, all show Ca spikes. Some neuron somata, such as *Aplysia* ganglion cells, produce both Na and Ca spikes. These Ca spikes seem to play an important role in excitation-secretion coupling. One interesting example of the spike is the one found in the membrane of a *Paramecium* by Naitoh and Eckert.¹⁴ This Ca spike is normally produced by a mechanical stimulus applied to the rostral portion of the animal. The positive-going change in the membrane potential during the Ca spike results in the reversal of the ciliary movement. In other words, the *Paramecium* swim backwards after the rostral part of the body hits an obstacle. In general, one can find Na spike wherever the conduction of impulse is important, and Ca spike whenever the action potential couples effective functions.

We consider the binding of Ca ions to a site of the Ca channel to be an important step in the permeation of Ca ions through the channel.^{15,16} The Ca channel has a site X, and the site is either in free state X or the bound state XCa, when Ca ions enter the channel. Since the internal Ca ion concentration is small, we can neglect the efflux of Ca ions. Therefore, the steps involved with inward flow of Ca ions may be roughly represented by

Then the inward Ca current at a given membrane potential $I_{\text{Ca}}(V)$ is given as a function of the external Ca ion concentration by the equation which is similar to the Michaelis-Menten equation in enzyme kinetics

$$I_{\text{Ca}}(V) = \frac{I_{\text{Ca max}}(V)}{1 + \frac{K_{\text{Ca}}(V)}{[\text{Ca}]_0}} \quad (1)$$

K_{Ca} is the apparent dissociation constant of the site to the external Ca ions and $I_{\text{Ca max}}$ is the current expected when all the sites are occupied by Ca ions. In order to test this relation, one can record Ca inward current by the voltage clamp technique and examine its amplitude with $[\text{Ca}]_0$. In the experiment shown in Fig. 7, the maximum rate of rise of the Ca spike was used since this parameter is proportional to the maximum amplitude of the inward Ca current during the spike. The experiment was performed with barnacle muscle fibers,¹⁵ and the calculated curve (a broken line) agrees quite well with the experimental data. Recently, Beiraos Lakshminarayanaiah¹⁷ examined barnacle muscle with the voltage clamp technique and obtained the same result. The above conclusion indicates that the Ca channel is a saturable system. This, however, is not the property which distinguishes the Ca channel from the Na channel. The external solution is usually very close to the isotonic NaCl solution. Therefore, the effect of $[\text{Na}]_0$ is examined for the range of $[\text{Na}]_0$ below the normal concentration. In contrast, $[\text{Ca}]_0$ is

small in the normal solution and hence the effect has been observed by raising $[\text{Ca}]_0$ above its normal value. If the observation is made below the normal value of $[\text{Ca}]_0$, the saturation will be less marked. In fact, some saturation phenomena have been observed for the Na channel of the Ranvier node when the concentration was increased to a very large value.¹⁸

Other polyvalent cations such as Mg, Ni, Co and La do not carry current through the Ca channel, but they appear to bind the same membrane site thereby resulting in a blockade of the Ca channel. The maximum rate of rise of the Ca spike of the barnacle muscle fiber at 41 mM Ca was observed when the concentration of the blocking cation was altered and plotted against the concentration of the blocking cation (Fig. 8). The maximum rate of rise obtained in the absence of blocking cations is taken as the unity. If Ca and blocking cations compete in the same site X, the Ca current will be expressed by the following equation which is similar to the one for the competitive inhibition in enzyme kinetics.

$$I_{\text{Ca}}(V) = \frac{I_{\text{Ca max}}(V)}{1 + \frac{K_{\text{Ca}}(V)}{[\text{Ca}]_0} \left(1 + \frac{[\text{M}]_0}{K_{\text{M}}(V)}\right)} \quad (2)$$

Broken lines in Fig. 7 were drawn according to the equation by assuming appropriate binding constants. The blocking effect of La is very high. 100 μM has already shown a significant effect. In order to obtain a similar effect, 5 ~ 10 mM is necessary for Co, Mn and Ni. The effect of Mg is very low, so that 100 mM or more is necessary to obtain a comparable effect.^{15,16}

The affinity of Ca to the site X of the Ca

Relation between $[\text{Ca}^{2+}]_0$ and the maximum rate of rise of the Ca-dependent action potential in a barnacle muscle fiber. A curve illustrated by a dotted line was calculated by Eq. by assuming $K_{\text{Ca}} = 24 \text{ mM}$.

Fig. 7. From references 4 and 14.

Fig.8. From references 4 and 14.

channel is not very large, but its mobility through the channel is large and therefore carries a significant current. In contrast, Co has a very high affinity to the site, but its mobility through the channel is almost zero. As a result of this, it carries little current. The amount of current carried by each ion species is thus determined by the two factors; they are the affinity to the site and the mobility through the channel.

The following experiments suggest that one has to consider both the affinity and the mobility factors in order to understand the Ca channel. Figure 9⁶ shows current-voltage relations at the peak of the early inward current obtained by voltage clamp. Three relations were obtained from the same muscle fiber at 20 mM Ca, Sr and Ba respectively. The result indicates that the amplitude of the inward Ba current is much greater than that of the inward Ca current. Therefore, one may simply conclude that Ba ions are more permeant through the Ca channel than Ca ions. In this experiment, Ca, Sr or Ba is the only divalent cation in the solution. When blocking divalent cations are present, the result could be different. In the experiment shown in Fig.10, the concentration of Ca, Sr or Ba was also 20 mM but the solution contained 20 mM Co in addition. The sequence among the Ca, Sr and Ba currents is now reversed; that is, Ba carries less current than does Ca. The revision of the sequence can be explained in considering that the Ca channel is a saturable system. One may assume that a cation having a higher affinity to the site is less mobile through the channel. For example, the affinity of Ca ions to the site X is

greater than that of Ba ions, but Ba ions are more mobile through the channel. When the concentration of permeant cations is relatively high, the sites would be nearly saturated with permeant cations. For example, if all the sites are occupied, the sequence of the current should be determined simply by the mobility of the cation through the channel. In other words, the Ba current

Fig.9. Relationship between the peak inward current and the membrane potential under voltage clamp of the barnacle muscle fiber⁶ 15

Fig.10. Similar to Fig.9, but the external saline contains 20mM CoCl_2 , 6, 15

would be greater than the Ca current. When a large amount of blocking cations are present, permeant cations have to compete for the site with blocking cations; therefore, the number of sites occupied by the permeant cation would vary greatly depending upon their binding con-

stants to the site. In other words, the number of sites occupied by Ca could be significantly greater than that occupied by Ba under this condition. This effect may overcome the difference in their mobilities. Therefore, the Ca current may become greater than the Ba current. The ratio between the Ca and Ba currents can be expressed by:

$$\frac{I_{\text{Ba}}(V)}{I_{\text{Ca}}(V)} = \frac{I_{\text{Ba max}}(V)}{I_{\text{Ca max}}(V)} \cdot \frac{C + \alpha K_{\text{Ca}}(V)}{C + \alpha K_{\text{Ba}}(V)}$$

$$\text{where } C = [\text{Ca}]_o = [\text{Ba}]_o \quad (3)$$

$$\text{and } \alpha = 1 + [\text{Co}]_o / K_{\text{Co}}(V)$$

The first and second terms of the righthand side of equation³ are mobility factor and affinity factor, respectively. From a series of experiments with barnacle muscle fibers, the affinity factor as expressed by $K_{\text{Ca}}/K_{\text{M}}$ and the mobility factor by $I_{\text{M max}}/I_{\text{Ca max}}$ were obtained for different divalent cations and plotted against the crystal diameter of each ion (Fig.11). It is very likely that different sequences may be obtained in different tissues. Unlike the Na channel, there is a good possibility that various different kinds of Ca channels are found among different tissues.

The Na and Ca spikes differ in their sensitivities to various pharmacological agents. Tetrodotoxin, which comes from a puffer fish, is ex-

Fig.11. Relative value of the affinity factor ($K_{\text{Ca}}/K_{\text{M}}$) and the mobility factor $I_{\text{M Max}}/I_{\text{Ca Max}}$ of the Ca channel obtained with barnacle muscle fibers.

D600·HCl (521 dalton)

PREPARATION	CONCENTRATION (50-80% reduction of I_{Ca})	OTHER EFFECTS	REFERENCES
SQUID AXON	200 μ M	Na current-substantially reduced Delayed K current- no effect	Baker, Meves & Rigway 1973
X-ORGAN (CRAYFISH)	100 μ M	Delayed K current-reduced equally Na current-reduced at 5-10X concentration	Kuroda & Iwasaki 1976
NERVE CELL (SNAIL)	30-100 μ M (Verapamil)		Akaike, Lee & Brown 1978
INTESTIN MUSCLE (GUINEA PIG)	10 μ M		Inomata & Kao 1976
PANCREATIC ISLET CELL (RAT)	10 μ M		Matthews & Sakamoto 1975
VENTRICULAR TRABECULAE (CAT)	1 μ M	Na current-no effect	Kohlhardt, Bauer, Krause & Fleckenstein, 1972
HEAR PURKINJE FIBER (CALF)	1 μ M	Na current-no effect Slow K outward current- reduced equally	Kass & Tsien, 1975

Fig.12. Effective doses of D 600 on Ca channels of different preparations.

tremely effective in blocking the Na channel but totally ineffective in blocking the Ca channel. Procaine suppresses the Na spike at a concentration of less than 10 mM. The same concentration has no effect on the Ca current. Since procaine suppresses the K current, the net effect is an enhancement of the spike. As mentioned already, Co, Ni, Mn, etc. block the Ca spike. No effect was observed on the Na spike at relatively low concentrations of these ions. However, various effects may appear even in the Na spike if the concentrations are high – for example, 50 mM. Tetraethylammonium ions suppress the K current, but have no effect on either the Na or Ca current.

Recently, various organic agents have been reported as blockers of the Ca channel. The most common ones are verapamil, its methyl

derivative D 600 and nifedipine. The mechanism of blocking of the Ca channel by these agents is not fully understood. Figure 12 shows concentrations of D 600 necessary to reduce the amplitude of the Ca current to 50 ~ 20% of the original value. It shows that the effective concentration varies greatly depending upon preparations. Effective concentrations are high in invertebrate preparations, and the effect is relatively non-specific. At this concentration of D 600, the Na current is also affected in the squid giant axon.¹⁹ In the X-organ of a crayfish, the K current of the delayed rectification is also suppressed.²⁰ In contrast, the effective concentration is low in vertebrate preparations, particularly in cardiac muscles. One μ M is sufficient to block the Ca current in cardiac fibers. At this concentration, the Na current is not affected at

Fig.13. Diagrams of intracellular perfusion of spherical cells.²³⁻²⁵

all. In this sense, D600 may be considered a specific blocker of the Ca channel. According to Kass and Tsien,²¹ 1 μ M of D600 will block the slow K outward current of the calf Purkinje fiber in addition to the Ca current. The Ca induced increase of the K conductance has been described in various tissues (Meech²²). When the Ca ion concentration inside the cell is increased as a result of the Ca influx through the Ca channel, an increase of the K conductance occurs. If the slow K outward current is a Ca-induced K current, D600 – which blocks the Ca channel – should eliminate this K current. Kass and Tsien have demonstrated that the slow K current is not a Ca-induced current. Thus, at present D600 or verapamil does not seem to be as specific to the Ca channel as tetrodotoxin is to the Na channel.

The lack of knowledge of pharmacology of the Ca channel is due to the number of difficulties in analyzing the properties of the Ca channel. It has been difficult to obtain preparations to which voltage clamp technique can be reliably applied. One of the most promising preparations is isolated ganglion cells of the snail. With this preparation, one can alter the intracellular as well as the extracellular ionic composition. Figure 13 illustrates three different methods of intracellular perfusion of spherical cells. The left diagram shows the technique used by Takahashi & Yoshii²³ for the egg cell of the tunicate. The cell is inside a glass tubing. The cell membrane is ruptured at the tip of the tubing and the internal solution enters from this region. The middle diagram is the method used with a suction pipette and developed by Lee, Akaike and Brown²⁴. The right diagram is the technique developed in Kiev, U.S.S.R.²⁵ In this method, two suction pipettes made with polyethylene tubings are used. Solutions flow from one pipette to the other. All of these methods are now successfully in use.

Last month we had a meeting of the American Biophysical Society in Atlanta, Georgia. At this meeting, I learned that Dr. K. Lee applied the suction pipette method to single rat cardiac muscle fibers isolated with collagenase and hyaluronidase. He did voltage clamp analysis with cardiac muscle fibers (about 30 μ m in diameter) after perfusing the interior of the cell with Cs-Aspartate. He could record inward Ca current in Na-free Tris media. The amplitude of the Ca current increased 3 ~ 4 times when 10^{-5} M adrenalin was added to the external solution. This effect of adrenalin has been

known and the effect is found to be on the β receptor of catecholamine. In contrast, adrenalin blocks the Ca current in spinal ganglion cells of a chick, and this effect occurs through the α receptor of catecholamine.²⁶ A similar effect was found also for enkephalin.²⁶ Thus, various transmitters and hormones may modify the Ca current, and this, in turn, alters the transmitter or hormone release. At this moment, however, the mechanism is little known. Understanding of this mechanism will shed some light in understanding a number of unsolved problems, not only in the cardio-vascular system, but also in many other systems such as brain functions.

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