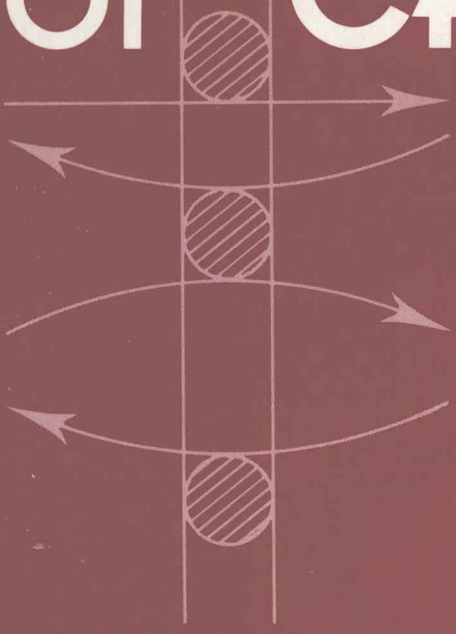
MEMBRANE TRANSPORT OF CALCIUM



edited by

ERNESTO CARAFOLI

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ERNESTO CARAFOLI

Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland

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Membrane Transport of Calcium

List of Contributors

Mordecai P. Blaustein, *Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201, U S A*

Felix Bronner, *The University of Connecticut Health Center, School of Dental Medicine, Farmington, Conn. 06032, U S A*

Ernesto Carafoli, *Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zürich, Switzerland*

Giuseppe Inesi, *Department of Biochemistry, University of Maryland, School of Medicine, Baltimore, Maryland 21201, U S A*

P. G. Kostyuk, *A.A. Bogomoletz Institute of Physiology, Bogomoletz str. 4, 252601 GSP, Kiev-24, U S S R*

Leopoldo de Meis, *Instituto de Ciências Biomédicas, Departamento de Bioquímica, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Cidade Universitária, Ilha do Fundão, 21.910, Rio de Janeiro, Brasil*

Mark T. Nelson, *Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201, U S A*

Barry P. Rosen, *Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201, U S A*

H. J. Schatzmann, *Universität, Veterinär-Pharmakologisches Institut, Länggass-Strasse 124 3000 Bern, Switzerland*

Preface

It is a lucky coincidence that this book should appear almost 100 years after the publication of the landmark contribution of Ringer on the role of calcium in the contraction of heart. This book is devoted essentially to the messenger function of calcium, a field which has now become immensely popular, and which has undoubtedly been opened by the remarkable observations made by Ringer in 1883.

An appropriate way of celebrating an anniversary, then. But also a way of telling what a long way the field of calcium-related research has come since the days – not too old, in fact – when the overwhelming interest of workers in the area was in the structural role of calcium. Against the background of this overwhelming interest stood a few scattered observations which suggested completely different possibilities. As is so frequent in science, most of them had come ahead of the times, and their implications were clear only to a handful of remarkably foresighted pioneers. At a meeting I attended in 1975, I remember Annemarie Weber quoting one of them, L. V. Heilbrunn, as once saying (or writing) "*Kalzium macht alles*". A remarkable comment, indeed. It may have sounded grossly exaggerated at the time it was made, but we can see today instead how close it was to reality.

In retrospect, it is difficult to trace back the present phenomenal explosion of knowledge and interest in the field of calcium biochemistry to a single factor. Whether it was the findings and the development of concepts in the field of nervous conductance, or the progresses made in the molecular mechanisms of endocrinology. Whether it was the rapid advances in the field of membrane transport of calcium, and the early work on sarcoplasmic reticulum. Or the discoveries in the area of high-affinity calcium ligation, with their implications for ionophoric transport and muscle contraction. Or was it, last but not least, the recent discovery of calmodulin? Reasonably, each one of these developments played a role, and it was a fortunate happening that situations favourable to important breakthroughs developed simultaneously in so many parallel directions. Not surprisingly, the

effect has been autocatalytic, and has been responsible for the atmosphere of great excitement the area enjoys today.

One obvious corollary of the messenger function of calcium is the necessity of controlling very precisely its activity in the intracellular environment, where the targets of the messenger function are located. So important is the messenger function, indeed, that a considerable number of systems for transporting calcium across biomembranes have been developed during evolution. These are independent systems, molecularly and mechanistically different from one another. Their multiplicity is probably a unique case in biology, and underscores the importance of calcium as a messenger. Using simple words, one could say that cells cannot afford significant fluctuations in the activity of calcium, and thus employ all possible ways and means to prevent it. The transport of calcium across biomembranes becomes thus a central component, perhaps *the* central component, of calcium biochemistry and physiology. One may object that this statement, which is reflected in the subject matter of this book, is somewhat restrictive, since it does not pay attention, at least in the traditional way, to the biochemistry of mineralization. This may well be so. But it certainly reflects the most exciting developments and the brightest prospects in the field.

The essays collected here review in detail the calcium transporting systems which are known today to operate in eukaryotic and prokaryotic membranes. Written by leading experts, they cover general properties, molecular aspects, mechanisms and function. The histories of the various calcium transport processes, however, have not been uniform. Out of necessity – the purification of the carrier proteins has generally come late – mechanistic and functional studies have frequently preceded molecular approaches. Thus, an important point in the book is the comparison of developments, both technological and conceptual, in neighbouring areas. Ideally, seasoned specialists as well as novices ought to find in it cross-fertilizing leads for future research endeavours. It is hoped that in the end significant advances in this beautifully exciting field may result.

October 1981 *Ernesto Carafoli*

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1

Penetration of Calcium Through the Membrane of Excitable Cells

P. G. KOSTYUK

A. A. Bogomoletz Institute of Physiology, Academy of Sciences of the Ukrainian SSR, Kiev, USSR

GENERAL ASPECTS OF THE PROBLEM

All cells maintain high gradients of Ca^{2+}, Na^+ and K^+ ions across the surface membrane but it is the Ca^{2+} gradient which is the most effectively maintained, due to powerful calcium binding mechanisms in the cytoplasm. This suggests that, if membranes possess ion-conducting molecular structures, Ca^{2+} ions may be highly effective carriers of electric current across the membrane and may be instrumental in recharging membrane capacity and in triggering active cellular reactions.

The classical work of Hodgkin and Huxley (1952) on perfused squid giant axons was the start of a number of investigations which showed the existence of molecular structures which allow Na^+ or K^+ ions to pass as a result of lowering of the membrane potential (membrane depolarization). The properties of electrically operated sodium and potassium channels have since been shown to be similar in many excitable membranes. Special 'grating" groups which can open or close a path in ionic channels for ionic movement under influence of external energy provide an exceptionally effective mechanism for the rapid release of potential energy accumulated by the cell in the form of Na^+ and K^+ gradients.

In 1953 Fatt and Katz showed in crayfish muscle fibres that a propagating action potential can occur in an extracellular medium even if sodium ions and hence sodium inward current are absent. The inward current necessary for membrane depolarization is due to divalent cations (Fatt and Ginsborg, 1958). Calcium-dependent action potentials in sodium-free solution could be obtained only if potassium conductance by the membrane was blocked by tetra-

ethylammonium (TEA); however, this treatment could be avoided if extracellular calcium was replaced by Sr^{2+} or Ba^{2+} ions, which appear to be more potent carriers of charges across the membrane than do Ca^{2+} ions.

More detailed information on the role of calcium ions in the electrical excitability of muscle fibres has been obtained by Hagiwara and his colleagues working on giant fibres of *Balanus nubilis* (up to 2 mm in diameter) (Hagiwara and Naka, 1964; Hagiwara *et al.*, 1964; Hagiwara and Nakajima, 1966). They showed that full-scale calcium-dependent action potentials in *Balanus* muscle can always be elicited by injecting calcium-chelating agents into the cell to cause an artifical decrease in the intracellular concentration of free calcium. The amplitude of action potentials in the concentration range of 20–100 mM extracellular calcium increased proportionally to the logarithm of $[Ca^{2+}]_o$, with a slope of about 29 mV for a 10-fold concentration increase. This dependence was close to the theoretical one for a calcium electrode according to the Nernst equation:

$$E_{Ca} = \frac{RT}{nF} \cdot \ln \frac{[Ca^{2+}]_o}{[Ca^{2+}]_i} \tag{1}$$

If at the peak of the action potential the membrane resembles the calcium electrode in its properties, then at two different concentrations of extracellular calcium, with its constant intracellular content, the difference between the peak amplitudes will be:

$$E_2 - E_1 = \frac{RT}{nF} \ln \frac{[Ca^{2+}]_2}{[Ca^{2+}]_1} \tag{2}$$

and at a temperature of 22° C this difference must be 29.5 mV with a 10-fold change of external calcium concentration. A good correlation between the theoretical and the experimental value was observed.

However, interpretation of these results was difficult. As mentioned, Hagiwara and colleagues showed that creation of a very low concentration of free calcium inside the cell (below 8×10^{-8} M) is a necessary condition for generation of the action potential in *Balanus* muscle fibre. This should correspond to extremely high positive values of the calcium e.m.f. (about + 150 mV), which exceeds by several times the observed values of the membrane potential during calcium spikes. This discrepancy can be accounted for if we assume:

(1) that intracellular calcium concentration near the membrane considerably exceeds its concentration in the bulk of the cell; (2) the potential of the calcium spike is determined not only by calcium conductance but also by potassium conductance and is dependent on the I_{Ca}/I_K ratio. In any case, the fact that the amplitude of the muscle fibre action potential spike is strongly dependent in sodium-free solutions on extra- and intracellular calcium concentration, clearly indicate the role of calcium ions in recharging the membrane.

Direct measurements of transmembrane ionic currents by a voltage clamp technique were carried out by Hagiwara *et al.* (1974). These authors' reversal potentials proved to be considerably lower than the expected values, probably because calcium and other transmembrane currents (potassium) were superimposed. When special measures were taken to suppress potassium conductance the equilibrium potentials shifted to more positive values (Keynes *et al.*, 1973). However, assessment of such potentials could be only approximated because direct current–voltage measurements at high positive membrane potentials proved to be virtually impossible and the values were extrapolated.

These authors confirmed that calcium ionic channels of muscle membrane allow other divalent cations to pass, namely Sr^{2+} and Ba^{2+}. In such cases, the quantitative changes in the amplitude of the action potential were also complex. The dependence of the action potential peaks on the logarithm of ion concentration did not show saturation effects with increasing concentration of Ba^{2+} ions, whereas such saturation was revealed with a change in Ca^{2+} concentration. In Ba^{2+}-containing solutions this dependence was nonlinear, its maximal slope being considerably higher than the theoretical value. Hagiwara (1973) proposed a model which could explain the following properties of calcium currents: (1) saturation of the current with increasing calcium concentration; (2) competition between calcium ions and other divalent cations (and also with some trivalent cations).

According to this model, the calcium ion, in order to pass through the membrane, must first be adsorbed by a certain membrane receptor, x:

$$\mathrm{Ca_o} + x \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{Ca}\,x \xrightarrow[k_3]{} \mathrm{Ca_i} + x \tag{3}$$

Since the total number of receptors, x, is constant at adsorption equilibrium:

$$K_{Ca} = \frac{([x] + [xCa])[Ca]_o}{[xCa]} \tag{4}$$

where K_{Ca} is the dissociation constant for calcium ions and receptor. From the equation (4) it follows:

$$[xCa] = \frac{[x]}{1 + K_{Ca}/[Ca]_o} \tag{5}$$

According to the model, $I_{Ca} \sim [xCa]$, and $I_{Ca}^{max} \sim [x]$. Then

$$I_{Ca} = \frac{I_{Ca}^{max}}{1 + K_{Ca}/[Ca]_o} \tag{6}$$

where I_{Ca}, I_{Ca}^{max}, K_{Ca} in the general case are potential-dependent values.

The quantitative description of this model completely fits the Michaelis-Menten equation (in the case of equilibrium) and the Langmuir isotherm. Equation (6) describes the saturation of calcium current with increase in external calcium concentration. If cation M^{2+} is present in the medium and binds to the same receptor with dissociation constant K_M but does not penetrate the channel, the equation which describes the calcium current will be:

$$I_{Ca} = \frac{I_{Ca}^{max}}{1 + (1 + [M]_o/K_M)K_{Ca}/[Ca]_o} \tag{7}$$

If I_{Ca} is the calcium current in the absence of other ion species and I'_{Ca} is the current in the presence of such species, then the ratio between these currents will be:

$$\frac{I_{Ca}}{I'_{Ca}} = \frac{1}{1 + [M]_o/K'_M} \tag{8}$$

where $K'_M = K_M (1 + [Ca]_o/K_{Ca})$.

This model does not take into consideration the potential-dependence of the measured values. Such dependence requires correction for the corresponding shifts in potential characteristics when K_{Ca} is determined. It can be achieved, for example, by creating a high concentration of impermeable divalent cations (e.g. 100 mmol Mg^{2+} ions) in external solution; however, these experimental conditions are largely artificial and can result in error.

As follows from the results of Hagiwara and Takahashi (1967), a series of divalent cations and one trivalent ion, lanthanum, can be arranged in the following order according to their ability to bind competitively to a presumed receptor in the calcium channel:

$$La^{3+}, UO_2^{2+} > Zn^{2+}, Co^{2+}, Fe^{2+} > Mn^{2+} > Ni^{2+} > Ca^{2+} > Mg^{2+}$$

The proposed model also allows evaluation of the relative ability of calcium channels to pass the above ions. So, for currents measured in equimolar calcium and barium solutions from the equation (7), one obtains:

$$\frac{I_{Ca}}{I_{Ba}} = \frac{I_{Ca}^{max}}{I_{Ba}^{max}} \cdot \frac{C + aK_{Ba}}{C + aK_{Ca}} \tag{9}$$

where $a = 1 + [M]_o/K_M$, $C = [Ca]_o = [Ba]_o$.

The ratio between maximal currents in this equation was called the "mobility factor" and the second term the "affinity factor". In the absence of blocking ions ($a = 1$) the following sequence of permeabilities has been obtained: $I_{Ca} : I_{Sr} : I_{Ba} = 1.0 : 1.05 : 1.3$ (Hagiwara *et al.*, 1974).

In 1956, from investigations of action potentials in mammalian heart muscle, it was observed that the plateau phase of these potentials is much less sensitive to removal of Na^+ ions from external solution than is the fast initial spike (Coraboeuf and Otsuda, 1956). Similar observations have also been made on frog heart (Niedergerke and Orkand, 1966). It became obvious that in heart muscle fibres, unlike nerve fibres, a considerable inward Ca^{2+} current appears during excitation. The detection of an increase in this current on application of adrenaline was of great importance. This conclusion was made first on the basis of the finding that adrenaline increases the amplitude of the action potential plateau in cases where sodium channels are blocked by tetrodotoxin (Stanley and Reuter, 1965). Increase in calcium influx was then confirmed by measuring ^{45}Ca entry into the muscle (Reuter, 1965). Counter to this, blockers of calcium currents (lanthanum, manganese, cobalt, nickel) caused a decrease in the amplitude of the plateau (Hagiwara and Nakajima, 1966). Results obtained in experiments with voltage clamping confirmed the presence of separate systems of sodium and calcium conductance in heart muscle fibre membrane; in sodium-free solution a slow inward current dependent on the external concentration of Ca^{2+} has been found (Reuter, 1967). This current was also seen in Na-containing solutions but in such cases it was complicated by inward flow of Na^+ ions (Vitek and Trautwein, 1971). Data have been obtained on the conductance of calcium channels in heart muscle for Ba^{2+} and Sr^{2+} ions, as well as on the measurable conductance for Mg^{2+} ions contrary to the results obtained in other membranes (Kohlhardt *et al.*, 1973). Sodium and calcium components

of the inward current in heart muscle fibre membranes have been partially separated by verapamil or its derivative D-600, which suppresses calcium conductance competitively (Kohlhardt *et al.*, 1972).

The reversal potential of the slow calcium inward current in heart muscle proved to be surprisingly low – about + 50 mV. A conditioning depolarization resulted in a small shift to more positive membrane potential values (for several millivolts – Bassingthwaighte and Reuter, 1972). Such low values of the reversal potential observed for calcium current in the heart muscle were explained by the low selectivity of the corresponding channels, which may pass Na^+ and K^+ ions together with Ca^{2+} ions. Rations P_{Ca}/P_{Na} and P_{Ca}/P_K were supposed to be $\sim 1:0.01$ (Reuter and Scholtz, 1977).

Studies on replacing extracellular ions and on the effect of specific blockers of ionic channels on the amplitude, rate of rise and duration of electrical responses in mammalian smooth muscle membrane gave largely similar results, despite individual differences in action potentials. In all smooth muscles investigated so far the action potentials are, on the one hand, highly sensitive to changes in concentration of external Ca^{2+} ions and to the blocking effect of Mn^{2+}, Co^{2+}, La^{3+} and D-600; on the other hand, they are resistant to the blocking action of TTX (Bülbring and Tomita, 1969; Anderson *et al.*, 1971; and others). Various smooth muscles are capable of generating action potentials in Na-free solution for a long period of time, their amplitude being dependent on the concentration of external Ca^{2+} ions (Holman, 1958; and others).

These findings led to the conclusion that calcium conductance in electrically excitable membranes of smooth muscle cells is not only an essential factor but the main mechanism of the generation of a propagating action potential.

Because of technical difficulties only a few measurements have been carried out on the analysis of ionic currents by voltage clamping in muscle fibres of taenia coli, uterus and portal vein (Kumamoto and Horn, 1970; Anderson *et al.*, 1971; Mironneau, 1974; Kao and McCullough, 1975; Inomata and Kao, 1976; Bury and Shuba, 1976). The results obtained in these experiments do confirm the above conclusion; however, the authors failed to perform a detailed analysis of calcium currents.

All these data called for a more thorough analysis of the question as to whether the mechanism of generation of the action potential in the nerve fibre also involves the activation of calcium conductance of the membrane together with sodium and potassium conductances. Hodgkin and Keynes (1957) investigated ionic fluxes in the squid

using labelled isotopes. They found that 0.006 pmol/cm^2 of Ca^{2+} ions actually enter the fibre in the course of generation of one nerve impulse. This quantity is negligible compared with inward-going Na^+ ions (4 pmol/cm^2). Obviously, in the nerve fibre Ca^{2+} ions are capable of transferring only a small proportion of the charge necessary for generation of the nerve impulse.

More detailed investigation of this question became possible due to the discovery of aequorin, a protein that emits light in the presence of traces of ionized calcium (Shimomura *et al.*, 1962). By introducing this protein inside the axon, Hodgkin and his colleagues identified two phases of calcium entry (Baker *et al.*, 1970; 1971a, b; 1973b). The first, fast, phase was comparable with sodium inward current in its time dependence and was correspondingly blocked by TTX. Therefore, Baker *et al.* concluded that this portion of calcium current is carried through sodium channels due to the imperfect selectivity of the latter. Later, it was possible to measure directly the selectivity of sodium channels and to show that the ratio between sodium and calcium conductance in sodium channels is close to 100 (Meves and Vogel, 1973).

The second, slow, phase of calcium entry in the axon was dependent on external concentration of these ions and was characterized by the presence of a region of negative resistance in the current–voltage curve. At constant depolarization, calcium entry during this phase decreased from a maximal value to a considerably lower level. The time course of this process was approximately exponential with a time constant of the order of several seconds (Baker *et al.*, 1971c). The slow phase of calcium entry into the fibre was insensitive to TTX and TEA but could be suppressed by the action of Mg^{2+}, Mn^{2+} and Co^{2+} on the outer surface of the membrane, as well as by the more active derivative of verapamil, D-600 (Baker *et al.*, 1973a). In the experiments described the authors did not manage to measure directly calcium current associated with the slow phase of calcium entry into the fibre. However, Tasaki and colleagues observed prolonged action potentials in giant axons in conditions when only divalent cations (Ca^{2+}, Sr^{2+} or Ba^{2+}) were present in the external solution and monovalent cations inside the fibre, which appeared to be caused by both fast and slow calcium entry into the axon (Watanabe *et al.*, 1967; Tasaki *et al.*, 1969). These authors tried to explain their data from a special viewpoint which was not confirmed experimentally. According to Tasaki, electrical excitation is provided by conformational changes of macromolecules in the membrane which results in an increased diffusion of cations across the membrane;