The Squid Axon as a Model for Studying Plasma Membrane Mechanisms for Calcium Regulation

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SUMMARY Calcium movement across plasma membranes occurs mainly by three routes: voltage-dependent calcium channels, adenosine 5'-triphosphate-driven calcium pump, and Na⁺-Ca²⁺ exchange. The regulation of the intracellular ionized calcium is the consequence of two parallel calcium transport mechanisms: a high affinity, low capacity system responsible for extruding calcium during resting conditions (calcium pump) and a low affinity and high capacity system (Na⁺-Ca²⁺ antiporter). This last system is designed to extrude calcium ions when intracellular calcium rises above certain levels and also to lead calcium ions into the cell under conditions that favor the reverse mode of operation of the exchanger. This short review provides an analysis of the most conspicuous features of the two membrane transport mechanisms determined in dialyzed squid axons with special emphasis on both the complexity of the Na⁺-Ca²⁺ exchange system and its marked asymmetry.

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KEY WORDS · calcium pump · Na⁺-Ca²⁺ exchange · squid axon · membrane

CALCIUM ions play a fundamental role in the regulation of cellular functions. On one hand, their large, inward, electrochemical gradient is used in the production of electrical signals, and on the other, the actual levels of internal ionized calcium (Ca²⁺) are responsible for triggering several cell processes such as neurotransmitter release, hormone secretion, and muscle contraction. Since the electrochemical calcium gradient favors net calcium entry into the cells, powerful mechanisms for maintaining a low resting Ca²⁺ must exist. This article concentrates on the general properties of the two plasma membrane mechanisms responsible for calcium extrusion: the calcium pump and the Na⁺-Ca²⁺ exchange.

Basic Properties of the Na⁺-Ca²⁺ Exchange and Calcium Pump Mechanisms

Na⁺-Ca²⁺ Exchange

Experiments carried out in cardiac muscle¹ and squid axons² led to the discovery of a countertransport mechanism (Na⁺-Ca²⁺ exchange) that is now recognized to be of importance in the contraction of cardiac muscle.³ Calcium exit from these preparations depends on the presence of extracellular sodium (Na⁺; forward Na⁺-Ca²⁺ exchange), while only a small fraction depends on Ca²⁺ (Ca²⁺-Ca²⁺ exchange). A critical test of the Na⁺-Ca²⁺ exchange hypothesis was the demonstration in squid axons that increasing Na⁺, or decreasing Na⁺ (Na gradient reduction) led to a reversal of the exchange and therefore to an increase in calcium influx.⁴ This point is of functional significance since drugs (cardiac glycosides), poisons (veratridine), or experimental conditions that elevate the internal sodium levels increase calcium entry through the exchange mechanism.

Experiments in squid axons⁵,⁶ and in membrane vesicle preparations from cardiac muscle⁷ permitted the characterization of important kinetic parameters of Na⁺-Ca²⁺ exchange. Figure 1A shows the Ca²⁺ dependence of the Na⁺-dependent calcium efflux in squid axons dialyzed under physiological conditions. The apparent $K_m$ for Ca²⁺, is in the order of 10 μM.⁸ The low affinity of the exchange for Ca²⁺, contrasts with its large capacity ($V_{max}$) for calcium translocation and this seems to be a generalized property of the Na⁺-Ca²⁺ exchange (see Reference 8 for a review).

Experiments carried out in squid axons demonstrated that the Na⁺-dependent calcium efflux is decreased by depolarization and increased upon hyperpolarization, thus suggesting that Na⁺-Ca²⁺ exchange is electrogenic.⁹,¹⁰ Recently, it was possible to measure the current generated by the exchange, thus confirming its
electrogenic nature.\textsuperscript{11,12} During each cycle, the translocation of one calcium ion is coupled to the transport of more than two sodium ions in the opposite direction, resulting in a net transfer of positive charge across the membrane. The system can promote a voltage-dependent calcium influx by a route different from the known voltage-dependent calcium channels.

**ATP-Dependent Calcium Pump**

An adenosine 5' -triphosphate (ATP)-dependent calcium transport mechanism responsible for extruding calcium was first demonstrated in red blood cells.\textsuperscript{8} This system was suggested to be specific of nonexcitable tissues since the erythrocyte is one of the few cells in which Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange is absent. Experiments carried out in dialyzed squid axons demonstrated the presence of an ATP-dependent net calcium extrusion in the absence of electrochemical ionic gradients,\textsuperscript{13} thus showing that an ATP-driven calcium pump is also involved in the regulation of Ca\textsuperscript{2+} in excitable tissues. Figure 1A shows the Ca\textsuperscript{2+}-dependence of the calcium pump in squid axons.\textsuperscript{8} In contrast to the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange system, the calcium pump has a much higher affinity for Ca\textsuperscript{2+}, but a lower capacity of transport. The apparent $K_m$ for Ca\textsuperscript{2+} activation is about 0.18 $\mu$M, with a $V_{max}$ of 250 to 300 fmol·cm\textsuperscript{-2}·sec\textsuperscript{-1}. The Mg\textsuperscript{2+} requirement, the apparent affinity for ATP (25 $\mu$M) and Ca\textsuperscript{2+}, and the sensitivity to inhibitors like vanadate are the same as for the calcium adenosine triphosphatase (ATPase) found in purified membrane fractions of the optic nerve of the squid.\textsuperscript{6,14} Furthermore, the sensitivity of the squid nerve Ca\textsuperscript{2+}-ATPase to calmodulin,\textsuperscript{15} a characteristic property of most Ca\textsuperscript{2+}-ATPases, suggests that it is of the type found in the red blood cell (E\textsubscript{1}-E\textsubscript{2} type).

The presence of two parallel systems cooperating in the regulation of Ca\textsuperscript{2+} appears to be a general feature of most excitable preparations. At the resting physiological Ca\textsuperscript{2+}, (10$^{-7}$ M or less) the calcium pump is the main system balancing the passive calcium "leak," whereas the countertransport mechanism becomes more relevant at higher Ca\textsuperscript{2+} (cell activity).

**The Squid Axon as a Model for Cell Calcium Regulation**

Although early evidence of the interaction between sodium and calcium ions came from force measurements in cardiac muscle, it appears that simple mechanical force determinations may not be sufficient for further detailed characterization of the kinetics of the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange system. This is particularly true when studying the kinetic properties of the exchange process in intact cells in which different factors may influence the level of Ca\textsuperscript{2+}, including voltage-dependent calcium channels, plasma membrane, and sarcoplasmic reticulum calcium pumps. Furthermore, measurements of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity in small cells that involve changes in Na\textsuperscript{+}, will affect the kinetics of the exchange\textsuperscript{5} as well as its voltage sensitivity.\textsuperscript{16}

Membrane vesicle preparations have shown to be of great importance in determining kinetic parameters of both Na\textsuperscript{+}-Ca\textsuperscript{2+} and calcium pump systems.\textsuperscript{7} Problems exist with vesicle preparations: difficulty in measuring true initial rates due to inhomogeneity in vesicle size, inhomogeneity of vesicle orientation (inside-in and inside-out), and alterations of the carrier system as a result of membrane preparation.\textsuperscript{7} However, this procedure has been very useful not only for kinetic studies but also in the isolating, purifying, and reconstituting the calcium transport mechanisms.\textsuperscript{15}

Another preparation that has unraveled several of the complexities of calcium homeostasis is the squid axon. The introduction of large plastic dialysis capillaries (9000 molecular weight cut-off) longitudinally into the axon allows a rapid exchange of solutes between the cytosol and the dialysis fluid. Furthermore,
the cylindrical geometry of the preparation allows precise longitudinal spatial control of the membrane potential using standard voltage clamp techniques. This report analyzes some recent findings on the Na⁺-Ca²⁺ exchange system obtained in the dialyzed squid axon preparation.

Internal Ligand Interactions with Na⁺-Ca²⁺ Exchange

One striking feature of Na⁺-Ca²⁺ exchange is its sensitivity to ATP. Although the nucleotide is not required for the exchange to operate, it markedly increases the rate of exchange both in the forward and reverse modes. ATP increases the affinity of the external and internal sites toward sodium and calcium ions, respectively. The stimulatory effect of ATP is 1) highly selective; other triphosphates (uridine 5′-triphosphate, guanosine 5′-triphosphate, inosine 5′-triphosphate) and diphosphates (adenosine 5′-diphosphate) inhibit by displacing ATP; 2) Mg²⁺ is an absolute requirement; and 3) only hydrolyzable ATP analogues can mimic ATP. In squid axons the ATP analogue [γ-thio]ATP, which can act as substrate for kinases but not for ATPases, activates the Na⁺-Ca²⁺ exchange in a Ca²⁺-dependent manner. This demonstrates that ATP regulates the rate of exchange by phosphorylating the Na⁺-Ca²⁺ exchange carrier. Studies on sarclemma vesicles also show that Na⁺-Ca²⁺ exchange is regulated by a Ca²⁺-activated, calmodulin-dependent protein kinase-phosphatase system responsible for the phosphorylation-dephosphorylation of the exchange.

An interesting ligand that strongly affects Na⁺-Ca²⁺ exchange is internal magnesium. This divalent cation is a partial noncompetitive inhibitor of the exchange (Kᵢ = 2 mM). At the physiological Mg²⁺ concentration found in squid axons (3–4 mM), the exchange is half inhibited. Besides the well-known inhibitory effect of Na⁺ on the forward Na⁺-Ca²⁺ exchange, another monovalent cation that modifies the rate of exchange is internal potassium (K⁺). Experiments at constant membrane potential show that K⁺ strongly activates the Na⁺ dependent calcium influx. Substitution of K⁺ with tris⁺ or N-methyl-D-glucamine⁺ drops the Na⁺ dependent calcium influx by 70%. Similarly, K⁺o appears to have an activatory effect on Na⁺-Ca²⁺ exchange, since a large fraction of the K⁺, stimulated, Ca²⁺ dependent sodium influx is due to potassium ions per se, and only a small fraction is due to membrane depolarization. Hydrogen ions also affect the rate of Na⁺-Ca²⁺ exchange; in dialyzed squid axons, decreasing pH₁ from its physiological value of 7.3 to 6.8 causes a 50% reduction in the forward exchange, while increasing pH₁ to 8.5 causes a 400% increase in the exchange rate. External alkalization does not affect Na⁺-Ca²⁺ exchange.

Figure 1B shows the known ligand interactions of the Na⁺-Ca²⁺ exchange system. The inner side of the antiporter is shown interacting with several ligands leading to two major states: a low-rate configuration (Na⁺, Mg²⁺, ATP, and P, are low) and a high-rate configuration (opposite ligand concentrations). These interactions, which normally occur in vivo and may occur in vitro depending on the experimental conditions, must be taken into account when analyzing kinetic variables of the Na⁺-Ca²⁺ exchange.

Modulation of Na⁺-Ca²⁺ Exchange by Ca²⁺;
An Asymmetrical Antiporter

Na⁺-Ca²⁺ exchange models are based on a symmetrical transport system in which cation translocation occurs when either sodium or calcium ions are present at opposite sides of the membrane. Although Na⁺-Ca²⁺ exchange occurs in the absence of Ca²⁺ and Na⁺, an interesting observation is that the reverse exchange reaction (Ca²⁺, Na⁺, exchange) requires Ca²⁺. Figure 2A shows that [Ca²⁺] stimulates Na⁺, dependent calcium influx. A further demonstration of the activation of the Ca²⁺, Na⁺, exchange by Ca²⁺ is shown in Figure 2B. In this experiment, the Ca²⁺,
dependent sodium efflux is measured as an expression of the reverse exchange. In the absence of Ca\(^{2+}\), no reversal of Na\(^{+}\)-Ca\(^{2+}\) change occurs even in the absence of Na\(^{+}\), (a favorable situation for Ca\(^{2+}\) exchange).

The physiological significance of the positive feedback of Ca\(^{2+}\), on the reverse Na\(^{+}\)-Ca\(^{2+}\) exchange is under investigation. Calcium entry through voltage-dependent calcium channels (increase in Ca\(^{2+}\)) could contribute to further activate calcium entry through Na\(^{+}\)-Ca\(^{2+}\) exchange. The fact that raising Ca\(^{2+}\), from 0.1 to 0.6 \(\mu\)M produces a 10-fold increase in the extra calcium entry induced by membrane depolarization favors this hypothesis.\(^\text{28}\) The catalytic effect of Ca\(^{2+}\), explains both the inhibition of its entry through Na\(^{+}\)-Ca\(^{2+}\) exchange induced by the chelating indicator quin-2,\(^\text{29}\) and the requirement of the outward Na\(^{+}\)-Ca\(^{2+}\) exchange current (reverse mode) for Ca\(^{2+}\).\(^\text{11}\)

**Na\(^{+}\)-Ca\(^{2+}\) Exchange and the Regulation of Tension of Vascular Smooth Muscle**

In cardiac muscle, strong evidence exists for the presence of a Na\(^{+}\)-Ca\(^{2+}\) exchange that is thought to be electrogenic and thus to affect not only the development of tension but also the membrane potential. Smooth-muscle physiologists have much less evidence for the role of Na\(^{+}\)-Ca\(^{2+}\) exchange in determining the contractile state of the muscle. Some studies on intact smooth muscle indicate the importance of calcium in its contraction, thus implying the existence of a Na\(^{+}\)-Ca\(^{2+}\) exchange process.\(^\text{30}\) This view has been strengthened by the demonstration of a sodium-dependent calcium efflux in arterial smooth muscle,\(^\text{31}\) which has led to the hypothesis that Na\(^{+}\)-Ca\(^{2+}\) exchange is involved in the etiology of hypertension.\(^\text{30}\) According to more recent studies by other groups, however, it appears that Na\(^ {+}\)-Ca\(^{2+}\) exchange does not play an important role either in the regulation of Ca\(^ {2+}\), or in smooth muscle contractility.\(^\text{32}\) The plasma membrane from smooth muscle seems to be equipped with both a calcium pump and a Na\(^{+}\)-Ca\(^{2+}\) exchange. Calcium pumping ATPases and calcium transport powered by ATP have been reported from several smooth muscles, including artery muscle, which possesses a high affinity Ca\(^{2+}\)-ATPase stabilized fivefold by calmodulin.\(^\text{33}\) As already discussed, the actual role of these two parallel systems in the control of the Ca\(^{2+}\), will depend on the transport kinetic parameters of both systems. The existence of a Na\(^{+}\)-Ca\(^{2+}\) exchange process in a cell membrane is not a criterion of its involvement in calcium homeostasis. This is the case in certain smooth muscles that can maintain low resting Ca\(^ {2+}\), in the absence of an inwardly directed sodium gradient. However, under certain conditions (inhibition of the Na\(^{+}\)-K\(^{+}\) pump or reversal of the Na\(^{+}\) gradient), it is possible to demonstrate calcium movements through the exchange system.\(^\text{34}\) It is possible that Na\(^ {+}\)-Ca\(^{2+}\) exchange may modify the Ca\(^{2+}\), levels when Na\(^{+}\) is increased due to inhibition of the Na\(^{+}\)-K\(^{+}\) pump, conditions that may occur in hypertension. Nevertheless, only detailed knowledge of the affinities and capacity of the two calcium transport systems present in smooth muscles will allow us to determine whether a given decrease in the electrochemical sodium gradient (as that observed in certain forms of hypertension\(^\text{35}\)) can increase Ca\(^{2+}\), in the presence of a working calcium pump. Much remains to be investigated both in vivo and in vitro smooth muscle preparations before assigning any role to Na\(^{+}\)-Ca\(^{2+}\) exchange in smooth muscle contractility.

**Conclusions**

The long-term regulation of cytosolic ionized calcium is the consequence of two separate calcium-transport mechanisms present in most eucaryotic cells: a high affinity, low capacity system, which is in charge of extruding calcium during resting conditions (the calcium pump), and a low affinity, high capacity system (Na\(^{+}\)-Ca\(^{2+}\) exchange), designed not only to extrude calcium ions when Ca\(^{2+}\), rises above certain levels, but also to let calcium into the cell under conditions that favor the reverse reaction of the exchange, such as increase in Na\(^{+}\), decrease in external sodium, and membrane depolarization. Experiments in dialyzed and voltage-clamped squid axons show that the Na\(^ { +}\)-Ca\(^ {2+}\) exchange system is a complex mechanism in which several ligands normally present in the cytosol interact with the exchange carrier. This should be taken into account when measuring kinetic properties of the exchange. Of great interest is the fact that Na\(^ {+}\)-Ca\(^ {2+}\) exchange is subject to enzymatic regulation (phosphorylation-dephosphorylation), which in turn is regulated by the levels of Ca\(^{2+}\). More evidence is needed from both isolated membrane preparations and in vivo systems to answer questions about the characterization of the partial reactions of the exchange system, the factors that modulate the affinity of the transport sites for sodium and calcium, the stoichiometry (constant or variable ?), the current generated by the exchanger, and the biochemical structure of the antipporter.

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