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Continuous Measurement of Calcium Influx in Mammalian Nonmyelinated Nerve Fibers: Effects of Na_o, Ca_o, and Electrical Activity

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Summary. A new technique for continuous monitoring of the cellular calcium was developed and used for studying the effects of external and internal Na (Na_o and Na_i), external Ca (Ca_o), Ca ionophore A23187, and electrical activity on membrane-bound and intracellular Ca in mammalian nonmyelinated nerve fibers. Increasing Ca_o increased both the membrane-bound and the intracellular Ca. Lowering Na_o increased the membrane-bound fraction of Ca indicating that lack of Na_o enhanced the capacity of the plasma membrane to bind Ca, and produced an increase of the internal Ca pool. Increasing Na_i by treatment with ouabain enhanced the Ca inflow in both, the presence and absence of Na_o, presumably by stimulating the Ca_o/Na_i exchange. The Ca ionophore A23187 produced a large and irreversible increase in the intracellular Ca without affecting the membrane-bound fraction. On the other hand, electrical activity, which is known to produce a large increase of the total Ca in squid axon, had no measurable effect on the total calcium content in our preparation. It is concluded that in mammalian nerve fibers a Ca load by exposition to Na-free solution or to A23187 produces an accumulation of Ca into the intracellular Ca stores, whereas during electrical activity the membrane-associated extrusion mechanisms are able to maintain the intracellular Ca^{2+} below the threshold for intracellular sequestration. Furthermore, the results indicate that the intracellular sequestration mechanisms are dependent on the internal concentration of Na.

Key Words mammalian nerve · intracellular calcium · membrane-bound calcium · calcium buffering · Na/Ca exchange · electrical activity · A23187

Introduction

The concentration of free calcium in the cytoplasm of virtually all living cells is maintained within the range of 10^{-8} to 10^{-7} M, compared to about 10^{-3} M in the extracellular fluid. It is thus not surprising that cells are endowed with systems, both intracellular and membrane-associated, that maintain and regulate the intracellular calcium ions at this extremely low concentration. Most of our knowledge on calcium regulation in excitable cells originates from studies in intact, injected, internally perfused or dialyzed giant axons or giant muscle fibers, and little is known about calcium homeostasis in mammalian nerve fibers.

We have therefore directed our attention to calcium fluxes across the cell plasma membrane in rabbit nerve fibers, in order to examine whether the mechanisms which have been described in giant axons are also found in mammalian nonmyelinated nerves.

The present study is based on results obtained by a method which allows continuous monitoring of the radioactivity of a preparation during superfusion with solutions containing ^{45}Ca (Jirounek et al., 1983). The results are compared to measurements of efflux of ^{45}Ca in the same tissue (Jirounek, Vitus, Pralong & Straub, *in preparation*) and of intracellular free calcium with the quin2 method (Pralong & Straub, 1985).

Materials and Methods

Preparation of Nerves; Apparatus for Recording the Radioactivity

Rabbits weighing between 2 and 3 kg were shot and their cervical vagus nerves rapidly removed and desheathed with scissors. The preparation was then inserted into a 5-cm length of plastic scintillator tubing (Nuclear Enterprises NE102A, internal diameter 0.7, external 0.9 mm), connected to a small chamber where the nerve fibers could be stimulated and the action potential recorded (*see* Fig. 1). The scintillating materials served to transform the weak beta radiation of ^{45}Ca into light flashes which were detected by a pair of photomultipliers placed on either side of the scintillator tube. The photomultipliers were connected via a coincidence circuit to a rate meter, and further to an integrator and pen recorder. After mounting, the preparation was first superfused during about 30 min with a nonradioactive solution of the same calcium concentration that was later used in the influx experiment. The flow of the solution was maintained at a rate of 1 ml/min by a peristaltic pump. After the period of equilibration in the

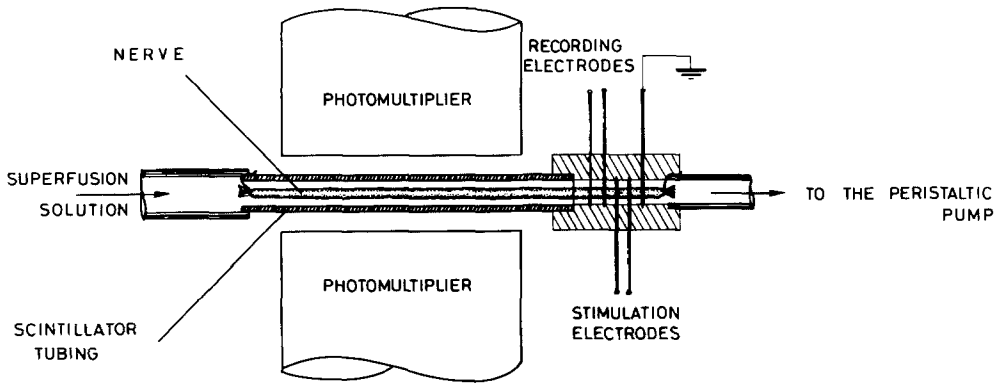

Fig. 1. Apparatus for recording the total radioactivity of the preparation and surrounding medium. A desheathed vagus nerve is inserted into a plastic scintillator tubing and a small chamber for stimulation and recording of action potentials. The tube with the preparation is continuously superfused with ^{45}Ca-labeled solution and placed between two photomultipliers for coincidence counting

nonradioactive solution, the superfusion solution was switched to a ^{45}Ca-labeled solution and the total radioactivity of the preparation and of the surrounding medium recorded. The whole recording system had a minimum time constant of about 0.5 sec, but longer time constants could be selected on the rate meter; 3.3 or 10 sec were generally used. This technique is a further development (for weak radiation) of the method of Anner et al. (1975) for continuous measurement of phosphate (^{32}P) and of the method described by Baker and Singh (1980) for measurement of the uptake of ^{85}Sr and ^{86}Rb by crab nerves.

Calculation of the Exchangeable Calcium of the Nerve

For the quantification of the influx experiments, the percentage of the volume inside the tube occupied by the nerve was first determined by superfusing the nerve with a ^{45}Ca-labeled solution of otherwise normal composition, but without unlabeled Ca, and with either La^{3+} (5 mM) or EGTA (1 mM). The radioactivity under these conditions reached a steady value after 5 to 10 min corresponding to the filling of the tube and of the extracellular space (*see* record *C* on Fig. 2). This level of radioactivity was then considered as the "base line" (*b*) for the calculation of the ^{45}Ca uptake. Before or after each experiment the radioactivity of the tube without nerve, perfused with the same solution that was used in the influx experiment was recorded (*t*). The fraction of the volume of the tube occupied by the nerve (without its extracellular space) is thus $(t - b)/t$, the total amount of calcium taken up by the nerve is $[Ca_o] \cdot (a - b)/t$, and the total calcium expressed in mmol/kg wet wt is $[Ca_o] \cdot (a - b)/(t - b)$, where a is the total recorded radioactivity of the preparation and the surrounding medium.

Solutions

The composition of the physiological solution was (in mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 1.8; $MgCl_2$, 0.5; Tris, 10; glucose, 5. Ca-free solution was prepared by omitting $CaCl_2$, Ca-free-EGTA solution by omitting $CaCl_2$ and by addition of 1 mM EGTA. Other calcium concentrations were prepared by addition of appropriate amounts of solid $CaCl_2$ to Ca-free solution. Lanthanum was added as the chloride salt. The pH of all solutions was adjusted with HCl to exactly 7.4 at 37°C. All salts were analytical grade. The labeled solutions contained 5 μCi ^{45}Ca/ml, added from a radioactive stock solution, obtained from the Institut National des Radioéléments, Fleurus (Belgium). The stock solution had a concentration of 2 mCi/ml and a specific radioactivity of about 15 mCi/mg. All experiments were carried out at 37°C.

Results

Effect of External Calcium on ^{45}Ca Labeling

Record *A* in Fig. 2 illustrates the uptake of radioactivity at 0.2 mM extracellular calcium. The record shows, immediately after the application of ^{45}Ca, a rapid increase in radioactivity, which corresponds to the filling of the tube, of the extracellular space and to the labeling of a superficial, probably membrane-bound fraction of calcium. Afterwards the increase in the radioactivity is much slower and after 1 to 2 hr reaches an almost steady level, indicating that the easily exchangeable calcium pools are then nearly completely equilibrated with the external radioactive calcium.

The same type of experiment was repeated at different external calcium concentrations. The results show that both the total amount taken up by the preparation, determined from the radioactivity at equilibrium, and the rate of labeling, increased with increasing extracellular calcium. The curves of ^{45}Ca uptake are, however, difficult to analyze quantitatively, since the measured radioactivity reflects a simultaneous labeling of at least two calcium pools: a superficial, rapidly filled membrane fraction of calcium and a much slower incorporation of ^{45}Ca into intracellular compartments. In the next

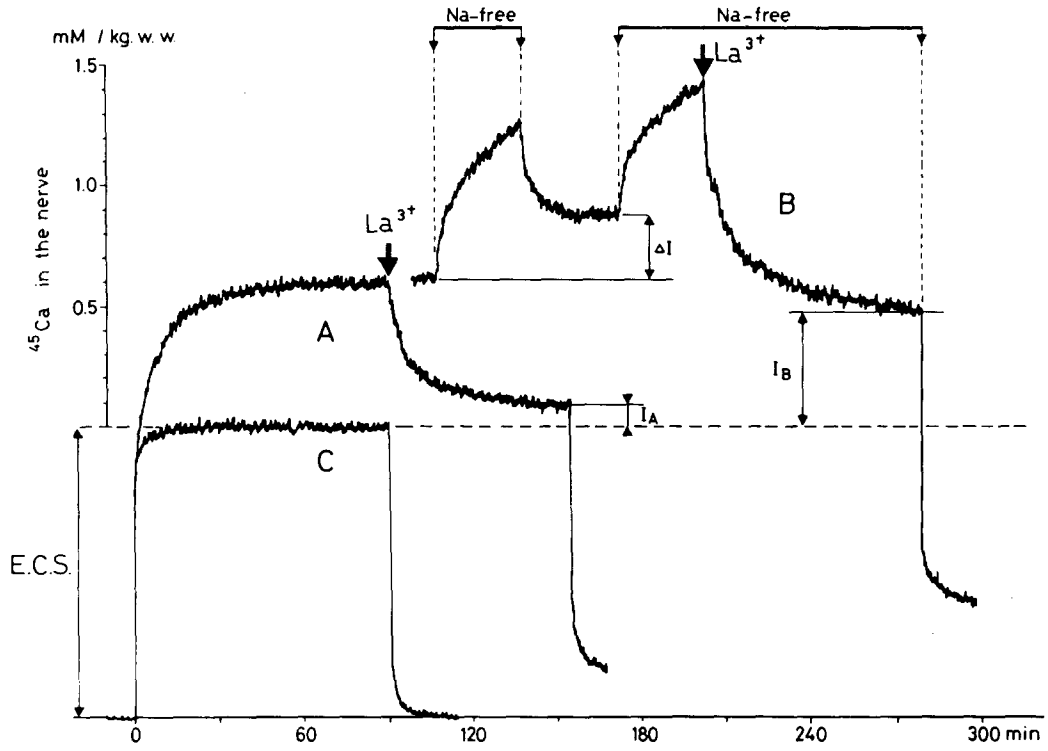

Fig. 2. Uptake of ^{45}Ca by vagus nerve. Records of radioactivity of the preparation and surrounding medium show, after application of ^{45}Ca-labeled solution, the filling of the tube and extracellular space (*E.C.S*), followed by the uptake of ^{45}Ca by the nerve. Record *A* was obtained by superfusion of the preparation with physiological solution; after equilibration, La^{3+} (5 mM) was added to the external medium. The increase of radioactivity of the preparation after Na withdrawal is shown on record *B*, where after equilibration (*not shown*), the external sodium was replaced by choline. When sodium was reintroduced, the radioactivity fell to a new equilibrium which was above the equilibrium before the Na withdrawal. During the second period of Na withdrawal, La^{3+} was added to the external solution. The La-induced decrease of radioactivity was much larger than when La^{3+} was added to the physiological solution (record *A*). Record *C* shows the radioactivity of the preparation superfused by physiological solution with lanthanum. The level at equilibrium is used for the estimation for the dead volume of the tube and of the extracellular space. All solutions contained 5 μCi of ^{45}Ca/ml. [Ca_o] was 0.2 mM, temp. 37°C, pH 7.4

series of experiments we have tried to identify these calcium pools.

Identification of Membrane-bound and Intracellular Calcium Pools

It has been found in a number of tissues that an important fraction of calcium is bound to negative fixed charges at the external surface of the membrane. Van Breemen and McNaughton (1970) have shown that this surface calcium can be displaced by lanthanum. In order to differentiate between the intracellular and the externally bound calcium, we have applied, on nerves previously equilibrated with ^{45}Ca, a solution containing 5 mM lanthanum. The effect of this trivalent cation is shown at the end of record *A* in Fig. 2. Immediately after the addition of lanthanum to the perfusion solution there was an important and rapid loss of radioactivity which was followed by a much slower decrease. We take this initial rapid fall to represent the liberation of the membrane-bound fraction, whereas the following slow decrease probably corresponds to the calcium efflux from intracellular pool, as observed in efflux experiments in nerves treated with lanthanum (Jirounek et al., *in preparation*). The amount of membrane-bound calcium can thus be determined by extrapolation of the slow exponential decrease to the beginning of the lanthanum effect.

It is thus possible to calculate the total exchangeable calcium, and, using lanthanum, to estimate the membrane-bound and the intracellular pools. The results of such determinations are presented in Fig. 3, where the amounts of these fractions are plotted for different extracellular calcium concentrations.

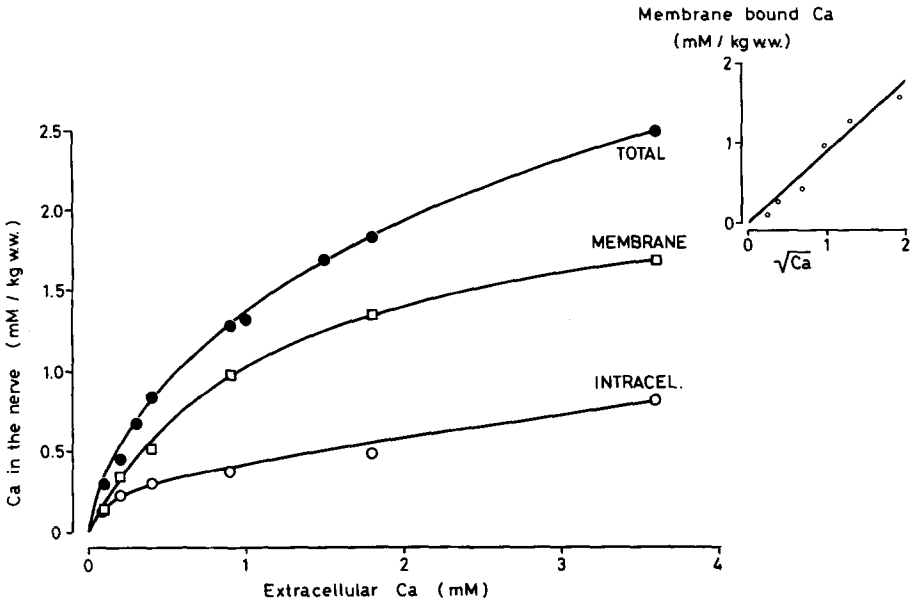

Fig. 3. Total, membrane-bound and intracellular calcium at different extracellular calcium concentrations. The total calcium (●) was calculated from the radioactivity after equilibration of the external radioactive solution with the cellular exchangeable calcium pools. The amount of calcium released during lanthanum treatment was considered as the membrane-bound fraction (□) and the difference between the total and the membrane-bound as the intracellular pool (○). Points are means ± SE of 4 to 26 separate experiments

Effect of Lowering Extracellular Sodium

Blaustein and Hodgkin (1969) have shown that a Na-coupled exchange mechanism is involved in a fraction of calcium efflux in squid axons. In order to examine if a similar Na/Ca exchange is present in mammalian nerves, we have tested the effect of Na-free solution on the ^{45}Ca labeling of our preparation.

Record *B* on Fig. 2 shows that replacement of the extracellular sodium by choline produces a large increase in radioactivity in a preparation that was previously equilibrated (*not shown*) with the radioactive solution. After reintroduction of Na into the external solution, the radioactivity falls to a new level along a time course that is somewhat faster than the onset of the effect. The new steady level of this and other experiments lies above the radioactivity before the Na withdrawal and is dependent on the external Ca concentration. At 0.2, 0.4, 1.8 and 3.6 mM Ca_o, the fractions of Ca bound during the Na-free period were 89, 140, 310 and 280 μM/kg wet wt, respectively. The record also shows that after the application of lanthanum in Na-free solution (end of record *B*), the amount of calcium lost by the preparation is much more marked than when lanthanum is added to a preparation superfused with normal Na concentration (record *A*). These results seem to indicate that the rise in radioactivity after Na withdrawal reflects a simultaneous labeling of a membrane fraction and a much slower increase in intracellular calcium.

In other experiments, intermediate Na_o concentrations were used. The ^{45}Ca uptake after lowering Na_o from the normal concentration (154 mM) to $\frac{1}{8}$, $\frac{1}{4}$, $\frac{1}{2}$ and $\frac{7}{8}$ was 95, 84, 60 and 7%, respectively, of the effect at zero external sodium.

The rise in internal Ca can be explained by either a decrease in the calcium efflux, by inhibition of the Na_o/Ca_i exchange, or by an increase in calcium influx, by stimulation of the Ca_o/Na_i exchange (Baker & Blaustein, 1968; Baker et al., 1969), or by a simultaneous action of both exchange mechanisms. To determine which of these two effects is predominant, we have measured the ^{45}Ca uptake in Na-free solution in nerves previously loaded with Na by treatment with ouabain.

Effect of Internal Sodium

Figure 4 shows the ^{45}Ca uptake produced by Na withdrawal at normal Na_i concentration and then after application of ouabain (100 μM). The effect of ouabain itself is also shown. These results indicate that an increase in internal sodium increases the ^{45}Ca labeling of the nerve, both in the presence and in the absence of external Na, suggesting that the effect of Na-free solution may be, at least partially, due to a stimulation of the Na_i-dependent Ca inflow.

Effect of Electrical Stimulation

Hodgkin and Keynes (1957) showed that excitation in squid axons is associated with a net calcium influx which in their experiments amounted to about 11 pmol/cm^2 sec with stimulation at 100 Hz. Brinley

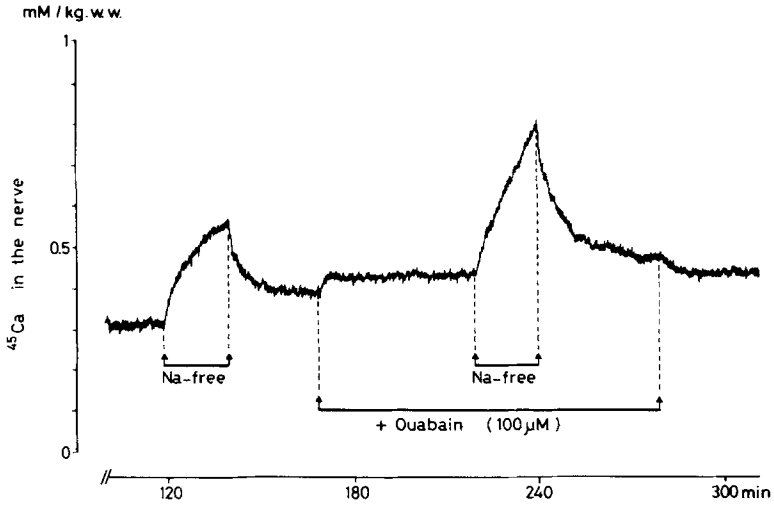

Fig. 4. Effect of Na_i on the ^{45}Ca uptake. The record shows the uptake of ^{45}Ca during the Na withdrawal at normal Na_i, the effect of ouabain (100 μM), and the increased effect of Na-free solution during treatment with ouabain. The curve is a representative record out of four similar experiments. $[Ca_o]$ was 0.2 mM, temp. 37°C, pH 7.4

et al. (1977) confirmed these results and found that the uptake of calcium during stimulation (100 Hz) increases linearly with duration of activity for at least 60 min. The increase in calcium content was about 50 μmol/kg axoplasm per minute of stimulation; only a small fraction of the uptake appeared as free intracellular calcium, the majority being incorporated into intracellular calcium buffers.

In a number of experiments we tested whether or not a similar calcium uptake can be observed in rabbit vagus nerve. The experiments were performed at extracellular Ca^{2+} concentrations varying between 0.01 and 5 mM and at stimulation of 2 to 15 Hz. In these experiments it was not possible to detect changes in either the rate of labeling or in ^{45}Ca content at equilibrium. Further, in experiments where the efflux of calcium was decreased by lowering the temperature to 22 or 10°C or by addition of lanthanum, or both, stimulation was also without detectable effect.

Effect of Ca Ionophore A23187

Compound A23187, a lipid-soluble carboxylic acid antibiotic is known as a fairly selective ionophore for divalent cations (Reed & Lardy, 1972). A typical effect of this drug on ^{45}Ca labeling at 0.9 mM external Ca is illustrated in Fig. 5. After equilibration with ^{45}Ca, addition of A23187 (10 μM) to the perfusion solution produced an increase in the recorded radioactivity which reached, with a half-time of about 30 to 40 min, a new steady level. Experiments with different calcium concentrations show that the time course of the A23187 effect was almost independent of the extracellular calcium concentration, indicating that the rate-limiting step for the effect

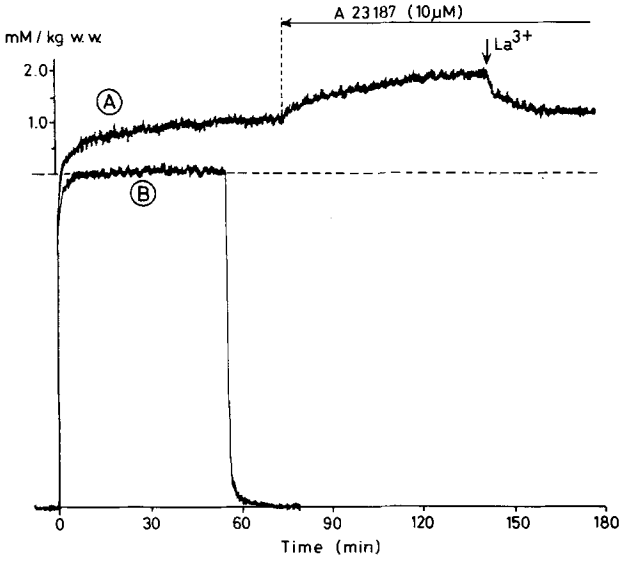

Fig. 5. Effect of A23187 on labeling. Record *A* shows the slow increase in radioactivity of the preparation after addition of A23187 (10 μM). For the determination of the membrane-bound fraction, La^{3+} (5 mM) was added to the superfusion solution at the end of the experiment. Record *B* was obtained by superfusion of the second nerve from the same rabbit with solution containing La^{3+} and shows the radioactivity of the dead volume and of the extracellular space. $[Ca_o]$ was 0.9 mM, temp. 37°C, pH 7.4

lies in the binding of the drug to its membrane sites rather than in the calcium translocation.

At the end of the experiment, when the effect of the ionophore was well developed, lanthanum (5 mM) was added to the superfusion solution in order to displace the membrane-bound calcium. In the experiment presented in Fig. 5, the amount of calcium just before the addition of lanthanum was 1.96

mmol/kg wet wt and the calcium lost by the preparation during the lanthanum treatment amounted to 0.92 mmol/kg wet wt, which is close to the mean value of the membrane-bound calcium determined on nerves in physiological conditions (Fig. 3). The difference between the total and the membrane-bound calcium which corresponds to the intracellular calcium pool is thus 1.04 mmol/kg wet wt, which is about 0.7 mmol/kg wet wt above the mean intracellular calcium determined in controls.

The effect of the ionophore was irreversible; the radioactivity taken up by the nerve remained in the preparation even during prolonged superfusion with ionophore-free solution.

Discussion

The Membrane-bound and the Intracellular Calcium Pools

Figure 3 shows, for different Ca concentrations, the total calcium and the intracellular and membrane-bound fractions. The total calcium, at 1.8 mM external Ca^{2+}, is 1.83 ± 0.15 mmol/kg wet wt (n = 6), which is slightly lower than the values measured in other cells, the mean total calcium of liver, muscle and nerve cells being 2.2, 2.1 and 2.7 mmol/kg wet wt, respectively (Borle, 1981). The intracellular calcium, calculated from our data is 0.48 for 1.8 mM, and 0.355 mmol/kg wet wt for 0.9 mM of external calcium. In kidney, liver, pancreas, muscle and pituitary cells, the average exchangeable cytosolic calcium pool at 1 mM external calcium measured by kinetic analysis of ^{45}Ca desaturation curves, averages 0.36 mmol/kg wet wt (Borle, 1981), which is very close to the 0.355 mmol/kg wet wt determined from our experiments at 0.9 mM external calcium. A similar amount of intracellular calcium has been found in squid axon by Blaustein and Hodgkin (1969), who reported a value of 0.4 mmol/kg wet wt for axons stored in 10 mM external calcium. On the other hand, a much lower value has been reported by Requena et al. (1979), who found, in axons stored in 3 mM of calcium, an internal concentration of only 0.068 mmol per kg of axoplasm.

The relation between membrane-bound calcium and external calcium concentration (Fig. 3) is in good agreement with data published by Baker and McNaughton (1978) who showed that during exposure of squid axons to a ^{45}Ca-labeled solution, an important fraction of calcium binds to the external membrane. This fraction increases roughly in proportion to $Ca_o^{1/2}$. The inset in Fig. 3 shows that for the rabbit vagus nerve this empirical half-power relation is also obeyed.

Experiments in Na-free solutions showed that the membrane-bound fraction of Ca increases considerably in absence of sodium. The effect can be explained by the fact that most surface charge compensation is due to Na (Bers et al., 1985).

Effect of External Na

The finding that in our experiments Na withdrawal produces a large increase in labeling indicates that in mammalian axons a Na/Ca exchange may operate, which is similar to that described in heart muscle (Reuter & Seitz, 1968) and squid axon (Blaustein & Hodgkin, 1969; Blaustein, 1974; Requena & Mullins, 1979; DiPolo & Beaugé, 1983). However, an analysis of the time course of the increase in radioactivity indicates that only part of the effect of lowering the external Na can be attributed to a Na/Ca exchange mechanism.

It can be assumed that the steady level of labeled intracellular calcium depends on the rate of influx and efflux and that it can be influenced by changing the input or the output rates. Either change will cause a shift to a new steady state with a rate constant determined *soley by the rate constant of the efflux* that is operating during the shift. The rate constant of the increase of radioactivity during the Na-free period should thus be equal to the rate constant of the remaining calcium efflux. Clearly this is not the case. In ^{45}Ca efflux experiments (Jirounek et al., *in preparation*), the rate constants of the calcium efflux, which could be separated into a rapid and a slow component by computer multiexponential analysis, were 0.03 and 0.008 min^{-1}. On the other hand, the rate constant for the increase of ^{45}Ca after Na withdrawal is about 0.2 min^{-1}. It is quite different from the efflux rate constants, but comparable to the diffusion of Na in the extracellular space (Keynes & Ritchie, 1965). This and the fact that an important part of the radioactive calcium accumulated in the nerve during superfusion with low Na is rapidly displaced by lanthanum (*see* Fig. 2, record *B*), suggest that lack of extracellular Na enhances the binding capacity of the external membrane to calcium (Bers et al., 1985). This does not mean that the lowering of external sodium has no effect on the intracellular calcium. It signifies only that the rapid and marked increase in membrane-bound calcium makes it difficult to detect a simultaneous and much slower modification of the intracellular pool. An estimation of such an increase in intracellular calcium during Na withdrawal can

be made from the reversibility of the effect. After reintroduction of the normal sodium concentration, the radioactivity decreases to a new steady level, which lies above the values before the Na withdrawal (*see* Fig. 2, record *B*), and remains constant even after prolonged perfusion at normal sodium concentration, indicating that this fraction of calcium is bound to some nearly stable intracellular stores. The amount of this bound calcium increases with increasing extracellular Ca concentration along a sigmoidal curve.

When ouabain was added to the superfusion solution (Fig. 4), the radioactivity rose rapidly to a new steady level, suggesting that the increase in the intracellular Na stimulates the Ca inflow. The effect, however, seems to be more rapid than the expected increase in Na_i. When Na was omitted from the external solution, the increase of labeling was greatly amplified, compared to the effect of Na-free solution in controls. These results show that the stimulation of the Ca influx by Na_i is enhanced in the absence of Na_o. It has been shown in squid axon that lack of external Na produces a large increase in Ca inflow through the Ca_o/Na_i exchange, which is very dependent on the Na content of the axon (Baker & DiPolo, 1984). Furthermore, in a previous paper we have shown that omission of Na_o produces an increase in phosphate efflux, which probably also reflects an increase in the rate of the Ca_o/Na_i exchange Jirounek et al., 1984).

A tentative explanation for our results could thus be that the lack of sodium in the external solution inhibits the Na_o/Ca_i exchange and simultaneously greatly stimulates the Ca_o/Na_i exchange, thereby increasing the internal free calcium above the threshold level for the mitochondrial uptake and that, as described by Brinley et al. (1978), this labile form of mitochondrial calcium then moves rapidly into the stable and metabolically insensitive state.

These observations are, however, somewhat different from those obtained by Requena et al. (1979), who showed in squid axons that all of an exogenous load obtained either by soaking in Na-free solution or by electrical stimulation is lost when the axon is transferred in seawater with a normal sodium and calcium concentration.

Effect of Electrical Stimulation

It has been shown by electrical stimulation or soaking in Na-free solution, that when a calcium load is imposed on a giant axon, only a fraction appears as ionized calcium, the majority being retained by intracellular buffers (DiPolo et al., 1976; Brinley et al., 1977; Requena et al., 1977). The former method produces a loading of the fibers at a rate of about 50 μmol/kg axoplasm per minute of stimulation at 100 Hz, which corresponds approximately to an entry of 10 nmol per impulse per kg axoplasm. If in the rabbit vagus nerve a similar influx of calcium occurred during activity, and if during the stimulation period this calcium were retained by the intracellular buffers, then after a train of 1000 impulses an increase of the total ^{45}Ca should be detectable by our method, which has a sensitivity of about 10 μM/kg wet wt.

As mentioned, stimulation up to 18,000 stimuli, at different Ca concentrations and in the presence of lanthanum or at 20°C did not produce a detectable increase in labeling. This could indicate that during electrical activity there is no Ca influx in mammalian axons or that the influx is too small to be detected by our method. However, results obtained with vagus nerve fibers loaded with quin2 (Pralong & Straub, 1985) showed a net increase in fluorescence during stimulation at 10 Hz. This increase was dependent on the external Ca concentration and abolished in the presence of cadmium, indicating that in rabbit vagus nerve, too, electrical activity produces an increase in Ca influx. Since, in the present experiments no increase in total ^{45}Ca was observed during stimulation, it can be concluded that the gain in calcium during activity is balanced by an increased efflux, so that the internal ionized calcium, though slightly increased (as indicated by experiments with quin2), remains below the threshold for intracellular sequestration. This interpretation is in agreement with findings of Medilanski and Straub (1984) who showed that in rabbit vagus nerve electrical activity results in an increased liberation of calcium.

The upshot of these observations is that in mammalian nerve the plasma membrane-associated extrusion mechanisms are able to maintain the internal ionized calcium at low levels during activity, whereas in the squid axon the intracellular calcium buffers must contribute to the control of the internal free calcium.

The following question, however, arises. Why, in the case of loading by exposure to Na-free solution, do the intracellular stores accumulate calcium, whereas during electrical activity they do not? The explanation probably is that the calcium-buffering capacity of the axoplasm is also dependent on sodium (Baker & Schlaepfer, 1978; Baker & Umbach, 1983), a rise in Na decreasing and a fall increasing the binding of the energy-dependent, presumably mitochondrial component. The findings that in our

experiments stimulation does not lead to a detectable accumulation of ^{45}Ca in contrast to experiments where nerves were loaded by incubation in Na-free solution may thus be due to the fact that stimulation increases both free calcium and sodium inside the nerve, whereas the Na-free solution leads to an increase in free calcium but to a decrease in internal sodium.

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