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Calcium Efflux and Intracellular Exchangeable Calcium in Mammalian Nonmyelinated Nerve Fibers

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Summary. Calcium efflux was measured in desheathed rabbit vagus nerves loaded with $^{45}Ca^{2+}$. The effects of extracellular calcium, sodium, phosphate, potassium and lanthanum ions on the calcium efflux were investigated and the distribution of intracellular calcium determined by kinetic analysis of $^{45}Ca^{2+}$ efflux profiles. The $^{45}Ca^{2+}$ desaturation curve can be adequately described by three exponential terms. The rate constant of the first component (0.2 min^{-1}) corresponds to an efflux from an extracellular compartment. The two slow components had rate constants of 0.03 and 0.08 min^{-1} and represent the efflux from two intracellular pools. The amounts of exchangeable calcium in these two pools, after a loading period of 150 min, were 0.170 and 0.102 mmol/kg wet weight, respectively. The total calcium efflux in physiological conditions amounted to about 24 fmol cm^{-2} sec^{-1}. The magnitude of the two intracellular compartments as well as the total calcium efflux were markedly affected by extracellular phosphate, sodium and lanthanum, whereas the corresponding rate constants remained almost unchanged. Phosphate reversed the effect of sodium withdrawal on the calcium efflux: in the absence of phosphate, sodium withdrawal increased the calcium efflux to 224%, but in the presence of phosphate, sodium withdrawal decreased calcium efflux to 44%. Phosphate also affected the increase in calcium efflux produced by inhibitors of mitochondrial calcium uptake, suggesting that two different mitochondrial pools contribute to the control and regulation of intracellular calcium and of the transmembrane calcium transport.

Key Words mammalian nerve · calcium efflux · exchangeable calcium · mitochondrial buffering · Na–Ca exchange

Introduction

In nerve fibers, as in virtually all animal cells, the concentration of intracellular free ionized calcium is much lower than in the external medium. Although the exact intracellular Ca^{2+} concentration is still a matter of speculation, a number of estimates indicate it to be not greater than 100 nM (DiPolo et al., 1976; Requena et al., 1977; Pralong, Jirounek & Straub, 1985). With an extracellular Ca^{2+} concentration of 2 mM and a membrane potential of about −50 to −70 mV, an inwardly oriented electrochemical gradient of several orders of magnitude therefore exists across the plasma membrane. It follows that in the regulation of intracellular Ca^{2+} the cell must be endowed with powerful mechanisms able to deal with a constant rate of passive basal calcium load coming from the outside, as a consequence of the large electrochemical gradient, and with the transient calcium influx during the process of conduction of action potentials. Numerous studies have provided evidence suggesting that both membrane extrusion mechanisms and intracellular buffering are involved in the regulation of the intracellular free calcium.

In our previous papers we have studied, in rabbit vagus nerve, the membrane-associated Ca^{2+}-transport systems and have shown that part of the Ca^{2+} efflux is mediated by a Na^+–Ca^{2+} exchange mechanism (Jirounek et al., 1986). We have also reported an interdependence between Ca^{2+} and phosphate fluxes (Jirounek et al., 1982), and suggested that the phosphate efflux may be linked to the operation of the Na^+–Ca^{2+} exchange (Jirounek et al., 1984*b*). Furthermore, we have shown that whenever the external Na^+ was decreased, there was a large increase in the labeling of the intracellular calcium pools (Jirounek et al., 1986). A similar effect was observed when the external phosphate concentration was increased (Jirounek et al., 1984*a*). These results show that specific changes in the composition of the external medium produce alterations in both intracellular and membrane-associated regulation mechanisms of the complex system of Ca^{2+} homeostasis. The experiments presented in this paper were carried out in order to further investigate the effects of external Ca^{2+}, Na^+ and phosphate on the intracellular Ca^{2+} distribution and to study the interdependence between the intracellular buffering and the Ca^{2+} transmembrane fluxes in mammalian nonmyelinated nerve fibers.

* Deceased 18 April 1988

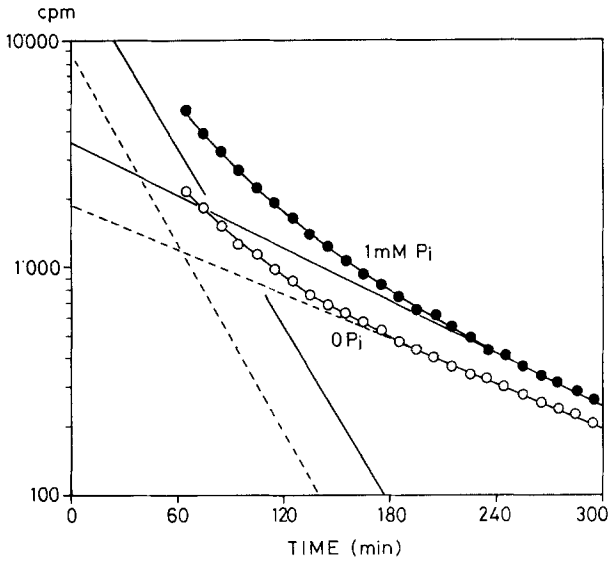

Fig. 1. Decomposition of the ^{45}Ca efflux profiles. After an incubation of 150 min in ^{45}Ca-labeled solution containing 0.2 mM phosphate, 0.9 mM Ca^{2+} and 154 mM Na^+, the efflux of ^{45}Ca was measured in solution where the phosphate was omitted (o), or increased to 1 mM. The effluents collected during the first 60 min, which were contaminated by the efflux of radioactivity from the extracellularly located ^{45}Ca pools, were discarded. The program for the computer multiexponential analysis of the effluents revealed two exponentials. The values of the parameters of the efflux in the presence of phosphate were: A_1 (t = 0) = 20918.1 cpm, k_1 = 0.03051 min^{-1}, A_2 (t = 0) = 3586.3 cpm, k_2 = 0.00902 min^{-1} (solid lines), and in the absence of phosphate: A_1 (t = 0) = 8979.7 cpm, k_1 = 0.03367 min^{-1}, A_2 (t = 0) = 1895.4 cpm, and k_2 = 0.00902 min^{-1} (dashed lines). The curves shown are those from one of eight experiments which gave similar results

We have found that the $^{45}Ca^{2+}$ desaturation can be adequately described by three exponential terms. The first component has a half-time which corresponds to an efflux from an extracellular compartment. The two slow components probably represent the calcium efflux from two intracellular Ca^{2+} pools. The magnitude of the two slowly exchangeable compartments were markedly affected by extracellular phosphate and sodium concentrations. Indirect evidence suggests that they represent two kinetically distinct mitochondrial pools.

Materials and Methods

Loading of the Tissue

For loading the tissue with $^{45}Ca^{2+}$, a desheathed cervical vagus nerve of a rabbit was mounted in a narrow polyethylene tube that was perfused at a rate of 1 ml/min with a physiological solution at 37°C. After a period of equilibration, a small amount of $^{45}Ca^{2+}$ (10 μC/ml) was added to the superfusion solution.

Measurement of the Efflux and Analysis of the Results

At the end of the labeling period, the radioactive solution was replaced by an inactive solution, the polyethylene tube connected to a fraction collector, the effluents collected during preset intervals and their radioactivity counted. At the end of the experiment the nerves were homogenized, the water soluble fraction extracted and counted.

The classical graphic method for analyzing the multiexponential functions showed, in experiments where there were no changes in the composition of the washing solution, that the time course of the loss of radioactivity by the preparation can be decomposed into several exponentials. However, this simple graphic method is somewhat subjective and prone to subtraction errors. For a more accurate analysis of our results we have therefore adapted a computer program originally developed by Tscharner (1982). The program is based on the nonlinear least-squares method, according to the Marquardt scheme (Marquardt, 1963). This iterative error-minimizing method is numerically stable in that the step size is purposely limited to avoid unpredictable increases in errors. Since initial parameter values must be chosen, a simple starting program was employed to compute a reasonably accurate estimate of the initial parameters. The program rapidly converges to a minimum and provides excellent results even in experiments where the radioactivity in the effluents was low and the noise level relatively high.

The results obtained by this type of analysis showed three components of $^{45}Ca^{2+}$ release. The rate constant of the first one corresponds almost certainly to the efflux from an extracellular pool. In order to characterize the components corresponding to the efflux from the intracellular compartments, the multiexponential analysis was applied to the effluents from the 60th min, where the contribution of the efflux from the extracellular pool becomes negligible. The two remaining components (Fig. 1) are characterized by the rate constant of desaturation, k_1 and k_2, and by the amount of exchangeable calcium, A_1 and A_2, extrapolated to the beginning of the washing period. In experiments where the composition in the external solution was modified during the efflux, the results were expressed as the fraction of the total radioactivity of the tissue lost during each collection period.

Solutions

The physiological solution contained (in mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 0.9; $MgCl_2$, 0.5, inorganic phosphate, 0.2; glucose, 5; Tris, 10. We have used Tris instead of HCO_3–CO_2 buffer, since Tris was used in almost all previous studies on nonmyelinated nerve fibers, particularly in the studies on oxygen consumption (e.g. Ritchie & Straub, 1979), where it was shown that Tris buffer does not affect nerve metabolism.

Solutions with higher calcium were prepared by adding solid $CaCl_2$, Ca^{2+}-free solution by omitting $CaCl_2$. Phosphate was added as the mono- and disodium salts. All experiments were carried out at 37°C and pH 7.4.

Results

Ca^{2+} Efflux in Steady State

In a first series of experiments, we have studied the kinetics of the $^{45}Ca^{2+}$ efflux without modification in

the composition of the extracellular solution, in order to avoid perturbations in the steady state of the complex intracellular Ca^{2+}-regulating system. After a loading period of 150 min, the efflux of $^{45}Ca^{2+}$ was measured during 5 hr. The multiexponential analysis of the efflux profiles showed three clearly distinguishable components of $^{45}Ca^{2+}$ release. The rate constant of the first one was about 0.2 min^{-1}, indicating that this initial rapid calcium efflux originates from the extracellular space and the membrane-bound fraction. Although this component represents about 80% of the total radioactivity found in the nerve at the end of the labeling period, more than 99.5% of this extracellular pool is desaturated after the first 60 min of washout. In order to obtain data for the efflux from the intracellular Ca^{2+} pools, the multiexponential analysis was applied to the effluents collected after the 60th min. The results showed that this part of the efflux is composed of only two components (Fig. 1), both presumably reflecting the efflux from intracellular calcium compartments.

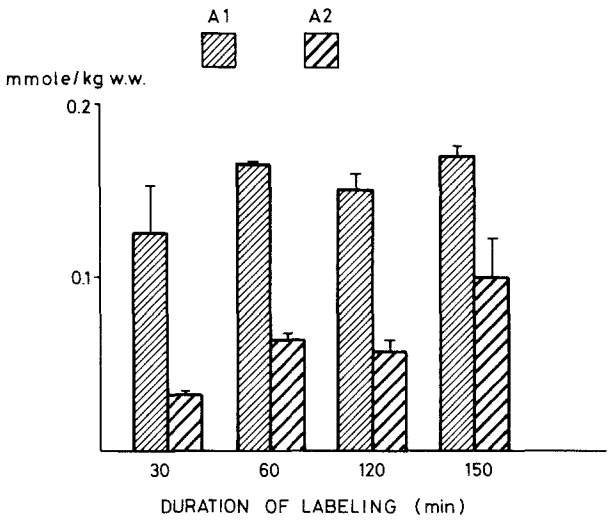

Fig. 2. Labeling of the two intracellular pools A_1 and A_2. Nerves were incubated in the radioactive solution during the times indicated, and then washed in nonradioactive Locke during 5 hr and the effluents collected. The radioactivity found in the effluents from the 60th min was submitted to a multiexponential analysis, in order to determine the amounts of exchangeable $^{45}Ca^{2+}$ in A_1 and A_2 and the corresponding rate constants k_1 and k_2. The values are means ± SEM of three to five separate experiments

Effect of the Time of Labeling on the Efflux

In these experiments the nerves were labeled by incubation in physiological solution containing 0.9 mM Ca^{2+} and 10 μCi/ml $^{45}Ca^{2+}$ during periods varying from 30 to 240 min. After the given labeling period, the efflux was measured from the 60th to the 300th min by washing the preparation with a solution of the same composition as was used for the labeling, but without the tracer $^{45}Ca^{2+}$. The results showed that the rate constants of both, the rapid and the slow component are almost independent of the duration of labeling. The mean values for k_1 and k_2 were 0.030 ± 0.001 and 0.0079 ± 0.002 min^{-1}, respectively (n = 11). On the other hand, the amounts of $^{45}Ca^{2+}$ incorporated into the two intracellular compartments increased with increasing time of incubation in the radioactive solution. Figure 2 shows that the labeling was much faster for pool A_1, where isotopic equilibrium was attained after 60 min of incubation, than for pool A_2, where the radioactivity continued to increase even after the 150th min. After 150-min incubation, which time corresponds to the labeling conditions used in the experiments described in the next sections, the amounts of labeled Ca^{2+} in pools A_1 and A_2 are 0.170 and 0.102 mmol/kg wet weight (w.w.), respectively. The total intracellular Ca^{2+} is thus 0.272 mmol/kg w.w., which can be compared to 0.355 mmol/kg w.w. found at isotopic equilibrium in the same preparation by a different method (Jirounek et al., 1986).

Effect of Na^+ and Phosphate

In these experiments the nerves were incubated in physiological solution containing 0.2 mM phosphate and 0.9 mM Ca^{2+}. The efflux was then measured either in the presence of phosphate (1 mM) or in its absence. Figure 1 shows that the apparent exchangeable Ca^{2+} in both A_1 and A_2 decreased considerably when the external phosphate was lowered. The rate constants k_1 and k_2, however, were not significantly affected by the external phosphate concentration (*see* Table 1). Similar experiments were then repeated in the presence of phosphate but in the absence of external Na^+. The results of these experiments are summarized in Fig. 3. In Ca^{2+}-free solution the effect of Na^+ omission, if compared to 0P and normal Na, produced an increase in both A_1 and A_2. In the presence of Ca^{2+}, however, the effect of the Na^+-free solution is comparable to the effect of low phosphate. This could be explained by the Na_o^+ dependence of the phosphate influx, described by Anner et al. (1976), who showed in the same preparation, that in Na^+-free solution the influx of phosphate is almost completely blocked. The decrease in A_1 and A_2, observed in Na^+-free solution, may thus be due to the inhibition of the phosphate influx by the lack of Na_o^+.

It is important to keep in mind that the values of A_1 and A_2 do not represent the total calcium in these

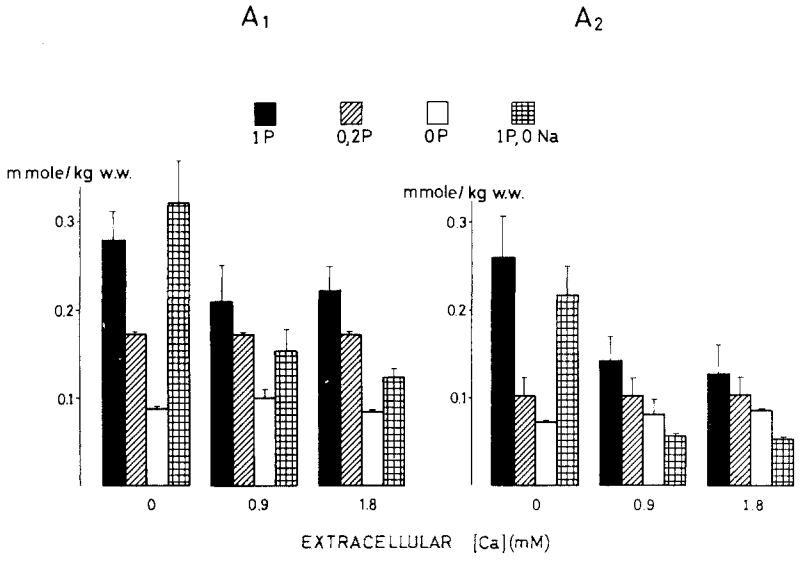

Fig. 3. Effects of phosphate and sodium on the exchangeable $^{45}Ca^{2+}$ in A_1 and A_2. The nerves were incubated 150 min in physiological solution (154 mM Na^+, 0.2 mM PO_4, 0.9 mM Ca^{2+}) with 10 μC/ml of $^{45}Ca^{2-}$, and then washed during 5 hr in solution containing indicated concentrations of phosphate, Na^+ and Ca^{2+}. The values of A_1 and A_2 were obtained by the multiexponential analysis of the radioactivity found in the effluents from the 60th min. The values are means ± SEM of four to eight separate experiments

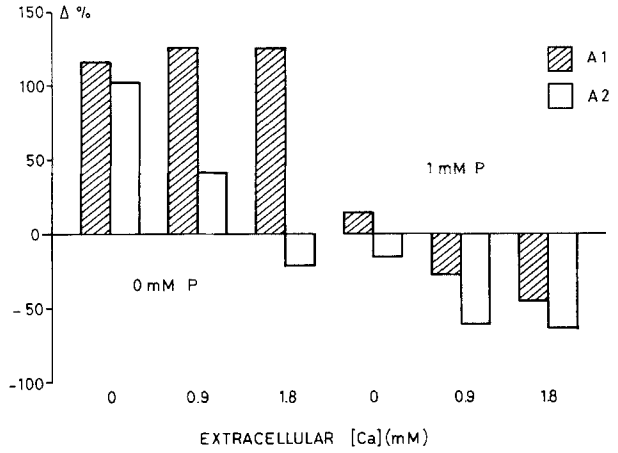

Fig. 4. Effect of Na^+-free solution on A_1 and A_2. The nerves were incubated as in experiments presented in Fig. 2 and washed either in the presence or in the absence of sodium in solutions containing indicated concentrations of phosphate and calcium. Plotted in this Figure are the differences between the results obtained in the presence and the absence of Na_o^+, expressed in percent of the value obtained in the presence of Na^+

pools, but only the exchangeable amounts that contribute to the measured efflux. According to the results presented in the first part of this paper (Fig. 2), the control amounts in A_1 and A_2 at the end of a labeling period of 150 min were 0.170 and 0.102 mmol/kg w.w., respectively. The fact that after 1 hr of efflux (the multiexponential analysis was applied to the effluents collected after the 60th min) these amounts, extrapolated to the beginning of the washing period, were significantly different from the controls, signifies that during the first hour of efflux the capacity of the intracellular buffers was altered.

The effects of Na^+-free solution on A_1 and A_2 are summarized in Fig. 4, where we have expressed the difference between the values obtained in Na^+ and in Na^+-free solution as the percentage of the values obtained in the presence of Na_o^+. It can be pointed out from this Figure that when Na_o^+ was omitted in the presence of phosphate, both A_1 and A_2 decreased, or remained unchanged (in Ca^{2+}-free solution). In the absence of phosphate, however, the effect on A_1 was inversed: omission of Na^+ produced an increase in A_1 by more than 100%. The effect on A_2 of Na^+-free solution in the absence of phosphate was Ca_o^{2+} dependent. In nominally Ca^{2+}-free solution the omission of Na_o^+ produced an increase of about 100%, at 0.9 mM Ca_o^{2+} the increase was only 40%, and at 1.8 mM Ca_o^{2+} there was a slight decrease.

The rate constants k_1 and k_2, determined by the multiexponential analysis of the efflux experiments are reported in Table 1 and show that the rate constants are almost independent of the external phosphate or sodium concentration.

The outflow, calculated from the values of k_1, k_2, A_1 and A_2, determined from these experiments are reported in Table 2. It is interesting to note the inhibitory effect on Ca^{2+} outflow of Na^+ omission in phosphate-containing solution in contrast to the stimulatory effect in the absence of phosphate.

Effect of Ca^{2+}

In these experiments the nerves were labeled during 150 min in a solution which had the same ionic composition as was then used in the efflux experiments. The results of the multiexponential analysis of the efflux profiles of nerves labeled and superfused at 0,

Table 1. Effect of calcium, sodium and phosphate on the amounts of exchangeable $^{45}Ca^{2+}$ in the two intracellular calcium pools (A_1 and A_2) and on the corresponding rate constants (k_1 and k_2)

Calcium (mM)	Sodium (mM)	Phosphate (mM)	A_1 (mmol/kg w.w.)	k_1 (min^{-1})	A_2 (mmol/kg w.w.)	k_2 (min^{-1})
0	154	1	0.279 ± 0.035	0.032 ± 0.003	0.257 ± 0.05	0.0074 ± 0.0005
0	154	0	0.088 ± 0.006	0.030 ± 0.003	0.071 ± 0.002	0.0072 ± 0.0006
0	0	1	0.321 ± 0.048	0.030 ± 0.002	0.215 ± 0.035	0.0070 ± 0.0003
0	0	0	0.190 ± 0.044	0.027 ± 0.002	0.144 ± 0.033	0.0058 ± 0.0006
0.9	154	1	0.211 ± 0.049	0.031 ± 0.006	0.140 ± 0.034	0.0078 ± 0.0017
0.9	154	0	0.099 ± 0.015	0.033 ± 0.004	0.078 ± 0.020	0.0084 ± 0.0004
0.9	0	1	0.154 ± 0.027	0.028 ± 0.004	0.055 ± 0.002	0.0070 ± 0.0006
0.9	0	0	0.225 ± 0.006	0.034 ± 0.005	0.111 ± 0.016	0.0079 ± 0.0005
1.8	154	1	0.220 ± 0.030	0.034 ± 0.006	0.132 ± 0.03	0.0089 ± 0.0018
1.8	154	0	0.084	0.027	0.086	0.0067
1.8	0	1	0.124 ± 0.011	0.027 ± 0.001	0.057 ± 0.001	0.0062 ± 0.0006
1.8	0	0	0.190 ± 0.096	0.026 ± 0.003	0.066 ± 0.027	0.0061 ± 0.0012

[a] The nerves were labeled 150 min in physiological solution (154 mM NaCl, 0.9 mM $CaCl_2$, 0.2 mM phosphate, 10 μC/ml $^{45}Ca^{2+}$, 37°C, pH 7.4), and then washed 5 hr with nonradioactive solution containing indicated concentrations of calcium, sodium and phosphate. The results were obtained by computer multiexponential analysis of the $^{45}Ca^{2+}$ profiles from the 60th min of the efflux. The values are means ± SEM of three to 10 separate experiments; the experiment where SEM is not indicated is a mean of two experiments.

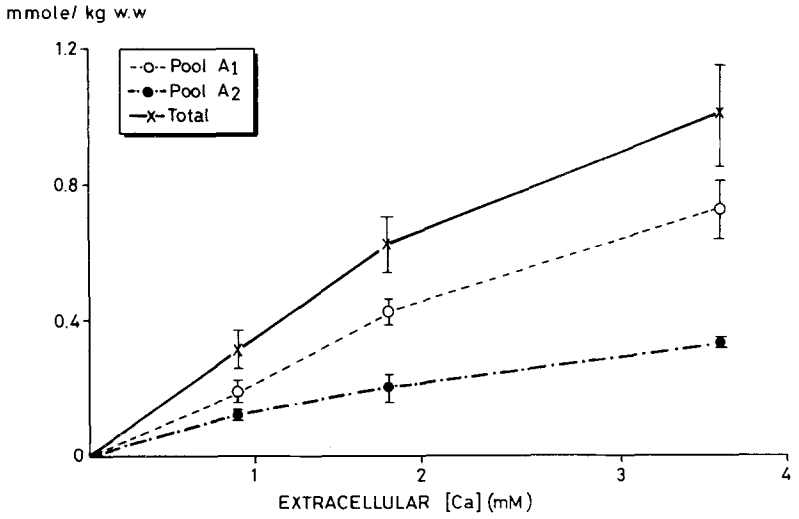

Fig. 5. Effect of external Ca^{2+} on A_1 and A_2. Nerves were incubated (150 min) and washed (5 hr) in physiological solutions with concentrations of Ca_o^{2+} indicated in the Figure. The results were obtained by the multiexponential analysis of the radioactivity found in the effluents collected from the 60th min. The values are means ± SEM of four to ten separate experiments

0.9, 1.8 and 3.6 mM Ca_o^{2+}, presented in Fig. 5, show that the amounts of $^{45}Ca^{2+}$ exchanged with the pools A_1 and A_2 during the labeling period increased considerably with increasing external Ca^{2+}, whereas the corresponding rate constants (Table 1) were either unaffected (k_1) or slightly increased (k_2).

Effect of La^{3+}

A series of experiments was undertaken to study the effect of lanthanum on the $^{45}Ca^{2+}$ efflux. After the initial labeling period, the $^{45}Ca^{2+}$ efflux was measured in the presence of 0.1, 0.5, 1 and 5 mM of La^{3+}. The results, presented in Fig. 6 show that low lanthanum concentrations enhanced the amounts of exchangeable Ca^{2+} in both A_1 and A_2. The maximal effect was observed at 100 μM; at higher La^{3+} concentrations the effect was less pronounced. The corresponding values of k_1 and k_2 were not significantly affected by low lanthanum concentrations, but at 1 and 5 mM there was a net decrease of about 40%.

Ca^{2+} Efflux after Sudden Changes in the Composition of the Extracellular Solution

The results described in the preceding sections were obtained by application of the multiexponential analysis to the $^{45}Ca^{2+}$ steady-state efflux profiles. In the next part of this paper we report results

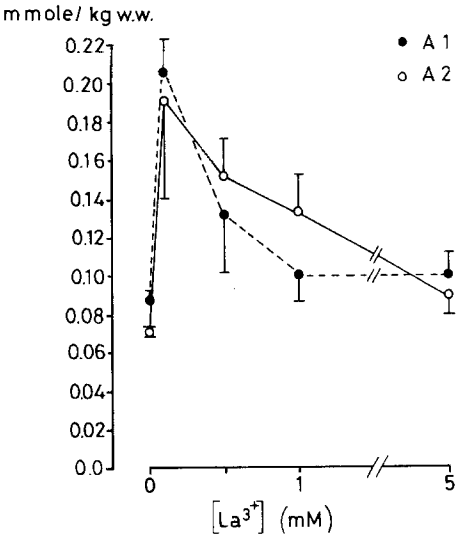

Fig. 6. Effect of lanthanum on exchangeable calcium in A_1 and A_2. Nerves were loaded with $^{45}Ca^{2+}$ during 150 min as in experiments presented in Fig. 2, and washed 5 hr in Locke or in Locke containing 0.1, 0.5, 1.0, or 5.0 mM La^{3+}. The graphic shows the values of A_1 and A_2 (means of three to five experiments ± SEM) in the absence of lanthanum and at the tested lanthanum concentrations. The maximal effect on both A_1 and A_2 was obtained at 0.1 mM and decreased with increasing the lanthanum concentration

obtained by measuring the Ca^{2+} efflux *during* the changes in the composition of the washing solutions. For the interpretation of these experiments the multiexponential analysis cannot be applied, since any modification in the extracellular medium will cause a modification in the kinetics of the Ca^{2+} efflux which cannot be approximated by a simple sum of exponentials. The results of these experiments are presented as the fraction of the total radioactivity lost during each sampling interval. The information obtained by this method is purely qualitative and does not allow the determination of the pool size. However, the results show how rapidly the changes in the internal Ca^{2+} distribution occur and how these changes alter the $^{45}Ca^{2+}$ efflux. Since Na_o^+ and phosphate have a marked influence on the distribution of intracellular calcium, it was interesting to measure the $^{45}Ca^{2+}$ efflux during these Na_o^+ and phosphate-induced alterations of the intracellular calcium.

Effects of Na^+ and Phosphate

Figure 7 shows that the effect of an increase in external phosphate concentration on the $^{45}Ca^{2+}$ efflux is Na_o^+ dependent. In the presence of external Na^+ the increase in phosphate concentration produced a slight but significant and reversible decrease of the $^{45}Ca^{2+}$ efflux, whereas in Na^+-free solution the effect was inversed. Similarly, the effect of Na^+ omission is phosphate dependent. Na^+ withdrawal in the presence of phosphate produced a decrease of the calcium efflux, whereas in the absence of phosphate there was a slight, but only transient increase (Fig. 8).

From Table 1, where we have summarized the results obtained by the multiexponential analysis, we can see, for 0.9 Ca_o^{2+}, that when phosphate was added to the superfusion solution in the presence of Na_o^+ there was an increase in A_1 and A_2 from 0.99 to 0.211 mmol/kg w.w. and from 0.078 to 0.140 mmol/kg w.w., respectively. On the other hand, an increase in phosphate in Na^+-free solution produced a decrease in both A_1 and A_2 from 0.225 to 0.154 mmol/kg w.w. and from 0.111 to 0.055 mmol/kg w.w., respectively. If we assume that an increase in intracellular buffering decreases the Ca^{2+} efflux, and vice versa, then the results presented in Fig. 7 are in concordance with the predictions of the multiexponential analysis. However, from the same Table we see that omission of Na^+ in the presence of phosphate produced a decrease in A_1 and A_2 from 0.211 to 0.154 mmol/kg w.w. and from 0.140 to 0.055 mmol/kg w.w., respectively, and that lack of Na_o^+ in the absence of phosphate increases A_1 and A_2 from 0.099 to 0.225 mmol/kg w.w. and from 0.078 to 0.111 mmol/kg w.w., respectively. Comparing these data to Fig. 8 we observe that there, in contrast to the effects of phosphate, a decrease in the intracellular buffering produces a decrease of the efflux. A possible explanation of this discrepancy is that the large influx of $^{40}Ca^{2+}$ which occurs in Na^+-free solution (Jirounek et al., 1986), dilutes the internal $^{45}Ca^{2+}$, producing thus an apparent decrease of the $^{45}Ca^{2+}$ efflux. We can, however, not exclude that at least a part of the effect is due to the inhibition of the Na_o^+–Ca_{in}^{2+} exchange.

Effects of Metabolic Inhibitors

The effects of phosphate on A_1 and A_2 show that the properties of these two intracellular compartments are similar to those of isolated mitochondria. We have therefore tested the effects of several mitochondrial Ca^{2+} entry blockers. Figure 9 shows results of experiments with mersalyl, known to block the phosphate-dependent mitochondrial Ca^{2+} uptake. The poison was added to the washing solution after 180 min of efflux either in the presence of phosphate during the whole efflux period or to nerves where phosphate was omitted at the beginning, or after 60 or 120 min of efflux. Figure 9 shows that mersalyl (100 μM) produced a net increase of

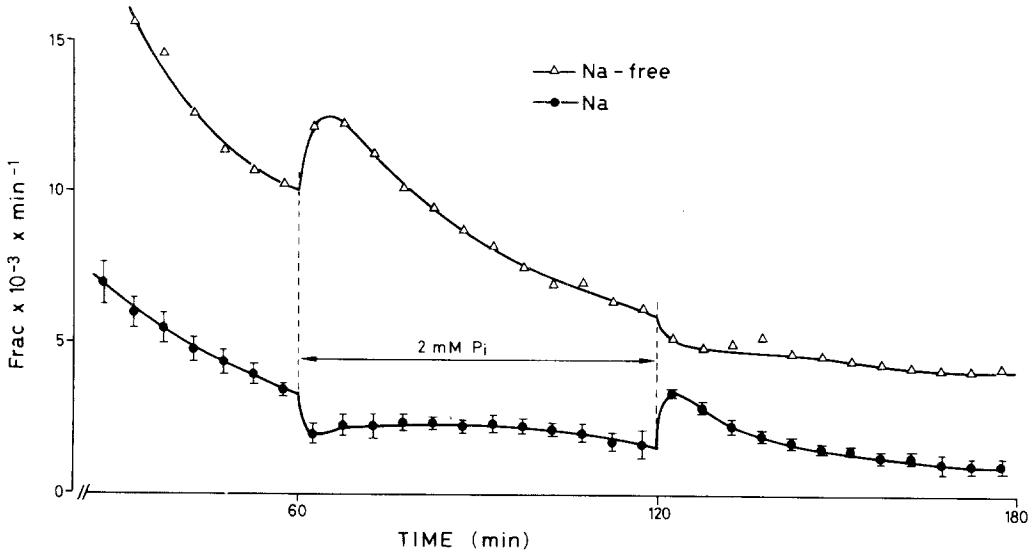

Fig. 7. Effect of phosphate on $^{45}Ca^{2+}$ efflux. The nerves were incubated 150 min as in experiments presented in Fig. 2 and then washed in phosphate-free solution either in the presence or the absence of Na^+. After 60 min, phosphate (2 mM) was added to the washing solution. The ordinate shows the fraction of radioactivity lost by the preparation during each collection period. Points with bars indicating SEM are means of three experiments; points without SEM are means of two experiments. Abscissa is time after beginning of washing

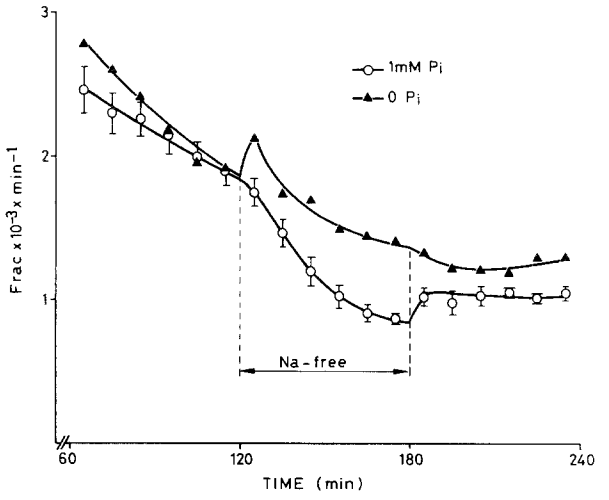

Fig. 8. Effect of Na^+ on $^{45}Ca^{2+}$ efflux. Nerves were loaded with $^{45}Ca^{2+}$ as in experiments presented in Fig. 2 and then washed in isotope-free Locke either in the presence or the absence of phosphate. After 120 min of efflux, the sodium was withdrawn from the washing solution. Ordinate represents the fraction of radioactivity lost by the preparation during each collection period. Points ± SEM are means of three, without SEM of two experiments. Abscissa is time after beginning of washing

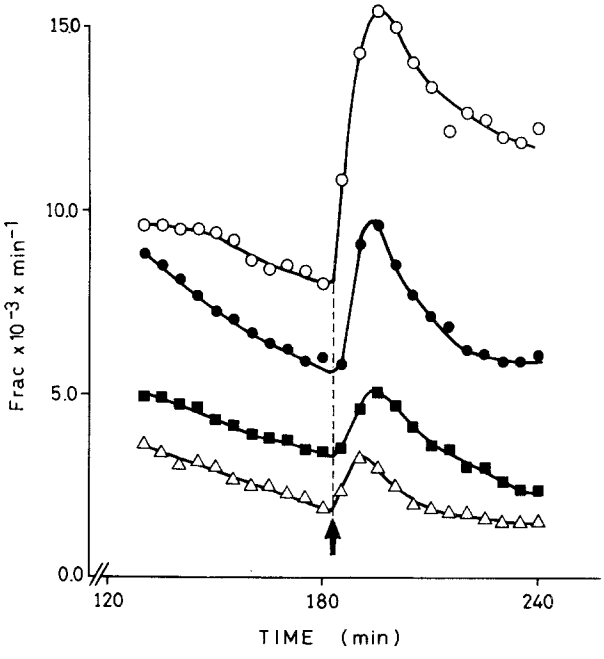

Fig. 9. Effect of mersalyl on $^{45}Ca^{2+}$ efflux. Nerves were labeled with $^{45}Ca^{2+}$ as in experiments presented in Fig. 2 and then washed in nonradioactive Locke. Phosphate was present during the whole efflux period (○), or during the first 120 min (●), or 60 min (■), or was absent during the efflux (△). Mersalyl was added at the 180th min. Abscissa represents the fraction of radioactivity lost by the preparation during each collection period, ordinate is time after beginning the efflux

the efflux which increased with decreasing time of washing with the phosphate-free solution. Moreover, after 2 hr of washout, there was a net inhibition of the rates of $^{45}Ca^{2+}$ efflux, proportional to the duration of the washout in phosphate-free solution.

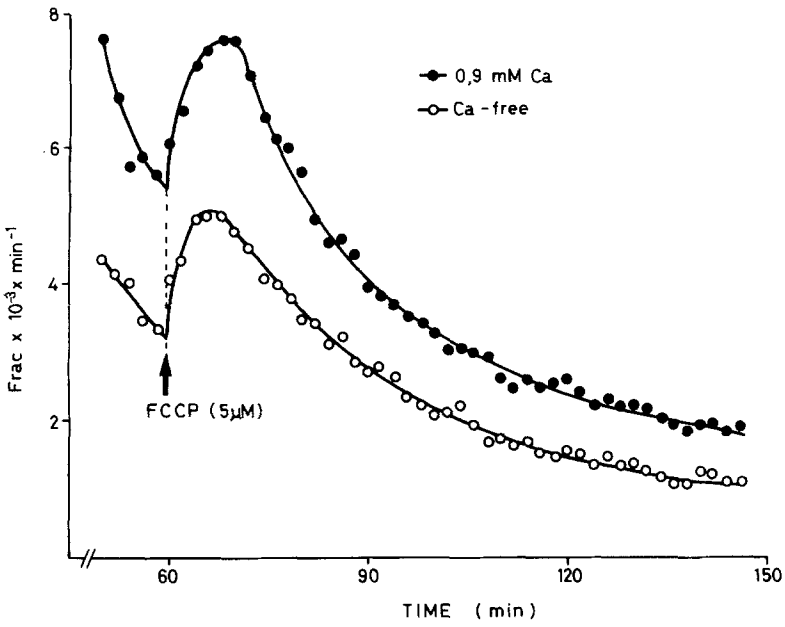

Fig. 10. Effect of FCCP on $^{45}Ca^{2+}$ efflux. Nerves were loaded 150 min with $^{45}Ca^{2+}$ as in experiments presented in Fig. 2, and then washed in isotope-free solution in the presence or the absence of Ca^{2+}. After 60 min of efflux, 5 μM FCCP were added to the washing solution. Abscissa represents the fraction of radioactivity lost by the preparation during each collection period; ordinate is time after beginning of washing

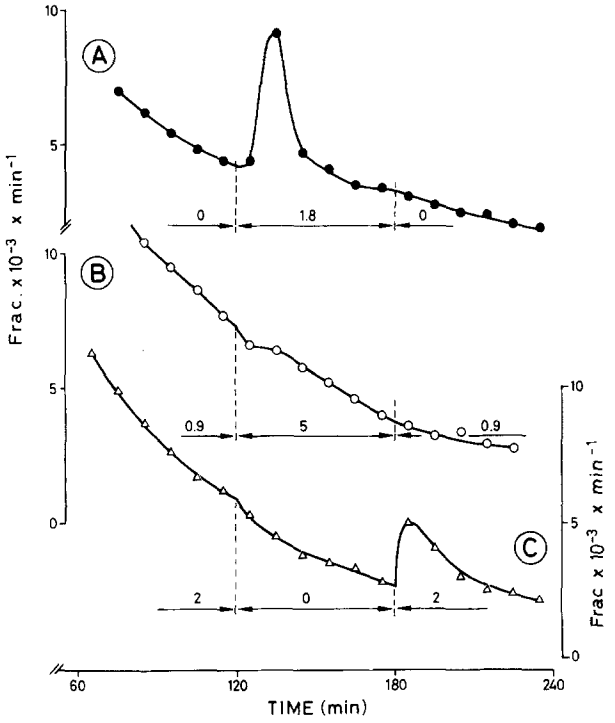

Fig. 11. Effect of external calcium on $^{45}Ca^{2+}$ efflux. Nerves were loaded with $^{45}Ca^{2+}$ as in experiments presented in Fig. 2. During the efflux period, external calcium was changed as indicated. Abscissa represents the fraction of $^{45}Ca^{2+}$ lost during each collection interval; ordinate is time after beginning of washing

These results bring additional evidences for an involvement of the mitochondrial phosphate-dependent calcium uptake in the regulation of the intracellular free Ca^{2+} concentration.

In eight experiments we have also tested the effects of *p*-trifluoromethoxyphenylhydrazone (FCCP). Because FCCP is thought to inactivate completely the Ca^{2+}-accumulating capacity of mitochondria, the presumption was that under these conditions the Ca^{2+} efflux will increase. The results presented in Fig. 10 confirm this prediction. The maximal effect was obtained at 5 μM, but a net increase of the efflux was observed already at a concentration of 1 μM. Similar results were obtained in 12 experiments with 5 mM cyanide.

Effect of Ca_o^{2+}

Curve *A* in Fig. 11 shows the effect of an increase in the external calcium concentration from a nominally Ca^{2+}-free solution to 1.8 mM. There was an initial transient increase in the efflux, followed by a rapid fall to values that are not significantly different from the efflux in controls. The initial transient effect disappeared completely when some calcium was present before the exposition of the nerve to high Ca_o^{2+}. This is illustrated by curve *B* in Fig. 11 which shows the results of an experiment where the external calcium was increased from 0.9 to 5 mM. On the other hand, lowering external calcium from 2 mM to nominally Ca^{2+}-free solution (curve *C*) produced a slow decrease of the $^{45}Ca^{2+}$ efflux to values slightly below the efflux in controls. After reintroduction of Ca^{2+} to the washing solution there was a small transient rise, followed by a decrease to the values of the controls.

Effect of La^{3+}

When, in the presence of 0.9 mM Ca_o^{2+}, La^{3+} was added to the washing solution, the efflux rapidly decreased. For 0.005, 0.05, 0.1, 0.5, 1 and 5 mM La^{3+} the inhibition of the efflux was of 4, 17, 28, 43, 48 and 51%, respectively. If, during the treatment with La^{3+}, calcium was removed, the efflux continued to decrease slowly.

When lanthanum was added to Ca^{2+}-free solution the decrease in $^{45}Ca^{2+}$ efflux was preceded by a rapid transient loss of radioactivity. Readmission of Ca^{2+} during the lanthanum treatment produced a slight increase in the $^{45}Ca^{2+}$ efflux.

Effect of K_o^+

The effect of extracellular K^+ on the Ca^{2+} efflux was studied by addition or omission of K^+ in the washing solution and in the presence and absence of Ca_o^{2+}. The results showed that depolarization of the nerve with 50 mM of potassium, either in the presence or absence of external calcium, does not produce any sustained modification of the $^{45}Ca^{2+}$ efflux; only a small and transient increase was usually observed just after the application of the high potassium to the perfusion solution. Omission of K^+, which hyperpolarized the nerve was also without significant effect on the $^{45}Ca^{2+}$ efflux. These results are in agreement with those reported by Stout and Diecke (1981), who showed, in myelinated nerve, that changes in K_o^+ did not affect $^{45}Ca^{2+}$ efflux significantly.

Discussion

A precise analysis of $^{45}Ca^{2+}$ desaturation curves is not easy, since the efflux of $^{45}Ca^{2+}$ from cells depends not only on the unidirectional transport from the cytosol to the cell environment, but also on the intracellular free calcium and on the specific activity of this pool. Moreover, an important fraction of calcium is bound to the cell surface and also contributes to the $^{45}Ca^{2+}$ of the effluents. We have therefore used two different, but complementary approaches for the interpretation of our results. The first one is based on the multiexponential analysis of the steady-state efflux kinetics, the second one consists of measurement of the radioactivity lost by the preparation during the period of the $^{45}Ca^{2+}$ transients. In spite of some difficulties in interpretation, both the multiexponential analysis and the transient efflux profiles give important information about the kinetic properties and behavior of the calcium efflux.

The Efflux in Steady State

The multiexponential analysis, applied to the effluents collected after the 60th min identified two clearly distinguishable components of $^{45}Ca^{2+}$ release, indicating that the calcium outflow comes from two different intracellular pools. The values of A_1, A_2, k_1 and k_2 indicate the apparent amounts of exchangeable calcium in these two pools and the rate constants of desaturating of these pools, respectively.

The Time Course of Labeling

Figure 2 shows the time course of labeling of both intracellular pools A_1 and A_2. If we assume that the system remains in a steady state during the whole experiment, i.e. during the influx and efflux periods, then the rate constants of labeling should correspond to those of the desaturation process. Thus after 30 and 60 min of labeling with a rate constant of desaturation of 0.03 min^{-1}, the pool A_1 should be labeled to 60 and 80%, respectively, whereas the pool A_2 with a rate constant of 0.0079 min^{-1} only to 21 and 37%, respectively. The results of the labeling process, presented on Fig. 2, are in good agreement with these predictions, suggesting that the steady state remains almost unaffected during the whole influx and efflux experiment.

The Effect of Phosphate and Na^+

The results presented in Table 1 and in Fig. 3 show that the amounts of $^{45}Ca^{2+}$ that contribute to the measured efflux increase with increasing external phosphate concentration. The findings that the exchangeable Ca^{2+} of the nerve was higher in the presence of phosphate than in its absence, and that there was no change in the rate constants k_1 and k_2 indicate that during the first hour of efflux in high phosphate, some intracellular calcium was mobilized and became exchangeable, but that this new pool continued to desaturate with the same rate constant.

Similar but less pronounced effects on A_1 and A_2 were observed when, in the presence of phosphate, Na^+ was omitted (Table 1 and Fig. 3). In our previous paper (Jirounek et al., 1986) we have shown that during incubation of rabbit vagus nerve in low Na^+ media there was an important gain in the total $^{45}Ca^{2+}$ which was due to the reversal of the Na^+–Ca^{2+} exchange. Brinley et al. (1977) have shown that during condition of net Ca^{2+} gain, the great majority of the Ca^{2+} entering axons is buffered and only 1/1000 or less is detectable as free calcium.

The axoplasmic components contributing to this buffering have been characterized in some detail by Baker and Schlaepfer (1975, 1978), Brinley et al. (1977), Brinley, Tifert and Scarpa (1978) and Requena et al. (1977). Their main findings are that the Ca^{2+}-binding capacity of the isolated axoplasm is the result of energy-dependent and energy-independent processes. The only energy-dependent process that has been clearly identified is the mitochondrion, although there is some evidence for an endoplasmic reticulum buffering system as well (Blaustein, Ratzlaff & Schweitzer, 1978*a*; Blaustein et al., 1978*b*; Rasgado-Flores & Blaustein, 1987). Since an extensive phosphate-dependent uptake is known to occur only in the mitochondria (Fiskum & Lehninger, 1982), it seems that the effects of phosphate on A_1 and A_2 are in relation with the Ca^{2+} uptake by the mitochondrion rather than by the endoplasmic reticulum.

The last column in Fig. 3 represents the exchangeable Ca^{2+} in A_1 and A_2, determined in the presence of 1 mM of phosphate and the absence of Na_o^+. When Ca^{2+} was present (0.9 and 1.8 mM), the values of A_1 and particularly A_2 were lower than in controls. This effect of Na^+ withdrawal can be explained by a decrease in the internal phosphate, since the influx of phosphate is strongly Na_o^+ dependent (Anner et al., 1976; Straub et al., 1977). This decrease of the intracellular phosphate will tend to decrease the buffering by A_1 and A_2. However, when the same experiment was performed in nominally Ca^{2+}-free solution, the amounts of exchangeable calcium in both A_1 and A_2 were increased. A possible explanation could be that in low Ca^{2+} the effect on the buffering of the intracellular Na^+ becomes predominant, and the decrease in the internal Na^+ (consecutive to the Na^+ withdrawal), increases the apparent binding capacity of the mitochondrion by inhibiting the Ca^{2+} efflux by the mitochondrial Na^+–Ca^{2+} exchange (Crompton, Capano & Carafoli, 1976; Carafoli & Crompton, 1978; Affolter & Carafoli, 1980; Nicholls & Akerman, 1982).

The real calcium efflux can be calculated by simply multiplying the rate constant by the amounts of exchangeable Ca^{2+} in the corresponding Ca^{2+} compartment. In Table 2 we have summarized the results of these calculations. The data show that the effect of Na^+ withdrawal on the $^{45}Ca^{2+}$ efflux is phosphate dependent. When in the presence of phosphate all the external Na^+ is replaced with choline there is either a slight stimulation of the Ca^{2+} efflux, (in nominally Ca^{2+}-free solution), or, at 0.9 and 1.8 mM Ca_o^{2+}, a decrease of 35 and 55%, respectively. On the other hand, whenever the external Na^+ was omitted in the absence of phosphate, the efflux of $^{45}Ca^{2+}$ greatly increased. It should be pointed out that these changes in the Na_o^+-dependent fraction of the $^{45}Ca^{2+}$ efflux result principally from modifications of the exchangeable internal Ca^{2+} pools and thus cannot be considered as directly reflecting changes in activity of the Na_o^+–Ca_i^{2+} exchange mechanism.

Effects of Sudden Changes in the Composition of the External Solution

Effect of Na_o^+

The results of the multiexponential analysis indicate that both A_1 and A_2 decrease when Na^+ is omitted in the presence of phosphate (Fig. 3), and that the effect is inversed in its absence (Table 1). The efflux transients observed after Na^+ withdrawal (Fig. 8) do not reflect these modifications of the intracellular exchangeable Ca^{2+} pools, since a decrease in the internal Ca^{2+} buffering should increase the $^{45}Ca^{2+}$ efflux and vice versa. The explanation is probably that in Na^+-free solution the Na_o^+–Ca_{in}^{2+} exchange is blocked and the Na_{in}^+–Ca_o^{2+} exchange greatly enhanced (Jirounek et al., 1986). Both the inhibition of the Na_o^+-dependent part of the Ca^{2+} efflux and the dilution of the internal $^{45}Ca^{2+}$ pool by the large $^{40}Ca^{2+}$ load result in an important decrease in the $^{45}Ca^{2+}$ efflux which probably masks the effect of Na^+ on the intracellular buffering.

Reintroduction of the standard (154 mM) Na^+ concentration to the superfusion fluid produced in some experiments an initial overshoot of the efflux. The cause of these transients is uncertain. They are, however, not limited to our preparation; similar transients were also observed in squid axons. This observation may indicate that at least a part of the Ca^{2+} efflux depends on the Na^+ gradient, since after a period of washing with Na^+-free solution, the internal Na^+ will be decreased. When Na_o^+ is then reintroduced, there is an important initial Na^+ gradient, which will slowly recover to the normal Na^+ condition. Another possible explanation, advanced by Blaustein (1977), is that the internal Na^+ just inside the axolemma slowly rises as a consequence of the Na^+ influx and will inhibit the Ca^{2+} efflux by competition between internal Na^+ and Ca^{2+} for the active sites of the Na^+–Ca^{2+} exchange system.

Effect of Phosphate

In analogy to experiments with Na^+ on the $^{45}Ca^{2+}$ efflux, where the effect was phosphate dependent, the effect of phosphate on the $^{45}Ca^{2+}$ efflux is Na^+ dependent (Fig. 7): in the presence of Na_o^+ the phos-

Table 2. Effect of sodium, phosphate and calcium on the calcium efflux[a]

Efflux I (f/cs)

	In presence of phosphate (1 mM)				In absence of phosphate			
Na Ca	154	0	Difference (f/cs)	(%)	154	0	Difference (f/cs)	(%)
0	24.55	26.83	+2.28	+9.3	7.32	14.44	+7.12	+97.3
0.9	17.65	12.49	−5.16	−29.2	9.12	18.11	+8.99	+98.5
1.8	20.1	9.24	−10.76	−53.5	6.30	15.24	+8.94	+141.9

Efflux II (f/cs)

	In presence of phosphate (1 mM)				In absence of phosphate			
Na Ca	154	0	Difference (f/cs)	(%)	154	0	Difference (f/cs)	(%)
0	5.16	4.21	−0.95	−18.4	1.41	2.38	+0.97	+68.8
0.9	3.33	1.00	−2.33	−70.0	1.78	2.32	+0.54	+30.3
1.8	3.53	0.99	−2.54	−72.0	1.12	1.34	+0.22	+19.6

Total Efflux (f/cs)

	In presence of phosphate (1 mM)				In absence of phosphate			
Na Ca	154	0	Difference (f/cs)	(%)	154	0	Difference (f/cs)	(%)
0	29.71	31.04	+1.33	+4.5	8.73	16.52	+7.79	+88.2
0.9	20.98	13.49	−7.49	−35.7	10.90	20.43	+9.53	+87.4
1.8	23.63	10.33	−12.21	−56.3	7.42	16.58	+9.16	+123.5

[a] The values of efflux were calculated by multiplying the amount of exchangeable calcium in A_1 or A_2 by the corresponding rate constant (k_1 or k_2) and expressed in fmol/cm^2 sec^{-1}, (f/cs), by assuming a mean membrane area of 60 cm^2 per 10 mg of nerve (*see* Jirounek et al., 1984*b*).

phate decreases the efflux, in Na^+-free solution it stimulates it.

The multiexponential analysis showed that phosphate in the presence of Na_o^+ increases the exchangeable calcium pools A_1 and A_2, whereas in Na^+-free solutions it decreased it (with an exception at zero Ca^{2+}). The effluxes measured during the changes in external phosphate concentration (Fig. 7), reflect well these changes in the intracellular sequestration of Ca^{2+} by A_1 and A_2. These results confirm our previous results (Jirounek et al., 1986), obtained by a method which allows continuous monitoring of the radioactivity of the preparation (Jirounek et al., 1983), which show a large increase in the total $^{45}Ca^{2+}$ after addition of phosphate in the presence of Na_o^+, whereas in Na^+-free solution the effect of phosphate was almost completely abolished (Jirounek et al., 1984*a*). A similar effect of phosphate on the $^{45}Ca^{2+}$ incorporation was reported by Borle (1972) in isolated kidney cells and in heart cells by Ponce-Hornos, Langer and Nudd (1982) and by Ponce-Hornos and Langer (1982).

Effect of Metabolic Inhibitors

Figure 9 shows the $^{45}Ca^{2+}$ efflux profiles in four different nerves to which 100 μM of mersalyl was added after 180 min of efflux, each of the nerves being washed in phosphate-free solution during a different time before the application of the drug. Figure 9 shows that the response to mersalyl decreases with the time that the nerve spent in the phosphate-free solution, indicating that it has inhibited the phosphate-dependent Ca^{2+} uptake. Mersalyl, however, is not a specific inhibitor of the mitochondria, since it inhibits also the endoplasmic reticulum Ca^{2+} uptake (Martonosi & Feretos, 1964; Blaustein et al., 1978*b*). In another series of experiments we have therefore measured the $^{45}Ca^{2+}$ efflux in the presence of the mitochondrial uncoupler FCCP. The results showed (Fig. 10) a large release of $^{45}Ca^{2+}$ after application of low concentrations of the drug, indicating that the mitochondrion represents an important intracellular Ca^{2+} sequestering organelle.

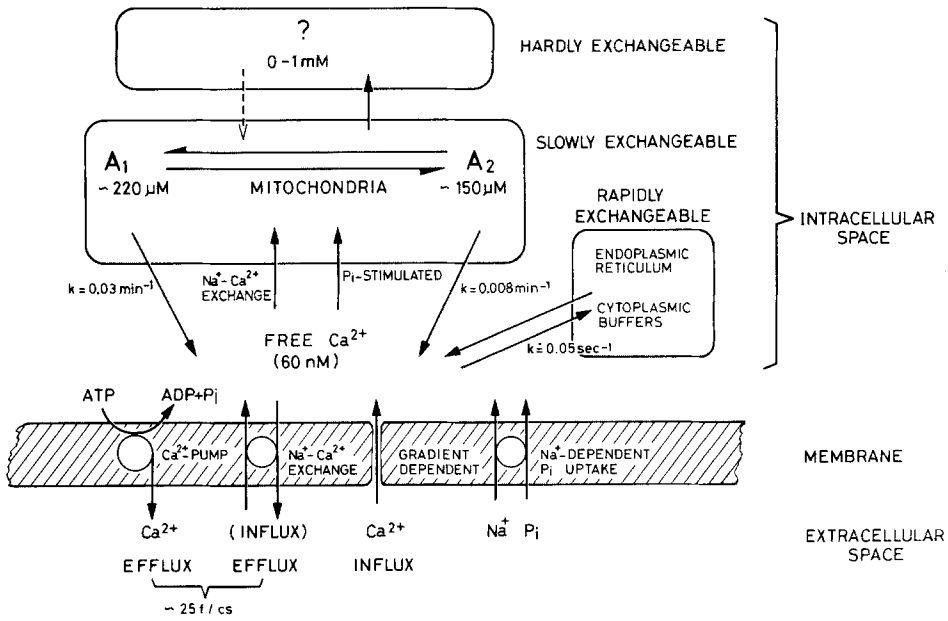

Fig. 12. Schematic diagram of calcium movements in rabbit vagus nerve. The calcium concentration of the free intracellular Ca^{2+} corresponds to the value obtained by Pralong and Straub (1985) in the rabbit vagus nerve and is expressed in nM per kg axoplasm. The rate constant of the rapidly exchangeable pool is an estimation from data reported by Rasgado-Flores and Blaustein (1987). The transfer of calcium from the (presumably) mitochondrial pool into a stable compartment was reported by Brinley et al. (1978) in squid axon and confirmed for rabbit vagus nerve (Jirounek et al., 1986). The amounts of Ca^{2+} in the hardly and slowly exchangeable pools are expressed as concentration per kg w.w.

Effect of Ca_o^{2+}

Increasing external calcium from nominally Ca^{2+} free to 1.8 mM produced a sudden sharp rise in efflux, which was followed by a rapid return to the control levels and then, in some experiments by a slow decrease below the efflux in controls (curve *A* in Fig. 11). The rapid initial calcium flush almost completely disappeared when a high Ca_o^{2+} concentration was added to a nonzero external calcium concentration or when the nerve was previously washed with EGTA or lanthanum. This strongly suggests that this rapid calcium liberation originates from the externally bound calcium. This is confirmed by the efflux *B* in Fig. 11 where the nerve was first washed in 0.9 mM and then in 5 mM of Ca_o^{2+} and where no significant changes in the $^{45}Ca^{2+}$ efflux were observed. It is, however, possible, that an increase of the efflux is masked by a dilution of the internal Ca^{2+} pool by an increased $^{40}Ca^{2+}$ influx.

Lowering Ca_o^{2+} produced a slow decrease in the liberation of the $^{45}Ca^{2+}$, (curve *C* in Fig. 11), which corresponds probably to a real lowering of the calcium outflow, because there the specific activity cannot be diluted by an influx of nonradioactive calcium. However, a dilution by some intracellular calcium stores cannot be excluded.

Identification of A_1 and A_2

The results of the multiexponential analysis of the calcium efflux in steady state shows that the simplest system which is consistent with our data is one in which two kinetically distinct compartments, A_1 and A_2, contribute to the measured efflux (Fig. 12). Surprisingly, both these compartments responded in a similar manner to the different experimental strategies, used in our study. This behavior makes it difficult to attribute these compartments to two different intracellular buffering systems. The modifications in the exchangeable calcium in these two pools in response to changes of external phosphate concentrations, suggest that both A_1 and A_2 represent the exchangeable calcium in the mitochondrion. This hypothesis is in agreement with the results reported by Barritt and Lamant (1982), who show, by kinetic analysis, two different pools of exchangeable Ca^{2+} in heart mitochondria. Similar results were also reported by Studer and Borle (1980) in isolated mitochondria from kidney, and by Barritt (1981) in mitochondria isolated from liver. We can, however, not exclude, that A_1 and A_2 represent two pools of mitochondria differently located, one in the axons and the second, for example, in the Schwann cells.

Although our results suggest that mitochondria are, in rabbit vagus nerve, the predominant Ca^{2+}-sequestring system, the homeostatic role of this organelle in physiological concentrations of free intracellular Ca^{2+} remains somewhat controversial. It has been shown (Blaustein et al., 1980), that in presynaptic nerve terminals, mitochondria take up little Ca^{2+} when the internal ionized calcium is low, and that at physiological Ca_i^{2+} concentrations the smooth endoplasmic reticulum is the most important ATP-dependent Ca^{2+} buffering system. More recently, Rasgado-Flores and Blaustein (1987) reported results which show that the nerve terminal mitochondria do not sequester Ca^{2+} from media with free calcium concentration below about 0.5 μM. It is thus, at first, surprising to find in our preparation, where the internal free calcium was almost probably around 0.1 μM (Pralong et al., 1985; Pralong & Straub, 1985), a large amount of $^{45}Ca^{2+}$ stored in the mitochondria. However, the results reported by Blaustein et al. (1978*a*) and by Rusgado-Flores and Blaustein (1987), were obtained by incubation of brain presynaptic nerve terminals (synaptosomes) in $^{45}Ca^{2+}$-containing medium during a few seconds. When the incubation time is increased to several minutes, Ca^{2+} uptake into mitochondria is readily demonstrable (Blaustein et al., 1978*a*). As in our experiments the incubation time was more than 2 hr and the mitochondrial Ca^{2+} compartment had largely the time to exchange its calcium with the external $^{45}Ca^{2+}$. Also the half-time of labeling and desaturation of A_1 and A_2 which are of about 25 and 90 min, respectively, indicate that these two compartments are different from the endoplasmic reticulum where the half-time of Ca^{2+} sequestration, estimated from the results of Rasgado-Flores and Blaustein (1987), is maximally 30 sec. It seems thus reasonable to conclude that most of the Ca^{2+} from a small load is rapidly sequestered by a compartment unaffected by FCCP, presumably the endoplasmic reticulum (McGraw, Somlyo & Blaustein, 1980), whereas most of the Ca^{2+} from a large load is sequestered by a FCCP-sensitive and slowly exchangeable compartment, presumably the mitochondrion (Fig. 12).

It is interesting to note that the changes in the ionic composition of the external solution affect the intracellular exchangeable pools rather than the corresponding rate constants. This signifies that the fraction of the total intracellular exchangeable calcium, released by the nerve remains almost constant whatever the magnitude of the exchangeable pools. In other words it means that the membrane-associated extrusion systems are able to adapt their transport rates proportionally to the intracellular Ca^{2+} load.

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