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Effects of Calcium and Lanthanum on Phosphate Efflux from Nonmyelinated Nerve Fibers

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Summary. Phosphate efflux was measured as the fractional rate of loss of radioactivity from rabbit vagus loaded with radiophosphate. The effects of changes in extracellular calcium and of lanthanum have been investigated. In Locke solution with normal, 0.9 mM, calcium and without phosphate, the fractional rate of loss was 1.62×10^{-3} min^{-1} at 120 min after the beginning of the washing period and fell slowly (9% hr^{-1}) during washing from 2 to 6 hr. Addition of calcium to the Locke solution produced a transient increase followed by a reversible maintained increase in phosphate efflux. The latter was 40 and 75% above efflux in normal calcium for 20 and 50 mM calcium, respectively. Removal of calcium, with or without addition of EGTA, produced only a transient increase in phosphate efflux, with no subsequent maintained change. Addition of low concentrations of lanthanum produced a reversible inhibition of phosphate efflux. Half-maximal inhibition was at 3.5 μM lanthanum and appeared to be due to binding of lanthanum to more than one, probably two, sites. Measurements of inhibition by lanthanum at different calcium concentrations did not indicate any competition between calcium and lanthanum. It is suggested that at least a part of phosphate efflux depends on internal calcium and that lanthanum acts by preventing release of phosphate from the phosphate transport mechanism.

Key words nerve fibers · membrane · transport · phosphate · calcium · lanthanum

Introduction

Previous studies of the transport of inorganic phosphate across the nerve cell membrane have shown that the influx of phosphate is mediated mainly by a saturable transport process, dependent on the presence of external sodium ions (Anner et al., 1976; Caldwell & Lea, 1978). The efflux of phosphate also shows a dependence on external sodium and phosphate, apparently by a *trans*-stimulation of the transport process, and probably depends as well on the internal phosphate and sodium concentrations (Anner et al., 1976; Maire & Straub, 1980).

The present experiments were carried out in order to investigate whether the phosphate efflux could be affected by calcium ions. In the course of this study, the action of lanthanum ions was also investigated, since lanthanum is known to enhance or inhibit the effects of calcium in many biological systems (Weiss, 1974). As will be shown here, the phosphate efflux is increased on increasing the extracellular calcium concentration. In contrast, lanthanum ions, at low concentrations, inhibit the efflux of phosphate from vagus nerve fibers. However, the inhibition is, surprisingly, independent of the extracellular calcium. A preliminary note on the effect of lanthanum ions has appeared elsewhere (Jirounek, Rouiller & Straub, 1980).

Materials and Methods

The details of the experimental method have been published previously (Anner, Ferrero, Jirounek & Straub, 1975). Briefly, desheathed rabbit vagus nerves were mounted in small polyethylene tubes, through which Locke or modified Locke solutions were perfused at a rate of about 1 ml min^{-1}. The nerves were loaded by perfusion during 2.5 hr with Locke containing trace amounts of radiophosphate and carrier phosphate at a concentration of 0.2 mM. Nonradioactive Locke was then applied and the effluent collected in glass vials and counted by Cerenkov radiation. At the end of the experiment, the preparation was removed and homogenized. The homogenate was mixed with chloroform, centrifuged, and the radioactivity of the water-soluble fraction counted (Ferrero, Jirounek, Rouiller & Straub, 1978). The efflux was then expressed as the fractional rate of loss of the extractable radioactivity per unit time during each collection period.

In the experiments reported here, the efflux was measured using Locke solutions containing zero phosphate, to avoid precipitation of calcium or lanthanum phosphate. For nerves loaded with radiophosphate from Locke containing 0.2 mM phosphate, and subsequently washed with zero phosphate solution, the rate of efflux of radioactivity fell rapidly during the first hour of washing (which time is sufficient to remove activity from the extracellular space and establish a new steady state for the phosphate balance of the cells; Anner et al., 1976). The rate of efflux settled during the next hour to follow fairly closely an exponential curve. When expressed as the fractional rate of loss, the efflux fell slowly during this period of time (*cf.* Ferrero et al., 1978). Experiments on the effect of changes in calcium concentration and on the effect of lanthanum were carried out during these times. In order to compare the efflux in modified Locke solution with the efflux which would

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have occurred without a change, the average rate of decrease of the fractional rates of loss of radioactivity was measured in control experiments. The decrease was found to be 9.0 ($\pm$1.2, n=13) percent per hour. The percent decrease was not significantly different for solutions which contained normal, 0.9 mM calcium, reduced calcium, with or without EGTA, or increased calcium.

Locke solution contained (in mM): NaCl, 154; KCl, 5.6; $CaCl_2$, 0.9; $MgCl_2$, 0.5; glucose, 5; TRIS, 10. For the radioactive solution, phosphate was added as the mono- and di-sodium salts, 0.2 mM, together with 2 μCi ml^{-1} of carrier-free ^{32}P phosphoric acid (NEN). Ca^{2+}-free solutions were prepared by omitting $CaCl_2$. Ca^{2+}-free-EGTA solutions were prepared by omitting $CaCl_2$, with the addition of EGTA, 1 mM. Solutions with high calcium were prepared by adding solid $CaCl_2$ to Locke solution. Lanthanum was added as the chloride salt.

All experiments were carried out at 37 °C and pH 7.4. When appropriate, results are expressed as mean values $\pm$ standard error.

Results

The Effect of Changes in External Calcium Ion on the Phosphate Efflux

A reduction in the calcium concentration of the Locke solution from the normal 0.9 mM by omission of calcium (Ca^{2+}-free solution) did not produce a significant maintained change in the phosphate efflux from vagus fibers, though usually there was an initial transient increase. The use of EGTA to further reduce the calcium concentration (Ca^{2+}-free-EGTA solution) was also without a maintained effect. Figure 1*A* shows a typical initial transient increase. On the other hand, an increase of the external calcium concentration increased the phosphate efflux, though a large addition was needed to produce this effect. Fig. 1*B* shows the effect of an increase from 0.9 to 20 mM calcium. There was an initial transient rise in the efflux, followed by a fall to a maintained fractional rate of loss of radioactivity, which was about 40% above the normal level. A subsequent return to Locke solution without calcium addition produced a return to the normal level within a short time interval. A larger increase was found when the calcium concentration was increased to 50 mM. The subsequent maintained level was somewhat variable, but, on average, about 75% above the efflux in normal Locke solution.

A part of the effect of increased external Ca might be due to an increase in the osmolarity of the Locke solutions. Indeed, addition of 20 or 50 mM $CaCl_2$ increased the osmolarity by 16 and 40%, respectively (measured by freezing point depression). To test for this possibility, in a separate series of experiments the phosphate efflux was measured during superfusion with Locke solutions of increased osmolarity produced by addition of appropriate amounts of sucrose or mannitol. The increases in efflux after 1 hr in solution with increased osmolarity equivalent to addition of 20 or 50 mM $CaCl_2$ were 11% ($\pm$4%, n=4) and 36% ($\pm$9%, n=4), respectively. Addition of lithium or choline chloride to the Locke solution produced either a much smaller rise (lithium) or a rise two times higher (choline) than for equivalent additions

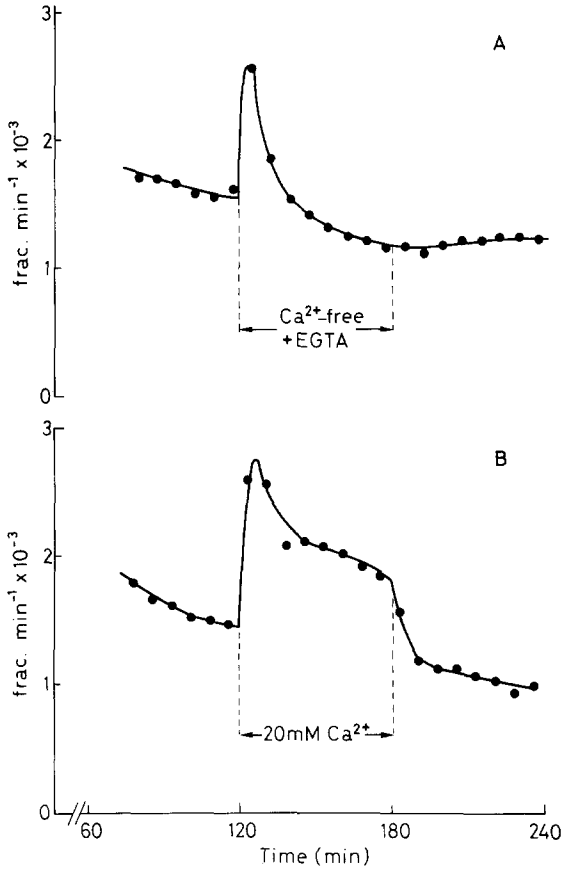

Fig. 1. The effect of changes in external calcium concentration on the phosphate efflux from vagus nerve fibers. (*A*): The Locke solution was changed during the period indicated to Locke solution without calcium and with 1 mM EGTA added. (*B*): The Locke solution was changed to Locke solution containing 20 mM calcium, by addition

of sucrose or mannitol. The reduced effect of lithium chloride addition can be explained by inhibition of phosphate efflux by the lithium ion (Ritchie & Straub, 1980*b*), whereas the increased stimulation by choline can be attributed to stimulation by choline, as previously shown for this and other cholinomimetics (Straub et al., 1978). In four experiments the solutions were then changed to 20 or 50 mM Ca Locke solutions to test the effect of increased external Ca without a change in osmolarity. The relative stimulation of phosphate efflux by Ca in these experiments after sucrose or mannitol addition was almost the same as reported in the rest of this article for 50 mM Ca (average 65%, n=2) or even higher for 20 mM Ca (average 56%, n=2). A marked stimulation was also found on introduction of increased Ca solutions after solutions containing added lithium or choline chloride.

Phosphate Efflux during Washing with Solutions with Modified Calcium

In some experiments, the calcium concentration was modified at the beginning of the washing period, and the phosphate efflux followed for periods from 2 to 6 hr. When calcium was omitted from the washing solution, with or without the addition of EGTA, the mean fractional rates of loss were similar to those

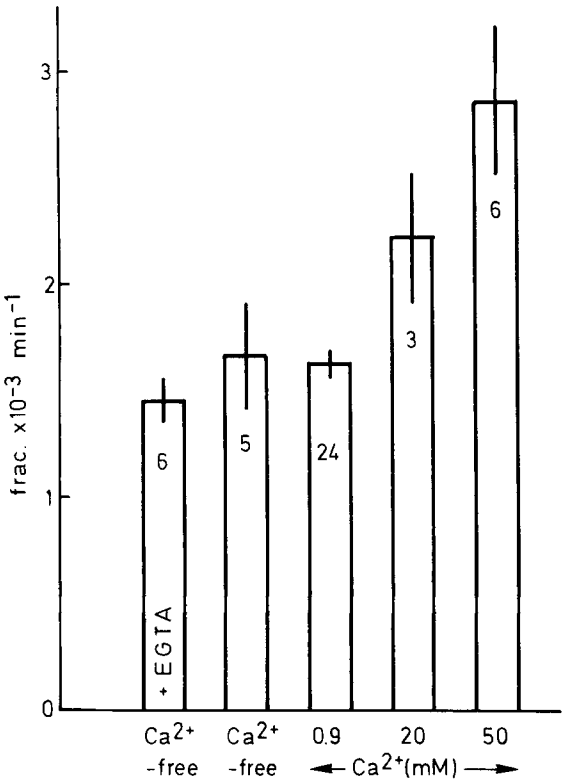

Fig. 2. The fractional rate of loss of radiophosphate at different external calcium concentrations. The columns indicate the mean values ±SE and the number of experiments. The fractional rates of loss were either measured at 120 min after the beginning of the washing or measured after a change in solution and then corrected back to 120 min (*see* text). *Ca^{2+}-free* indicates Locke solution from which calcium was omitted. *Ca^{2+}-free-EGTA* indicates Locke solution without calcium to which EGTA, 1 mM, was added

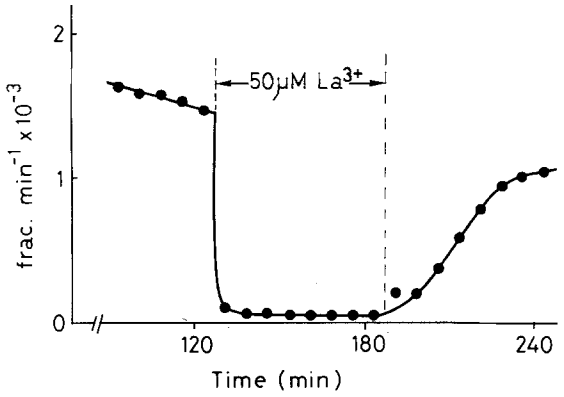

Fig. 3. Inhibition of the phosphate efflux from vagus nerve fibers by lanthanum. Each point shows the fractional loss of radiophosphate at different times during washing with Locke solution without phosphate, which was changed during the time indicated to a Locke solution containing 50 μM lanthanum

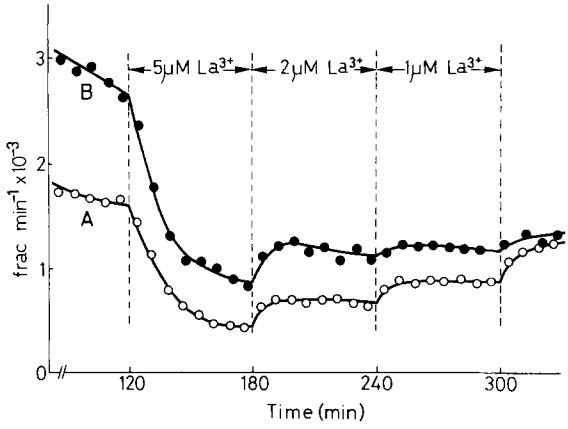

Fig. 4. Partial inhibition of the phosphate efflux by lanthanum. The fractional loss of radioactivity was measured during washing with Locke solutions without phosphate and, during the times indicated, different low concentrations of lanthanum. For experiment *A*, the wash solutions contained normal (0.9 mM) calcium. For experiment *B*, the wash solutions contained 50 mM calcium (by addition). The initial rapid decrease in *B* reflects the end of the transient effect on increase in calcium (the 50 mM calcium solution was introduced 20 min after the beginning of the washing period)

found at corresponding times for normal Locke solution, confirming the observation that removal of external calcium produces only a transient change in phosphate efflux. The phosphate efflux was also measured with Locke solutions containing 50 mM calcium. In this case, the efflux was higher than in normal Locke (*see*, for example, Fig. 4), but was not significantly different from the maintained increase in efflux, at corresponding times, after a change to 50 mM calcium during the washing period.

Figure 2 shows mean values of the fractional rates of loss of phosphate at different calcium concentrations, measured either at 120 min after the beginning of the washing period or measured after a change in calcium concentration and then corrected back to 120 min, using the known mean decrease in fractional rates of loss during washing (*see* Materials and Methods).

Inhibition of Phosphate Efflux by Lanthanum

The effect of the addition of lanthanum ions to the washing solution on the efflux of phosphate from vagus nerve fibers is shown in Fig. 3. In this experiment, the Locke solution superfusing the nerve was changed during a period of 1 hr to a Locke solution containing 50 μM $LaCl_3$. The efflux of radioactivity rapidly fell to a low level, which remained constant during washing in the presence of lanthanum. On removal of lanthanum, the efflux returned slowly to the normal level. At lower concentrations, a partial inhibition was found. Figure 4, experiment *A* shows the result of an experiment in which three different concentrations of lanthanum during successive periods of 1 hr were applied. It is clear from this figure that low, micromolar, concentrations significantly inhibit the phosphate efflux.

The inhibition of the phosphate efflux at different lanthanum concentrations was measured by comparing the fractional rate of loss of radioactivity after 1 hr in the lanthanum containing solutions to the fractional rate of loss before application of lanthanum. The percent inhibition was calculated and then corrected for the slow fall in the fractional rates of loss found in control experiments (*see* Materials and Methods). The average percent inhibition at different lanthanum concentrations is shown as the solid line of Fig. 5*A*. The concentration for half-maximal effect was 3.5 μM.

Inhibition of the Phosphate Efflux by Lanthanum at Different Calcium Concentrations

To investigate whether there exists an interdependence of the effect of changes in calcium and the inhibition by lanthanum, experiments were carried out during which the efflux was measured in either Ca^{2+}-free or 50 mM calcium solutions containing different concentrations of lanthanum (the inhibition could not be measured in Ca^{2+}-free-EGTA solution, since lanthanum, as well as calcium, is complexed by EGTA). The results of a typical experiment are shown in Fig. 4, experiment B. This curve shows the fractional rate of loss of radioactivity, at different concentrations of lanthanum in Locke solution containing 50 mM calcium. As for the experiments with normal calcium (*cf.* experiment *A* of the same figure), at these concentrations of lanthanum, a partial inhibition of the phosphate efflux was obtained.

Measurements were made of the fractional inhibition at different lanthanum concentrations, as described previously. That is, the fractional rate of loss after 1 hr in the lanthanum containing solutions was compared to the fractional rate of loss before lanthanum, and the result then corrected for the slow fall of the fractional rates of loss found in control experiments. In addition, in a number of experiments, the calcium concentration was changed *after* the application of lanthanum. The subsequent fractional rate of loss, which increased when the calcium concentration was increased to 50 mM, was then calculated as a percentage of the fractional rate of loss for controls in the absence of lanthanum. This change in procedure did not produce significantly different results for the percentage inhibition, as was expected from the reversibility of both the calcium and lanthanum effects (*see* above). Mean values for the percent inhibition at different lanthanum concentrations in Ca^{2+}-free or 50 mM calcium solutions are shown in Fig. 5*A*. It is clear from this figure that the inhibition of the phosphate efflux by lanthanum is independent of the external calcium concentration.

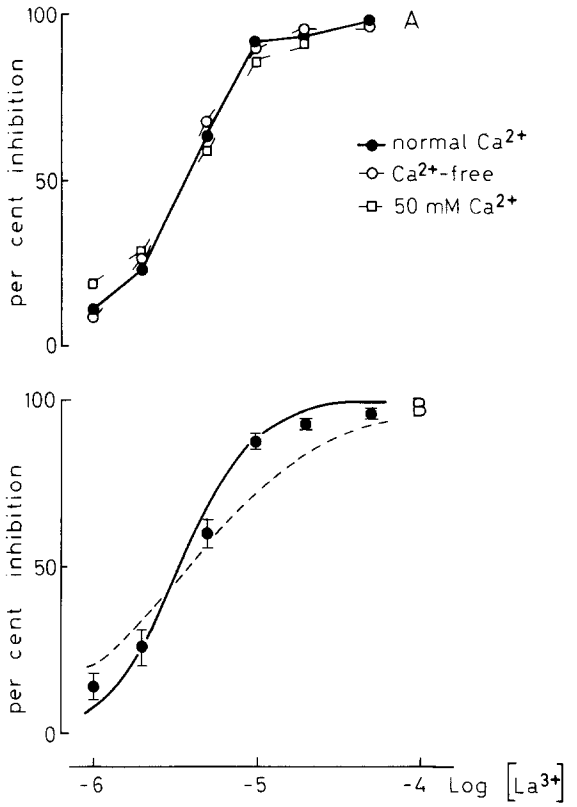

Fig. 5. (*A*): The inhibition of the phosphate efflux as a function of the external lanthanum concentration, measured at different calcium concentrations. The filled circles (solid line) show the percent inhibition in normal Locke solution (0.9 mM Ca^{2+}). The percent inhibition was also measured in Ca^{2+}-free solution (open squares) and in Locke solutions which contained 50 mM calcium by addition (open circles). Each point represents the mean value of 3–7 separate experiments, except for the highest lanthanum concentration, where the points represent a single experiment and the mean of two experiments. (*B*): Lanthanum inhibition of phosphate efflux. Combined data from *A* showing mean values (±SE). Each point is the mean of >9 experiments or of 3 experiments (50 μM La^{3+}). The curves are fits of these points to a function of the form $C^n/(C^n+K)$ where C is the lanthanum concentration, K is a constant, and $n=1$ (dashed curve) or $n=2$ (solid curve)

The inhibition of the phosphate efflux as a function of the external lanthanum concentration, as shown in Fig. 5*A*, does not correspond to a simple binding scheme. In Fig. 5*B*, the results of Fig. 5*A* have been combined and plotted with error bars corresponding to the standard errors of the means. Also shown are fits of these mean values to a function of the form $C^n/(C^n+\mathrm{K})$, where C is the lanthanum concentration, and K is a constant. Inhibition of phosphate efflux according to this formula would correspond to lanthanum binding to n independent sites. The curves for $n=1$ and $n=2$ are shown. The data fit considerably better the curve for $n=2$. A Hill plot of the data (not shown) also indicated an exponent value for the lanthanum concentration much closer to 2 than to 1.

Discussion

The experiments on the efflux of phosphate from vagus nerve fibers described in this paper were carried out using Locke solutions without phosphate ions. This condition is necessary for studies using increased calcium concentrations, or with lanthanum addition, in order to avoid precipitation of calcium or lanthanum phosphate salts. The absence of external phosphate, however, has the additional advantage of eliminating the possibility that observed changes in phosphate efflux are due to a trans-effect on the phosphate transport mechanism (*cf.* Ferrero et al., 1978). We have found that a maintained increase in phosphate efflux is produced by an increase in external calcium. The increases in external calcium in these experiments were made by adding $CaCl_2$ to the Locke solutions, which increased the osmolarity. Since the phosphate efflux is thought to be dependent on internal phosphate and sodium concentrations (Anner et al., 1976; Maire & Straub, 1980), the increase in efflux might have been due to increased internal concentrations of these ions, consequent on shrinkage of the nerve fibers. However, control experiments using addition of sucrose or mannitol, or addition of lithium or choline chloride, showed that only a fraction of the stimulation by Ca can be explained in this way.

The effect of increased external calcium is small, considering the large changes in external calcium that are required for its detection. On the other hand, removal of external calcium has no maintained effect on the phosphate efflux. No direct evidence is at present available, but one way to explain these results would be to postulate an involvement of internal calcium in the mechanism of phosphate efflux, since changes in the internal free calcium can be expected to be small, if not zero, on removing external calcium, and to be an increase on increasing external calcium. Though little is known about the state of the internal calcium in vagus nerve fibers, it appears that a large fraction is tightly bound (Kalix, 1971), and the ionized calcium is probably very well buffered, as is the case for squid axons (Baker & Schlaepfer, 1978). External calcium probably acts on internal calcium, as has been demonstrated for squid axons, where up to half the calcium efflux may depend on external calcium (Baker, 1976; *but see* Baker & McNaughton, 1978). Further, using aequorin in squid axons, it has been shown directly that the internal free calcium can be increased by an increase in the external calcium (Baker, Hodgkin & Ridgway, 1971; Requena, DiPolo, Brinley & Mullins, 1977). The response was small, however; only a two- or threefold increase in light response was found for tenfold changes in external calcium (Baker et al., 1971; Requena et al., 1977).

If the maintained increase in phosphate efflux is caused by an increase in internal free calcium, the question remains open as to whether the response is due to a direct stimulation of the phosphate transport, or to an indirect mechanism. Again, a simple possibility is that calcium can replace sodium in the sodium-dependent phosphate transport system that we have previously described for vagus nerve (Anner et al., 1976; Ferrero et al., 1978). Recent, preliminary experiments in this laboratory have shown that, with low external sodium, phosphate *influx* can be stimulated by addition of calcium, an effect which does not occur in normal sodium. This observation is consistent with the idea that calcium can replace sodium for phosphate transport. It seems unlikely that the effect of calcium on the phosphate efflux is mediated by changes in internal sodium, since an increase in external calcium is expected to produce a decrease in internal sodium through sodium-calcium exchange, and thus a decrease in phosphate efflux.

One effect of a calcium load which could explain the increase in phosphate efflux, would be a change in metabolic activity of the nerve, resulting in an increase in internal phosphate levels. Indeed, an increase or decrease of calcium does produce small changes in metabolism, as measured by the oxygen consumption (Ritchie & Straub, 1980*a*); these, however, are in the wrong direction to explain the results reported here. A tenfold increase in calcium, for example, produces a fall in oxygen consumption, which indicates more likely a fall in internal phosphate, rather than an increase (*see* Ritchie & Straub, 1980*a*).

It was hoped, at the beginning of the work reported here, that the use of lanthanum, acting as a calcium substitute or antagonist, would help to elucidate some of the details of the mechanism whereby external calcium can stimulate phosphate efflux from vagus nerve fibers. It is known, for example, that lanthanum inhibits the calcium transport system of red blood cells (Sarkadi, Szász, Gerlóczy & Gárdos, 1977) and of squid axons (van Breemen & DeWeer, 1970). It has been found, however, that external lanthanum, at micromolar concentrations, has a specific inhibitory action on the phosphate efflux, which is independent of the external calcium concentration. Thus, any effect of lanthanum on the changes due to calcium cannot be detected due to the direct and much more sensitive inhibition. The sensitivity of the phosphate efflux to lanthanum may be related to one of the actions of lanthanum on the red cell calcium pump. It has been shown that application of lanthanum to the active site of this (Ca+Mg)ATPase prevents the release of phosphate subsequent to the hydrolysis of ATP (Schatzman & Bürgin, 1978; Szász, Hasitz, Sarkadi & Gárdos, 1978). It is possible that

a similar process is operating here; that lanthanum is acting by preventing the release of phosphate from the carrier of the phosphate transport mechanism of vagus nerve fibers, blocking the carrier at the external surface of the cell membrane, and thus providing a very efficient, and so far unique, method of inhibiting the phosphate release.

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