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Role of Potassium in the Phosphate Efflux from Mammalian Nerve Fibers

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Summary. The efflux of phosphate was measured in rabbit vagus nerve loaded with radiophosphate. The efflux was found to depend on the K concentration of the bathing solutions; increasing the K from 5.6 up to 150 mM produced a maximal lowering of 28%; K-free solution produced a transient increase whose peak was 86% above the normal efflux. In the presence of Na, the K-free effect could be repeated; in Na-free solution, it was found only for the first application of the K-free solution. The phosphate efflux was not altered when K was replaced by Rb; replacement with Cs showed that this ion only partially mimics the effect of K.

The results suggest that the transient increase in phosphate efflux is due to release of label from a K-dependent saturable binding site, which is distinct from the main intracellular pool. The binding site appears to be labeled from the inside by the Na-dependent phosphate efflux previously described. It may correspond to the phosphorylation of membrane phospholipids. A mathematical model of this system is developed and curves simulated by an analog computer are compared to the experimental results.

Measurements of the membrane potential and the internal inorganic phosphate showed that the effect of K on the phosphate efflux could not be explained by changes in the membrane potential or in the internal phosphate pool.

We have shown previously that a large proportion of the transmembrane fluxes of inorganic phosphate in nonmyelinated nerve fibers is mediated by a saturable Na-dependent mechanism (Anner *et al.*, 1976; Ferrero *et al.*, 1978). This mechanism is different from the phosphate transport in other tissues, e.g., erythrocytes (Rothstein, Cabantchick & Knauf, 1976), mitochondria (Banerjee *et al.*, 1977) or *Escherichia coli* (Rosenberg, Gerdes & Harold, 1979).

In the course of further investigations on the phosphate efflux, a transient release of phosphate was observed when the potassium of the incubation solution was withdrawn. Conversely, when the potassium was increased, the efflux of phosphate was diminished. In the present study, we have analyzed these phenomena in more detail. Our results suggest that the phosphate released in the absence of K originates from a specific K-dependent phosphate binding site.

Materials and Methods

Desheathed rabbit vagus nerves were mounted in a polyethylene tube which was perfused with ^{32}P-phosphate Locke for 150 min at 37°. The preparation then was washed with inactive Locke solution, and the effluent was collected and counted. At the end of the experiment the preparation was homogenized in 0.1 M triethanolamine buffer (pH 8). The homogenate was mixed with chloroform, centrifuged at $3{,}000 \times g$ for 15 min and the activity of the water-soluble fraction was then counted. These counts were used for the calculation of the efflux rate constant. The composition of the Locke was (mM): 154 NaCl, 5.6 KCl, 0.9 $CaCl_2$, 0.5 $MgCl_2$, 5 glucose, 0.2 Na_2HPO_4-NaH_2PO_4, 1 Tris. Potassium isethionate (Eastman-Kodak) was added to prepare K-rich Locke. In Na-free solutions, sodium was substituted by equimolar concentration of Tris. For the preparation of the labeled solution, 2 μCi/ml of ^{32}P carrier free phosphoric acid (NEN) was added. All solutions were adjusted to pH 7.4.

In some experiments, the membrane potential was measured by the sucrose-gap method (Jirounek & Straub, 1971).

Results

Experiments with Na-Locke

During the first two hours of washing the preloaded preparation with nonradioactive Locke, the rate constant of the phosphate efflux decreased and then settled to a nearly steady value. After this initial period the effects of modifying the washing solutions could easily be studied.

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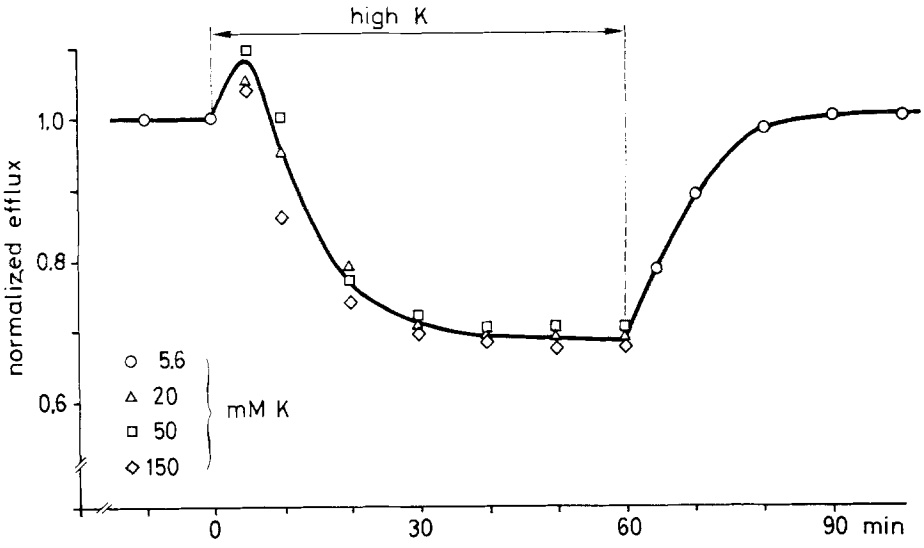

Fig. 1. Inhibition of phosphate efflux by high K (20 mM, △, 50 mM, □, and 150 mM, ◇). Results of different experiments were normalized to efflux rate in normal K (5.6 mM, ○). The K-rich solutions were applied after a period of equilibration of at least 100 min in normal nonradioactive Locke. Abscissa: time after change to K-rich concentration; temperature, 37 °C

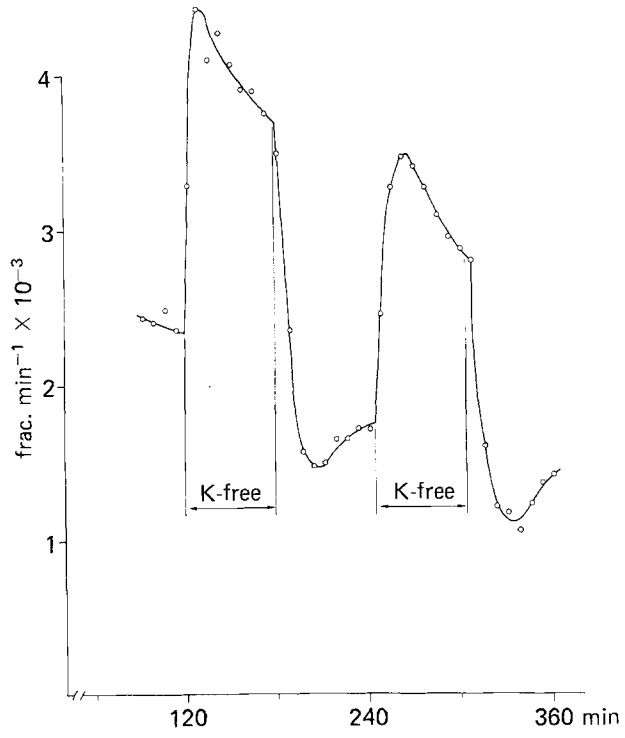

Fig. 2. Changes of phosphate efflux produced by K withdrawal. Effect of K-free solution can be repeated after a period of recovery (65 min in this case) in normal Locke. Abscissa: time after beginning of washing. Phosphate concentration, 0.2 mM; temperature, 37 °C

Effect of raising the K concentration. Figure 1 shows an experiment where the potassium concentration was raised from 5.6 to 20, 50 and 150 mM K. Efflux gradually decreased over a 30-min period to a new steady level. The maximal effect is already obtained with 20 mM K. After returning to Locke with 5.6 mM K, the efflux rapidly recovered to its normal value.

The mean decrease of the efflux in high K^+ concentrations was 28% of the normal efflux in 5.6 mM K ($n=14$).

Effect of K-free solution. Withdrawal of potassium from the washing solution produced a rapid increase in phosphate efflux (Fig. 2). The peak increase amounted to 85.7 ± 5.9 ($n=22$) of the efflux in Locke and the time to the peak was 13.4 ± 1.6 min ($n=22$). The increased efflux was followed by a slow decrease towards the initial value. When the normal potassium concentration was re-established the rate of phosphate efflux rapidly decreased below the normal value and then slowly recovered.

In some experiments the effluent collected during the potassium withdrawal was analyzed by column chromatography. More than 95% of the radioactivity was found in the inorganic phosphate fraction (maximal sensitivity of the method, Ferrero *et al.*, 1978).

The K-free effect on phosphate efflux was prevented when the KCl of the Locke was replaced by an equimolar amount of RbCl. This ion therefore seems to affect the phosphate efflux in a similar way as does potassium. On the other hand, Cs^+ ion replaced the K^+ ion only partially.

Effect of repeated application of K-free solution. A second application of K-free solution produced an effect similar to that of the first application (Fig. 2). However, the amplitude of the second effect was found to depend on the time of recovery in the presence of 5.6 mM K. Recovery during 50 min in 5.6 mM K was sufficient for the restoration of the full K-free effect.

The results described so far suggest the presence of a phosphate compartment distinct from the main

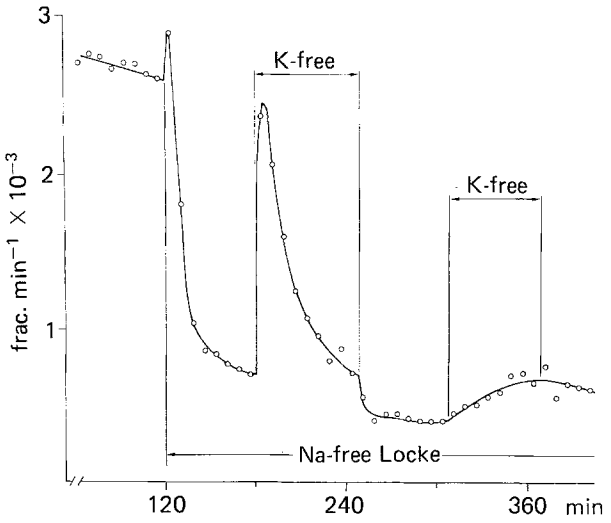

Fig. 3. In Na-free Locke (Na replaced by Tris), the phosphate efflux rapidly decreases. If then K is omitted from the solution, an increase in phosphate efflux is observed. A second withdrawal of K after 60 min recuperation, however, fails to produce the same effect. Abscissa: time after beginning of washing. Phosphate concentration, 0.2 mM; temperature, 37 °C

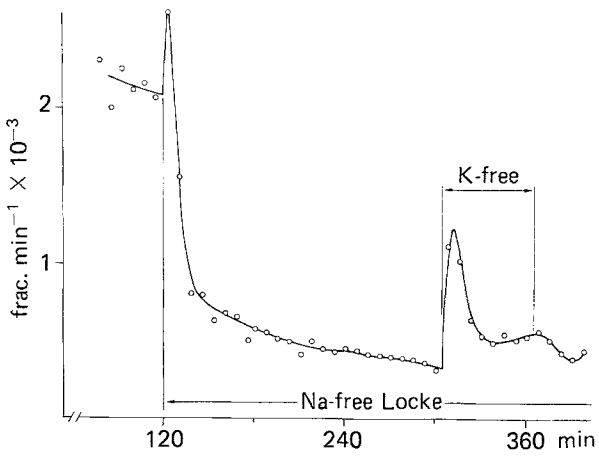

Fig. 4. After a long period of washing in Na-free solution, the K-free effect is still present, but the amount of liberated phosphate is significantly reduced. Abscissa: time after beginning of washing. Phosphate concentration, 0.2 mM; temperature, 37 °C

pool. The phosphate retention of this compartment appears to depend on the presence of K. If the potassium concentration of the solution is reduced, the content of the compartment is liberated, and an increase in efflux of radiophosphate is seen. The finding that the effect of K-free solution can be fully repeated after a short period of recuperation in normal nonradioactive Locke suggests that the compartment is filled with radiophosphate from the inside of the axon.

Measurements of intracellular phosphate. In order to test whether extracellular K alters the intracellular phosphate content, the water-soluble phosphates of nerves were extracted and separated by column chromatography (Ferrero *et al.*, 1978). For these experiments nerves were loaded with ^{32}P, washed with Locke for 120 min and then for 30 min with either Locke or K-free or K-rich Locke, and finally analyzed. The sum of the Pi + CrP content was $47.8 + 4.5$ ($n = 4$), 46.7 ± 7.4 ($n = 3$) and 46.6 ± 2.6 ($n = 3$) %, respectively, of the total intracellular water-soluble phosphates.

Experiments in Na-Free Locke

If the K-dependent phosphate compartment is filled by the Na-dependent phosphate efflux mechanism, it should be possible to modify the content of the compartment by modifying the rate of basic efflux from the axons. We have shown previously that the efflux of phosphate is considerably reduced when preparations are exposed to Na-free solution (Ferrero *et al.*, 1978). In the next series of experiments, the effects of potassium were studied in Na-free solution.

Effect of raising the K-concentration. In contrast to the results obtained in the presence of Na, raising the potassium in Na-free solution did not decrease the phosphate efflux. There was either no change in efflux or, in some experiments, a small increase.

Effect of K-free solution. Figures 3 and 4 show results obtained in experiments where the effect of K^+ withdrawal was tested. In these experiments, the efflux was first measured in normal Locke. The Na^+ ions of the Locke were then replaced by Tris and, as previously described (Ferrero *et al.*, 1978), a rapid decrease in the phosphate efflux was seen. In the experiment of Fig. 3, K-free-Tris-Locke was applied when the efflux approached a new steady value. Application of this solution produced a rapid transient increase in phosphate liberation. The amount of phosphate liberated during 60 min was comparable to the response in the presence of Na, but the kinetic parameters of the time course of the phosphate efflux were modified. The mean time to peak, 5.17 ± 0.44 min, was shorter than in Na-Locke and the following decrease was significantly faster.

After a prolonged exposure of the preparation to Na-free Locke (Fig. 4), the amount of liberated phosphate in K-free medium decreased.

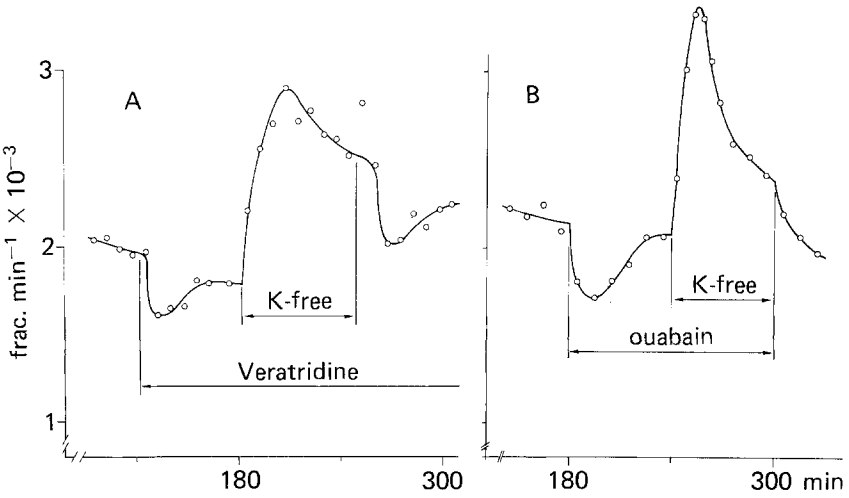

Fig. 5. (*A*) Veratridine (10^{-5} mg/ml) and (*B*) Ouabain (0.1 mg/ml) produce a similar transient decrease of phosphate efflux. These drugs do not significantly modify the K-free effect. Abscissa: time after beginning of washing. Phosphate concentration, 0.2 mM; temperature, 37 °C

Effect of repeated application of K-free solution. The effect of a second application of K-free solution in Na-free Locke was different from that seen in the presence of sodium. In Na-free solution, a second application of K-free failed to produce a rapid transient increase in phosphate efflux, even when the preparation had recovered during the 60 min in the presence of potassium (Fig. 3). At best a small increase in phosphate liberation was then observed.

The finding that the phosphate efflux does not increase after a second application of K-free solution is in agreement with the hypothesis that the K-dependent phosphate pool is charged by a Na-dependent mechanism. If the affinity of this compartment to the phosphate is sufficiently high, it will retain phosphate even in a very low rate of "input". Therefore when its affinity to phosphate is reduced by the first withdrawal of potassium, its content is liberated and an increase of efflux is observed. Then, after the re-introduction of normal potassium concentration in Na-free medium, the loading of this compartment by the Na-dependent mechanism is practically abolished and the next withdrawal of potassium liberates only a small amount of phosphate.

The results presented in Fig. 4 show that the absence of increase in phosphate efflux during the withdrawal of potassium depends on a previous emptying of the K-dependent phosphate pool, and not simply on the time of incubation in Na-free Locke.

Phosphate efflux during application of veratridine. In order to test whether the effects of varying the potassium concentrations on the phosphate efflux could be due to alterations in the membrane potential, preparations were exposed to veratridine, 10^{-5} mg/ml, which is known to decrease the resting membrane potential (Straub, 1956). In 5 experiments application of veratridine for 60 to 180 min produced only a transient decrease of phosphate efflux, which amounted to 17% of the efflux during the control period.

The effect of K withdrawal on the poisoned nerve was not significantly affected by the drug (Fig. 5*A*).

Phosphate efflux during the application of ouabain. When ouabain was applied at a concentration of 0.01 mg/ml (13.7 μM) the phosphate efflux did not show any measurable alteration, although at this concentration ouabain leads to a 90% loss in the potassium content and a corresponding accumulation of sodium (Wespi, 1969). These changes in the potassium and sodium content therefore do not seem to affect the phosphate efflux. At higher concentration (Fig. 5*B*) ouabain produced a decrease in phosphate efflux which subsequently recovered towards the normal efflux. Further, the effect of a subsequent withdrawal of potassium was not significantly modified by this pretreatment with ouabain.

Discussion

In order to understand the effects of potassium on the phosphate efflux, the different physiological actions of this cation, as well as the kinetic aspect of the phosphate efflux changes, are discussed.

A. Metabolic Effects of Potassium

Lack of extracellular potassium inhibits the activity of the Na-K-ATPase and presumably the turnover of the intracellular ATP. It might thus displace the intracellular ATP/P_i equilibrium. However, the determination of the intracellular P_i content shows that

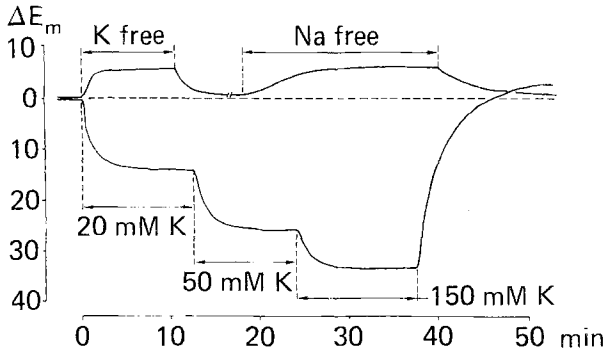

Fig. 6. Deviations from the resting membrane potential measured by the sucrose-gap method on three different nerves (high K concentration, K-free, Na-free solutions). Curves were traced from the original pen recordings; they are not corrected for the short circuiting. To obtain the real changes of the membrane potential, the values of the ordinate must be divided by the appropriate short-circuiting factor. In this case, its value is 0.86 (Jirounek, 1978); temperature, 37 °C

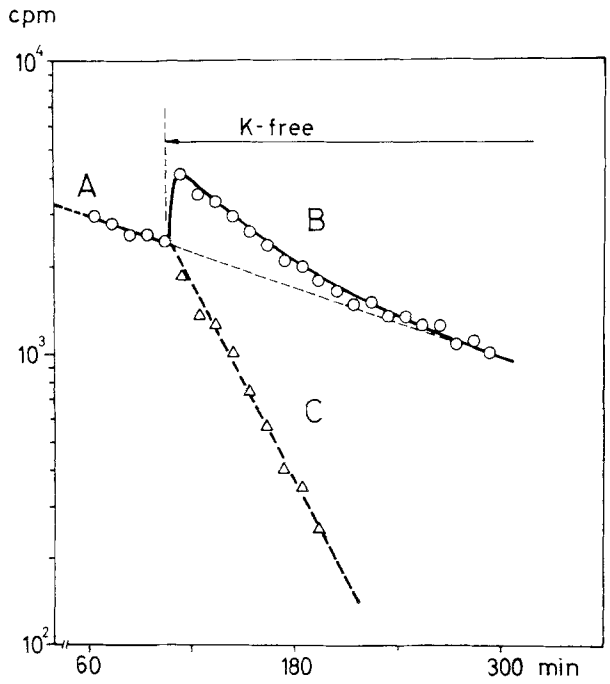

Fig. 7. Radioactivity of ^{32}P phosphate, in cpm, found in efflux (○) is plotted in semi-logarithmic scale. Section *A* of the curve and its prolongation (thin dotted line) correspond to the efflux in normal Locke. By substracting *A* from curve *B*, obtained after application of K-free solution, we get the straight line *C* (△). It suggests that the extra efflux of phosphate in K-free solution originates in a pool distinct from the main phosphate compartment. The rate constants calculated from the slope of *A* and *C* are 4.95×10^{-3} min^{-1} and 2.66×10^{-2} min^{-1}, respectively. The rate constant of the efflux in normal Locke obtained by this method is somewhat greater than the fraction *B* calculated in our experiments by dividing the efflux by the activity of the water-soluble extract of the nerve. This difference indicates that some of the phosphate of the water-soluble extract does not contribute to the efflux. Moreover, the higher external phosphate concentration in this experiment (0.6 mM instead of 0.2 mM) contributes to an increased efflux (Straub *et al.*, 1977); temperature, 37 °C

the extracellular K does not modify the intracellular phosphate. Moreover, recent results of Chmouliovsky *et al.* (1979) have shown that neither K-rich nor K-free solutions significantly alter the intracellular ATP. Thus, the observed changes of phosphate efflux do not seem to be attributable to a K-induced modification of the intracellular phosphate.

Another factor that might influence the phosphate efflux is the change in intracellular Na. Indeed, as a result of the inhibition of the Na-K-ATPase in K-free solution, the intracellular potassium content of the rabbit vagus nerve (Wespi, 1969) falls in 45 min to approximately half its initial value, and this fall is accompanied by a concomitant increase in sodium. However, the intracellular changes of potassium and sodium as measured by Wespi (1969) are too slow to explain the rapid rise of phosphate efflux after the withdrawal of extracellular potassium. Moreover, in the presence of ouabain (0.1 mg/ml), which produces a more pronounced increase in intracellular sodium, the phosphate efflux does not increase but transiently decreases. This, and the fact that after 60 min of incubation in ouabain the effect of K-free solution on the phosphate efflux is maintained (Fig. 5*B*) exclude the possibility that K-withdrawal acts on the phosphate efflux by increasing the intracellular sodium.

B. The Effect of Membrane Potential

Another effect of potassium ion in nerve is its action on the membrane potential (Fig. 6). Thus the hyperpolarization produced by the K-free solution would tend to increase the efflux of the negatively-charged phosphate ions and an opposite effect is expected from the depolarization in K-rich solution. Qualitatively, the effects of K on the phosphate fluxes could thus be explained by these changes in the membrane potential. Quantitatively, the effect of the membrane potential on the efflux of phosphate can be estimated by the following formula:

$$J_i = C_i \cdot \text{Permeability} \cdot \frac{zE_mF/RT}{1-\exp(-zE_mF/RT)}$$

where J_i is the efflux of the anion, C_i its intracellular concentration and z, E_m, F, R, T have their usual meanings. Assuming that C_i and the permeability do not change, the effect of a change in membrane potential can easily be calculated. With a hyperpolarization of 10 mV (Fig. 6) the efflux of the monovalent phos-

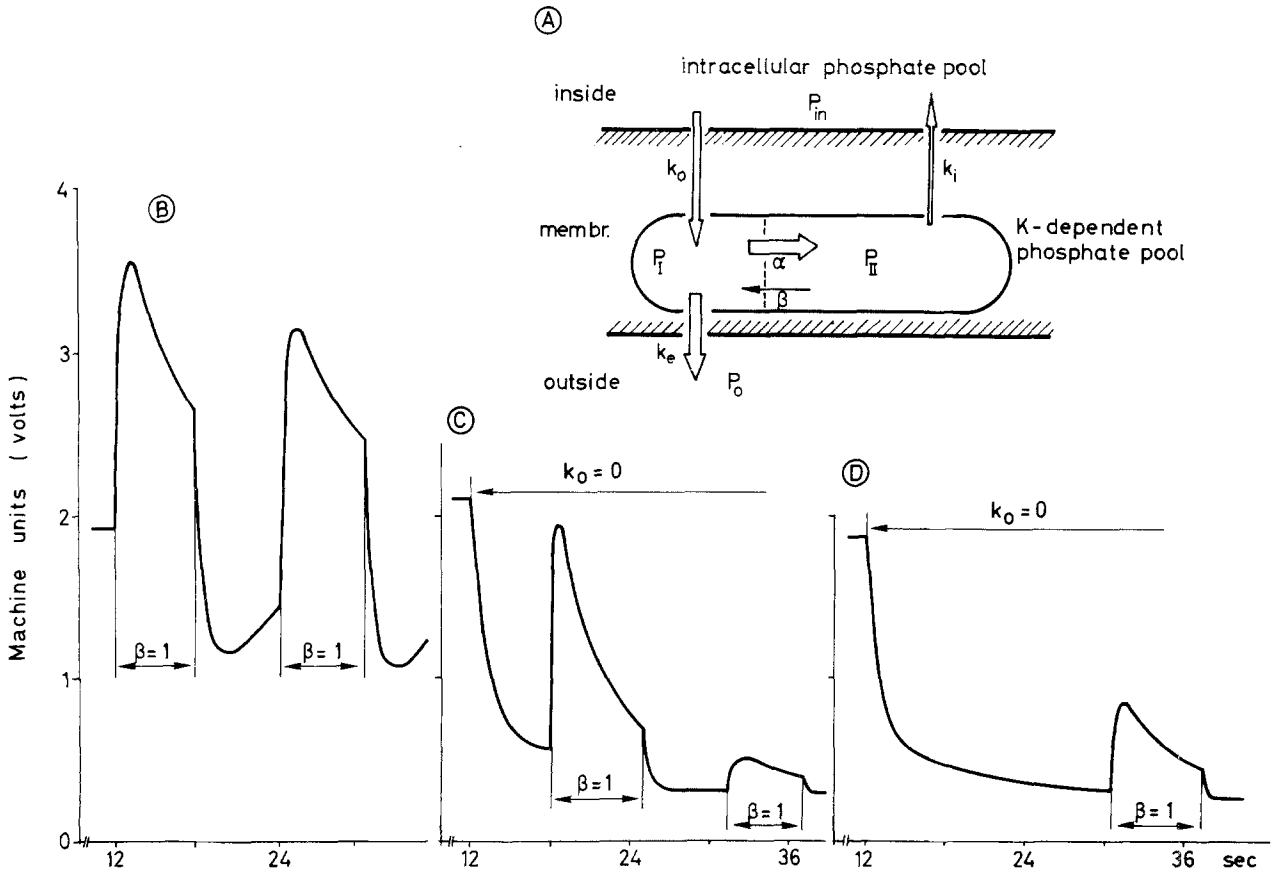

Fig. 8. Hypothetical model for the effect of K on phosphate efflux. (*A*): intracellular radiophosphate pool (P_{in}) amounts to 1 mM and corresponds approximately to the sum of the labeled phosphates in the water-soluble extract of nerves loaded for 150 min in ^{32}P Locke; k_o represents the rate constant of the Na-dependent transfer of phosphate from P_{in} to P_I pool; the affinity of the saturable pool P_{II} for the phosphate is modulated by the external concentration of K which modifies the ratio between the rate constants α and β; the rate constants of efflux (k_e) and of re-uptake (k_i) are assumed to be constant. Using an analog computer to solve Eqs. (1) to (4) for different experimental conditions, curves *B*, *C* and *D* are obtained (*compare* with Figs. 2, 3 and 4, respectively). For more details, *see* text

phate should increase by 14.6%; the corresponding value for the divalent is 19.3%. Since the activity of these ions is about equal at pH 7.4, the mean effect should be approximately 17%. Further, from earlier experiments, it is known that only 25% of the efflux is not dependent on Na, so that the final effect of the hyperpolarization is probably not more than an increase of 4–5% compared to the 86% found on removal of K. A similar argument can be applied to the depolarization; with 20 mM K the membrane potential is lowered by 20 mV, producing a 26% decrease for the monovalent and a 36% lowering for the divalent phosphate. The mean effect would thus be 31%, and the effect on the diffusional efflux would be a decrease of 8% against the observed 28% lowering. Further, the maximal inhibition of the phosphate efflux is found with 20 mM K, while the membrane is still more depolarized when higher K concentrations are used.

It thus seems very difficult to explain the effect of K on the phosphate efflux by the modification of the membrane potential.

C. Kinetic Analysis

The efflux of phosphate in our experiments was usually expressed in fractions b of the total water-soluble radiophosphate content of the nerve, effluated per minute. If the water-soluble phosphate pool represents the real and unique exchangeable phosphate reserve of the nerve, the value of b should correspond to the rate constant of the phosphate efflux, and the radioactivity of the effluated phosphate must decrease exponentially. Figure 7, where the efflux in cpm is represented in semi-logarithmic coordinates, shows that in normal Locke solution (curve *A*) this is actually the case. When the K-free solution is applied,

we observe an important transient increase of the phosphate efflux. The semi-logarithmic representation of the efflux in this case (curve *B*) shows that the total efflux can no more be approximated by a simple exponential function, as should be the case if the K-free solution produces a simple increase of the efflux rate constant from the intracellular phosphate pool. A straight line, however, is obtained if one plots, in semi-logarithmic coordinates, the difference between the efflux in K-free medium and normal Locke solution (curve *C*). This observation suggests a new phosphate pool, which becomes visible only when extracellular potassium is varied.

The effect of K-free solution on the phosphate efflux can be fully repeated after approximately 30 min of recovery in nonradioactive Locke containing physiological potassium and sodium concentrations. This means that the K-dependent phosphate pool is filled from the intracellular phosphate compartment. However, if the experiment is done in Na-free solution, the second withdrawal of potassium produces only a very small effect (Fig. 3). This finding indicates that the mechanism by which the K-dependent phosphate pool is refilled is Na-dependent.

The fact that even in Na-free solution the first withdrawal of potassium produces a liberation of phosphate shows that the K-dependent phosphate pool retains its content even when the filling is highly reduced. Thus, in normal potassium concentration (5.6 mM), the rate of output from this compartment must be low. Indeed, experiments with different potassium concentrations give for this compartment an apparent inhibition constant of 1.4 mM. It means that at 5.6 mM extracellular potassium, which corresponds to the physiological concentration, the efflux from the K-dependent compartment is strongly inhibited. We have summarized these different observations schematically in Fig. 8. In this model, sodium is required for the filling of a phosphate pool P_I from which phosphate is liberated into the extracellular space. This pool is in equilibrium with a saturable compartment P_{II} which, in physiological potassium concentration, has a high affinity for the phosphate of the P_I pool.

According to this model, the kinetics of the phosphate efflux corresponds to the following system of differential equations:

$$\frac{dP_{in}}{dt} = -k_0 \cdot P_{in} + k_i \cdot P_{II} \tag{1}$$

$$\frac{dP_I}{dt} = k_0 \cdot P_{in} - k_e \cdot P_I + \beta \cdot P_{II} - \alpha \cdot P_I(P_T - P_{II}) \tag{2}$$

$$\frac{dP_{II}}{dt} = \alpha \cdot P_I(P_T - P_{II}) - \beta \cdot P_{II} - k_i \cdot P_{II} \tag{3}$$

$$E = k_e \cdot P_I \tag{4}$$

where P_I and P_{II} is the amount of radiophosphate in membrane compartments I and II, respectively, P_T is the total number of binding sites in compartment II, and E is the measured efflux. The other symbols correspond to those of the schema in Fig. 8*A*. This mathematical model was solved using an analog computer (ALPAM 200). The results are presented in Fig. 8*B*, *C* and *D*. The changes of the phosphate efflux produced by K-withdrawal were simulated by increasing the rate constant β from its steady-state value (0.05 sec^{-1} in *A* and 0.069 sec^{-1} in *B* and *C*) to 1, and the effect of Na-free solution by decreasing the rate constant k_o from 0.01 sec^{-1} to zero during the time intervals indicated in the figures. The other factors were kept constant during the computation. They have the following values: $k_e = 0.95\ sec^{-1}$, $k_i = 0.03\ sec^{-1}$, $\alpha = 1.0\ sec^{-1}$ and $P_T = 0.05$ mM/kg wet wt. The time scale factor used for the computation of curves presented in Fig. 9 was 600; (1 sec machine time corresponds to 10 min real time).

The initial conditions which best fit our experimental results were: 1 mM/kg wet wt and 0.045 mM/kg wet wt for the total intracellular labeled phosphate and the labeled phosphate of the K-dependent phosphate pool, respectively. The first value corresponds to the amount of radiophosphate found by Ferrero *et al.* (1978) in the water-soluble fraction of nerves loaded for 150 min in ^{32}P Locke. The content of the K-dependent phosphate pool (45 μM/kg wet wt) was estimated by the computation procedure. It is close to the value found in the efflux experiments by integrating the area under the extra phosphate efflux during K-withdrawal (48.0 ± 5.7 μM/kg wet wt, $n = 4$). The theoretical rate of efflux calculated from this model for physiological steady-state conditions is 2.85 μM/min·kg wet wt which corresponds to our experimental results.

Some other models were tested but none of them gave a better correlation with the experimental results.

At this stage of our knowledge it is difficult to give a biochemical interpretation of the proposed model. One possible candidate for the K-dependent phosphate pool are the membrane phospholipids. Preliminary results show that ^{32}P incorporated into phospholipids after an incubation of 60 min amounts to 36 μM/kg wet wt. Similar values were obtained in the same tissue by Salway and Hughes (1972). In nerves loaded for 150 min and then washed 120 min with nonradioactive Locke, the labeling increases to about 180 μM/kg wet wt (*unpublished results*). The phosphate liberated during the K-withdrawal thus seems to involve a fraction of the total lipids only. For example, it has been shown that the phosphoryla-

tion rate of phosphoinositides and often also of phosphatidic acid is modified a few minutes after the application of different external stimuli (for review, *see* Michell, 1975). The obvious common feature of these stimuli is that most of them exert their effect on their target cell through interaction with receptor sites on cell surfaces, rather than by entering and directly affecting intracellular processes.

Effects of potassium ions on the phosphatidylinositol turnover were studied by Nagata, Nikoshiba & Tsukada (1973). They have shown in sympathetic ganglia that a high potassium ion concentration (80 mM) increase ^{32}P incorporation into phospholipids, particularly phosphatidylinositol and phosphatidylcholine. Jafferji and Michell (1976) have found similar results in ileum smooth muscle. Novotný, Živný and Saleh (1977) have confirmed these observations in frog *sartorius* muscle. If these results are interpreted in terms of the model presented in Fig. 8, the K-dependent phosphate pool will correspond to the inositol lipids. Although this would be an attractive explanation for our results, much more experimental information is still needed to allow such a conclusion.

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