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RELEASE OF INORGANIC PHOSPHATE DURING ACTIVITY IN MAMMALIAN NON-MYELINATED NERVE FIBRES

BY J. C. MAIRE AND R. W. STRAUB

From the Département de Pharmacologie, Ecole de Médecine, 1211 Genève 4, Switzerland

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SUMMARY

1. The efflux of labelled phosphate was measured in desheathed rabbit vagus nerve at rest and during activity.

2. In solutions with 2 mM-phosphate and 1 mM-K the rate constant of the resting efflux was $2{\cdot}7 \times 10^{-3}$ min^{-1}; stimulation caused an extra fractional loss of $2{\cdot}8 \times 10^{-6}$ impulse^{-1}.

3. Lowering the phosphate concentration decreased the resting and the stimulated efflux; with 0·2 mM-phosphate the corresponding values were $1{\cdot}9 \times 10^{-3}$ min^{-1} and $1{\cdot}8 \times 10^{-6}$ impulse^{-1}, respectively.

4. Increasing the K to 5·6 mM decreased both resting and stimulated efflux.

5. Lowering the temperature decreased the resting efflux with a Q_{10} of 2·9 and the stimulated efflux with a Q_{10} of 8·1.

6. Chromatography of the effluent showed that at rest and during activity at least 96 % of the radiophosphate was in the orthophosphate fraction.

7. Replacing the Na of the solution by Li lowered the rate constant of the resting efflux to $0{\cdot}8 \times 10^{-3}$ min^{-1} and abolished the extra release during activity, without reduction of the action potential.

8. The presence of ouabain did not affect the resting efflux, except at 100 μM, when a transient reduction was found. The extra fractional loss was not affected with 0·001 μM; with 0·01–0·5 μM, it was reduced without much change in the action potential, and abolished at higher concentrations.

9. The results agree with the hypothesis that the extra release results from an increase in internal inorganic phosphate caused by increased break-down of ATP during recovery.

10. Comparison with the O_2 consumption shows that about 1 % of the inorganic phosphate liberated at the inside of the axons escapes to the outside.

INTRODUCTION

In garfish olfactory nerve an increased release of labelled phosphate has been observed during electrical activity (Ritchie & Straub, 1978). The time course of the increase in phosphate efflux parallels the increase in O_2 consumption (Ritchie & Straub, 1979), and for different frequencies and durations of stimulation the extra release of phosphate is proportional to the extra O_2 consumption. These and other

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observations suggest that the extra release of phosphate may result from an increase in intracellular inorganic phosphate caused by an increased utilization of ATP by the Na–K-pump. Only a small fraction of the inorganic phosphate liberated from ATP seems to escape to the outside of the axons (see Ritchie & Straub, 1979).

A number of experimental observations on the phosphate efflux and the O_2 consumption in rabbit vagus nerve have recently been described (see Ritchie & Straub, 1980*a*, *b*). In the present experiments we have further studied the phosphate efflux during activity in this tissue. In order to analyse the effluent we have tried to increase the extra liberation by varying different factors, such as the phosphate or the potassium concentrations and the temperature (Maire, Medilanski & Straub, 1979). In appropriate conditions a relatively large extra efflux during activity was regularly found so that the effluent could be analysed chromatographically; it consisted mainly of inorganic phosphate. We have also studied the cause of the extra release by inhibiting the increased utilization of ATP after activity by the application of small amounts of ouabain, or by replacing the Na ions of the solution by Li ions. The results of both series suggest that the extra efflux results from an increased utilization of ATP after activity.

METHODS

The methods for measuring the resting and stimulated efflux were similar to those used by Ritchie & Straub (1978). In brief, desheathed cervical vagus nerves of rabbits were loaded with radiophosphate by incubation during 2 hr in [^{32}P]phosphate Locke with 0·2 mM-phosphate at 37 °C. After a short wash in non-radioactive Locke they were mounted in an apparatus where they could be stimulated and the action potential recorded. The apparatus was continuously perfused with non-radioactive Locke. The efflux from the part of the nerve that lay under the stimulating electrodes was discarded (see Ritchie & Straub, 1978), the remaining efflux was collected in glass vials and counted. At the end of the experiment the nerve was removed and the region from which the efflux had been collected was identified and cut in two. One half was used for the determination of the total phosphates, the other was homogenized in 2 ml. distilled water and centrifuged. The supernatant which contained the water-soluble phosphates was removed and counted. These counts were used as the basis for the calculation of the rate constant (see Ferrero, Jirounek, Rouiller & Straub, 1978). Unless otherwise stated, the preparations were maintained at 37 °C.

Chromatography of efflux. In a number of experiments the effluent from the apparatus was analysed by chromatography as described elsewhere (Anner, Ferrero, Jirounek & Straub, 1975). In these experiments half of the collected effluent was counted as usual; the other half was passed through a Dowex 1 column in order to separate the different phosphates.

Solutions. The Locke solution had the following composition (mM): NaCl, 154; KCl, 1; $CaCl_2$, 0·9; $MgCl_2$, 0·5; Tris, 1 or 10; glucose, 5. Na orthophosphate was present in the concentrations indicated in the text. The pH was adjusted to 7·4. Solutions with 5·6 mM-KCl were prepared by adding KCl; Li-Locke by replacing NaCl by LiCl. G-strophanthin (ouabain) Merck was used.

Whenever possible means and their S.E. are given.

RESULTS

A large effect of activity on the phosphate efflux was observed when solutions with 2 mM-phosphate and 1 mM-K and a temperature of 37 °C were used. Fig. 1 shows the resting phosphate efflux and the effect of activity in these conditions. It can be seen that stimulation raises the efflux and that the increased efflux then slowly returns towards the resting rate before stimulation. The mean extra fractional loss was

$2{\cdot}77 \pm 0{\cdot}30 \times 10^{-6}$ impulse^{-1} (thirty-five determinations in seventeen nerves) after stimulation at 10/sec during 3 min. In these preparations, the rate constant of the resting efflux immediately before stimulation was $2{\cdot}70 \pm 0{\cdot}01 \times 10^{-3}$ min^{-1}. The recovery of the increased efflux was nearly exponential. In seven experiments with 5 min collection periods the mean time constant was $6{\cdot}8 \pm 0{\cdot}6$ min; in two other experiments with 2 min collection a mean time constant of 7·1 min was found.

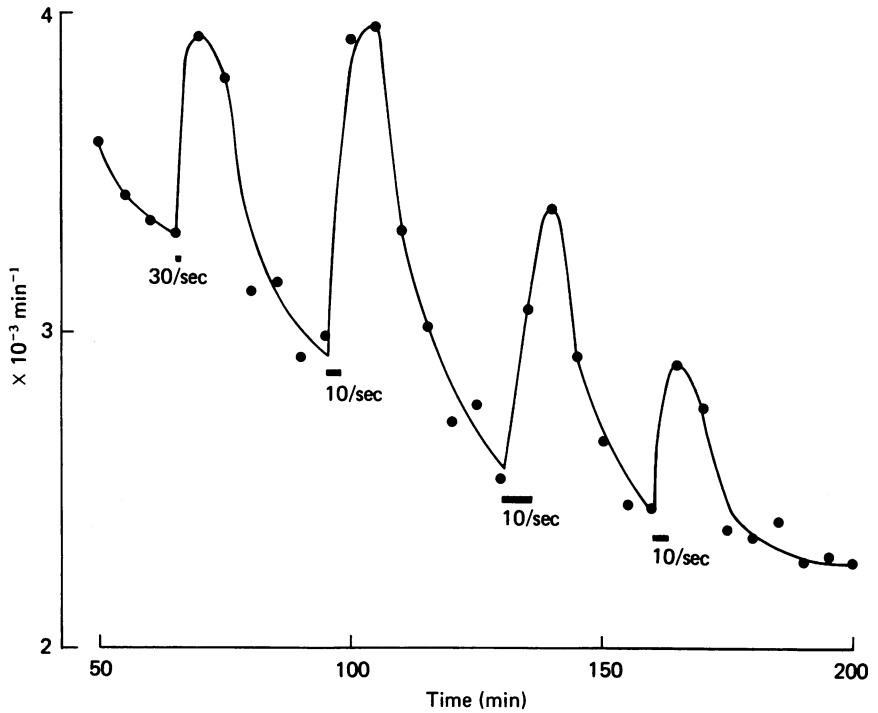

Fig. 1. Effect of electrical activity on the phosphate efflux in rabbit vagus nerve. The ordinate indicates the fractional loss of radiophosphate, the abscissa is time after end of loading with radiophosphate. Washing solution contained 2 mM-phosphate and 1 mM-K; the preparation was stimulated as indicated. Temperature, 37 °C.

Effect of phosphate

A number of experiments were done with solutions containing 0·2 mM-phosphate, a concentration used for most of the experiments on resting efflux. In a solution with 0·2 mM-phosphate and 1 mM-K, the resting efflux was smaller (see Ferrero *et al.* 1978) and the extra fractional loss was also decreased. The mean fractional loss was $1{\cdot}81 + 0{\cdot}38 \times 10^{-6}$ impulse^{-1} (eight determinations in seven nerves) stimulated at 10/sec during 3 min at 37 °C. The corresponding resting efflux was $1{\cdot}95 \pm 0{\cdot}13 \times 10^{-3}$ min^{-1}. In other experiments phosphate concentrations of 0·02 and 0·002 mM were used. Fig. 2 shows that the increase in stimulated efflux parallels the increase in resting efflux as the phosphate concentration is raised.

The resting efflux and the fractional loss of phosphate during activity found with 0·2 mM-phosphate are smaller than the corresponding values observed in solutions with the same phos-

phate concentration by Ritchie & Straub (1980*a*). The reason for this difference may lie in the use of Tris buffer for the present experiments, since Tris, at high concentrations anyway, tends to lower the phosphate efflux (see Ferrero *et al.* 1978).

Effect of K

With 5·6 mM-K the effect of stimulation was smaller than the effects of Fig. 1. In four nerves kept in a solution with 2 mM-phosphate the extra fractional loss was $1{\cdot}32 \pm 0{\cdot}27 \times 10^{-6}$ impulse^{-1} and the resting efflux $2{\cdot}14 \pm 0{\cdot}34 \times 10^{-3}$ min^{-1}.

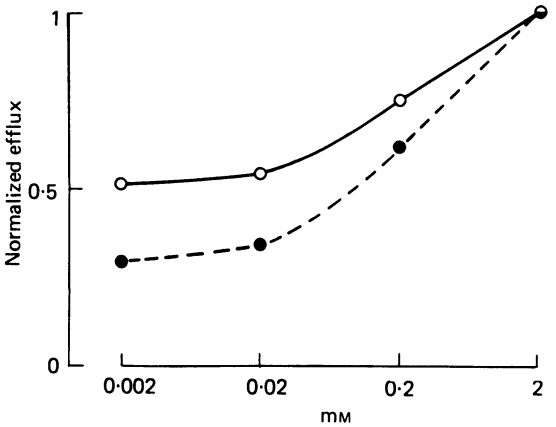

Fig. 2. Normalized values of rate constant of resting efflux (○) and extra fractional loss (●) at different phosphate concentrations. The points at 2 and 0·2 mM represent means of seven experiments, at 0·02 mM the mean is based on four experiments, and one determination was made at 0·002 mM. Temperature, 37 °C.

TABLE 1. Chromatography of effluent from resting and stimulated nerve

	Resting (%)	Stimulated (%)
P_i + CrP	98·5 ± 0·4	98·4 ± 0·4
ADP	0·6 ± 0·2	0·9 ± 0·4
ATP	0·8 ± 0·2	0·7 ± 0·2
	n = 6	n = 6

Effect of temperature

Lowering the temperature decreased both resting and stimulated efflux: in a solution with 2 mM-phosphate and 5·6 mM-K the resting efflux at 22 °C was 0·55 ± 0·05 min^{-1} and the extra fractional loss $0{\cdot}12 \pm 0{\cdot}06 \times 10^{-6}$ impulse^{-1} (twelve determinations in three nerves stimulated at 10/sec for 3 min). Compared to the corresponding values at 37 °C the change corresponds to a Q_{10} of 2·9 for the resting and of 8·1 for the stimulated efflux.

Chromatography of efflux

Since the stimulated efflux was relatively large it could be analysed by column chromatography. The experiments consisted in first collecting the efflux from the resting preparation immediately before stimulation. During the next 5 min collection period the nerve was stimulated at 10/sec for 3 min. A count of both samples was

then taken and an aliquot of each was passed through the columns. Table 1, which shows the results of the analysis, suggests that there is little difference between the resting and the stimulated efflux. It must be kept in mind, however, that the efflux of the stimulated samples was diluted by the efflux from the nerve at rest. The mean counts of the stimulated sample were $16 \pm 3\%$ higher than those of the samples at

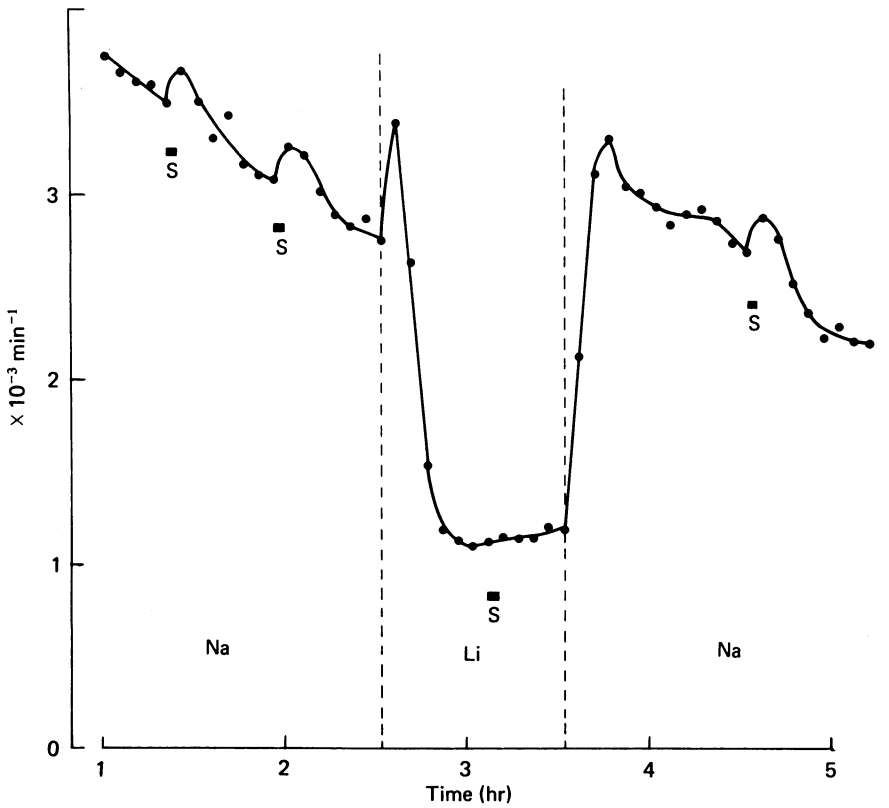

Fig. 3. Abolition of extra release of phosphate during activity by Li-Locke in rabbit vagus nerve. The preparation was first kept in Locke and stimulated as shown (S); Li-Locke was then applied and the preparation re-stimulated, later the preparation was returned to Locke. Ordinate indicates fractional loss of radiophosphate, abscissa is time after end of loading. Temperature, 37 °C.

rest (six experiments). Enzymic tests, kindly performed by Dr M. Chmouliovsky, showed that at most 2 % of the phosphate of the P_i + CrP fraction could be accounted for by CrP, so that the main component of this fraction must have been inorganic phosphate.

The results of Table 1 suggest the presence of small amounts of ATP and ADP. This seems to be due to an imperfect selectivity of the column. Thus, when [^{32}P]phosphate solution was passed through the column, $96{\cdot}1 \pm 0{\cdot}5\%$ of the ^{32}P was found in the monophosphate fraction (ten experiments).

Lithium

Fig. 3 shows an experiment in which the effect of Li-Locke was tested. It can be seen that stimulation produced the usual extra release of phosphate when the preparation was kept in Locke with 2 mM-phosphate. After the application of Li-Locke the effect of activity on phosphate efflux was abolished, although conducted action potentials were still present. Li-Locke also lowered the resting efflux. Both effects of the Li-solution were reversible. The lowering of the resting efflux was preceded by a transient increase (Fig. 3). This increase was found only when the lithium solution contained 1 mM-K; it was not seen in the earlier experiments with 5·6 mM-K (Ferrero *et al.* 1978).

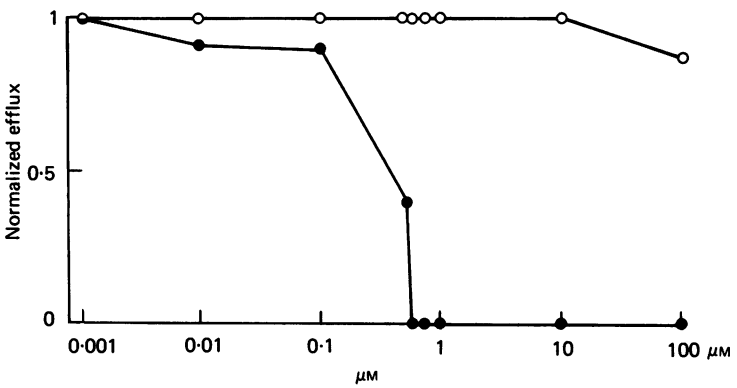

Fig. 4. Normalized values of rate constant of resting efflux (○) and extra fractional loss (●) at different ouabain concentrations. The points are means of two (0·001, 0·01, 0·1 and 0·5 μM), three (1 and 10 μM) and twelve experiments (100 μM); the remaining points are single determinations. Temperature, 37 °C.

The effects of Fig. 3 were confirmed in three other preparations. In Locke, before the application of the Li-Locke, the mean extra fractional loss was $2{\cdot}27 \pm 0{\cdot}30 \times 10^{-6}$ impulse^{-1} (ten determinations in the four nerves), and the rate constant of the corresponding resting efflux $2{\cdot}25 \pm 0{\cdot}25$ min^{-1}. In Li-Locke the extra fractional loss was abolished in all four preparations and the resting efflux lowered to $0{\cdot}85 \pm 0{\cdot}10 \times 10^{-3}$ min^{-1}. After returning to Locke the extra fractional loss recovered to $2{\cdot}53 \pm 0{\cdot}92 \times 10^{-6}$ impulse^{-1} (four determinations) and the resting efflux to $2{\cdot}17 \pm 0{\cdot}26 \times 10^{-3}$ min^{-1}.

The abolition of extra release in Li-Locke is probably due to the decreased activation of the Na–K pump after activity in Li-Locke (see Discussion); an additional effect may come from the lowering of the efflux of phosphate.

Ouabain

The effect of ouabain was studied because this drug, as seen in preliminary experiments, inhibits the activity of the Na–K pump, without much effect on the resting phosphate efflux. Since ouabain, at high concentration, causes inexcitability of the nerve fibres, a large range of concentrations had to be tested. Fig. 4 shows normalized values of resting and stimulated fluxes at different ouabain concentrations. At con-

centrations below 10 μM, ouabain did not alter the resting efflux; at higher concentrations the resting efflux showed a small decrease (Fig. 4). The decrease was transient; during prolonged exposure to ouabain the efflux recovered to the value before the application of the drug. The extra efflux during stimulation was decreased at low concentrations of ouabain, and abolished at higher concentrations. It is interesting to note that with 140 μM-ouabain, Wespi, Mevissen & Straub (1969) found a rapid decrease in K and an Na accumulation in this tissue; after 15 min exposure to the drug the contents of these ions were already markedly changed.

Phosphate content

The content in water-soluble phosphates of forty nerves, analysed at the end of the experiments, amounted to $9{\cdot}17 \pm 0{\cdot}47$ m-mole/kg.wet wt.

DISCUSSION

On the basis of indirect evidence, Ritchie & Straub (1979) suggested that the extra efflux of radiophosphate during and after activity corresponds to the release of inorganic phosphate. The present experiments confirm this suggestion: the chromatographic analysis of the efflux showed that the released radiophosphate is in the inorganic phosphate fraction and that ATP and ADP are practically absent from the efflux of resting and stimulated nerve.

The extra release of phosphate during activity may result from an increase in intracellular inorganic phosphate (see Ritchie & Straub, 1978, 1979). Such an increase has been observed in rabbit vagus nerve (Chmouliovsky, Schorderet & Straub, 1969). The results show that the intracellular phosphate is approximately doubled after 750 impulses given at a frequency of 50/sec. This should, on the assumption of direct proportionality between intracellular phosphate and phosphate efflux, double the efflux. In the present experiments a lower frequency was used and the observed effects were also smaller.

The hypothesis that the extra release is caused by an increase in intracellular phosphate accounts for the observations with Li-Locke and ouabain. In Li-Locke the extra efflux was absent, which is in line with the observations that lithium ions, while maintaining normal action potentials, activate the internal site of the Na-K pump much less than Na ions (see Ritchie & Straub, 1980*b*) and thereby produce only a small breakdown of ATP. In Li-Locke, therefore, activity presumably does not increase the intracellular inorganic phosphate, which would explain the absence of extra phosphate efflux. The lowering of the resting phosphate efflux in Li-Locke shows that in addition, Li ions inhibit the phosphate efflux from the fibres (see Ritchie & Straub, 1980*b*). This effect probably also contributes to the abolition of phosphate liberation after activity.

The results with ouabain also are in line with the general hypothesis. This drug slows the working of the Na–K pump, as shown for rabbit non-myelinated fibres, by the abolition of the extra oxygen consumption (Ritchie, 1967), recovery heat (Howarth, Keynes & Ritchie, 1968), and post-tetanic hyperpolarization (Ritchie & Straub, 1957; Rang & Ritchie, 1968). In this case the decrease in extra liberation of phosphate is not connected with a lowering of the resting efflux, which is unaltered.

The resting efflux is only lowered by high concentrations of ouabain (Fig. 4), maybe by a transient decrease in internal inorganic phosphate.

The increase in internal phosphate necessary to account for the observed release can be calculated from the internal inorganic phosphate, the resting efflux, the extra release and its rate of decline after activity (see Ritchie & Straub, 1979). Based on the phosphate fluxes at 0·2 mM-phosphate, the initial increase would correspond to 0·22 μmole.kg^{-1} impulse^{-1}; a similar calculation based on the O_2 consumption (Ritchie & Straub, 1980*a*) shows a value of 0·23 μmole.kg^{-1} impulse^{-1}.

As, judged from the extra O_2 consumption, one impulse increases the phosphate turnover by 1·40 μmole.kg^{-1} impulse^{-1}, only 16·6 n-mole.kg^{-1} impulse^{-1} are liberated, which is 1·2 %. The amount that is actually liberated appears to depend on the working of the Na-dependent phosphate transport system which is stimulated by external phosphate (see Ferrero *et al.* 1978). Thus, in 2 mM-phosphate, 25·4 n-mole.kg^{-1} impulse^{-1} are liberated as compared to the 16·6 n-mole in 0·2 mM.

The increase in liberation in low K may have a different cause. The effect of low K on the extra efflux is probably due to the larger action potential resulting from membrane hyperpolarization.

While the present observations are in line with a liberation of inorganic phosphate, the possibility of a release of ATP must also be examined. In this case it must be assumed that the liberated ATP is quickly hydrolysed by extracellular enzymes so that inorganic phosphate is collected. Indeed, liberation of ATP has been found at nerve endings (Silinsky & Hubbard, 1973; Silinsky, 1975). Our experiments, though not particularly designed to test this hypothesis render it however unlikely. The abolition of extra release in Li-Locke, and the decrease in ouabain, are not in line with an ATP liberation during the action potential, and the effect of external phosphate on the extra efflux also is not easily accounted for by the ATP hypothesis. Further, we did not observe a release of ADP, which might have been found if ATP had been hydrolysed.

Our experiments do not exclude, however, a spurious liberation of ATP during activity. Indeed, with more refined techniques such a liberation may also be detected. The possibility could probably be examined most easily by using the Li solution, where the resting and extra liberation of inorganic phosphate are much reduced or absent.

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