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TERMINATION OF TRANSMITTER RELEASE BY STIMULATION OF SODIUM-POTASSIUM ACTIVATED ATPASE

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SUMMARY

1. The release of acetylcholine (ACh) from Auerbach's plexus of guinea-pig ileum has been measured in eserinized Krebs solution using longitudinal muscle strip preparations.

2. Removal of the external K ions enhanced both the resting and stimulated release of ACh from the plexus. This effect was not affected by tetrodotoxin.

3. On readmission of K^+ to tissues which had been suspended in K-free Krebs solution the release of ACh was promptly reduced in both stimulated and unstimulated tissues. The extent of the reduction of ACh release depended on the exposure time to K-free solution, the recovery being delayed by longer exposure.

4. The ACh releasing effect of (1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP) was completely inhibited by the readmission of K ions to tissue which had been kept in K-free Krebs solution.

5. Rb^+ substitution for K^+ produced no change in ACh release and addition of 5·9 mM-Rb after K removal reduced the release of ACh as K did readmission. When the K ions were substituted by Cs^+, both the resting and stimulated release were enhanced. The amount of ACh released by a stimulus was enhanced both at low and high frequency of sustained stimulation.

6. Removal of the external K ions increased the release of tritiated noradrenaline (NA), from isolated rat iris; however, when K^+ (5·9 mM) was readmitted the release was reduced even below the control value.

7. It is concluded that the stimulation of (Na^+–K^+)-activated ATP-ase in the membrane inhibits the release of transmitter, and under physiological condition Ca-fluxes and the subsequent inhibition of membrane ATP-ase may be involved in triggering the release of transmitter.

INTRODUCTION

It has been shown that different experimental conditions known to lead to inhibition of (Na^+–K^+)-ATPase are capable of promoting ACh release from nerve terminals of Auerbach's plexus (Paton, Vizi & Zar, 1971; Vizi, 1972) and rat cortex (Vizi, 1972, 1974). Among other things it was found that ACh release from the nerve terminals of Auerbach's plexus was increased when the K was omitted from Krebs solution (Paton *et al.* 1971) as a consequence of the inhibition of membrane ATPase. In addition, Baker & Crawford (1975) succeeded in showing on frog neuromuscular junction that the superfusion of an end-plate with K-free Ringer causes an immediate exponential rise in the miniature end-plate potential (m.e.p.p.) frequency.

It has been reported by different authors (Rang & Ritchie, 1968*a*; Bolton, 1973) that the removal of K followed by a readmission of K might produce a stimulation of Na pump (Na^+–K^+)-activated ATPase (Skou, 1965). Since it might be expected that the removal of K^+ ions does not influence axonal nerve conduction this procedure, removal and readmission of K^+ ions, provides a possibility for studying the effect of stimulated activity of (Na^+–K^+)-activated ATPase on ACh release induced by nerve stimulation.

A brief report of some of the results presented in this paper has been communicated to the British Pharmacological Society (Vizi, 1973).

METHODS

ACh release. Guinea-pigs of 250–400 g body weight were used. The longitudinal muscle strips of guinea-pig ileum weighing 50–80 mg were prepared as described by Paton & Vizi (1969). Two strips were set up in an organ bath of 3·5 ml. volume in Krebs solution at 37° C. Physostigmine sulphate in a concentration of 2×10^{-6} g/ml. was added and the bath was bubbled with a constant stream of 95 % O_2 and 5 % CO_2. Before collecting the first samples for assaying the ACh released, the strips were allowed to equilibrate under resting conditions for 60 min. The ACh obtained in this way was assayed on a length of guinea-pig ileum suspended in 3·5 ml. Krebs solution at 36° C. Otherwise, the ileum was set up as described by Paton & Vizi (1969). In some experiments the release of ACh was measured in the presence of DMPP. To eliminate the possibility of interference from traces of DMPP carried over in the sample, hexamethonium (300 μg/ml.) was added to the Krebs solution for assay.

The strip was stimulated by means of two platinum electrodes using 'field' stimulation technique (Paton & Vizi, 1969). The electrical stimuli (10 V/cm), which were about 2–3 times the intensity required to stimulate supramaximally the most excitable fibres, were 1 msec in duration.

NA release. To measure the release of NA, isolated rat iris was used as described by Farnebo & Hamberger (1971). The irides were incubated for 30 min with 50 μCi [^{3}H]NA in 2·0 ml. Krebs solution bubbled with 5 % CO_2 in O_2. At the end of the

incubation period the irides were washed 10 times by placing them for 1 min into each of ten beakers containing 50 ml. fresh Krebs solution, also bubbled with 5 % CO_2 + 95 % O_2. The irides were placed into a chamber of 1·5 ml. containing K-free Krebs solution. 2·5 min fractions of superfusate were collected in counting vials. The superfusate was added to Insta-Gel scintillation solution (5 ml.) and the radioactivity was determined with a Packard Scintillation spectrometer (3300 TriCarb). After the tissue had been loaded in K-free Krebs solution for 10 min 5·9 mM-K^+ was added to the K-free Krebs solution.

The composition of the Krebs solution was (mM): NaCl 113; KCl 4·7; $CaCl_2$ 2·5; KH_2PO_4 1·2; $NaHCO_3$ 25; glucose 11·5. K was removed from solutions by omitting KCl and KH_2PO_4 without compensating for changes in tonicity.

Determination and calculation of intracellular content of ions. The changes in intracellular concentration of Na^+, Ca^{2+} and Mg^{2+} have been measured in desheathed isolated rabbit cervical vagus trunk since it has been shown to contain a great proportion of non-myelinated nerve fibres. After the rabbit had been killed by air embolism both vagus nerves were removed and desheathed. The nerves were immersed in Krebs solution of different ionic composition at 35° C and bubbled with carbogen (95 % O_2 + 5 % CO_2). After loading for different times the nerves were immersed for 7 min in an isotonic solution of choline chloride at 7° C. It has been shown by Keynes & Ritchie (1965) that K^+ and Na^+ disappear from the extracellular space. The nerves were then blotted on wet filter paper and weighed. Thereafter they were dried at a temperature of 70° C for 5–6 hr and reweighed. The ratio between dry and wet weight proved to be 0·194 ± 0·008 mg/mg (S.E. of mean, n = 72), which value is not significantly different from that (0·222) published by Rang & Ritchie (1968*b*). To the dry preparations double distilled water containing 10 % Co_2O_3 was added for 24 hr. Co^{3+} was used as a competing metal ion for emission spectroscopy. Then Na^+, Ca^{2+} and Mg^{2+} concentrations of the solution were determined by emission spectroscopy (Zeiss PGS II). The wave-lengths used were 589·6 nm for Na^+, 422·7 nm for Ca^{2+} and 279·6 nm for Mg^{2+}. The intracellular concentrations of ions were determined as described by Wespi (1969).

Immediately after dissection the intracellular concentrations of Na^+ and Ca^{2+} were found to be 31·5 and 3·2 m-mole kg^{-1} wet weight, respectively (see Table 3). However, when the nerves were incubated in normal Krebs solution both the Na and Ca concentration increased with time.

Drugs. The drugs used were: ACh iodide (B.D.H.); ouabain (B.D.H.); physostigmine sulphate (Burroughs Wellcome); tetrodotoxin(Sankyo); 1,1-dimethyl-4′-phenyl-piperazinium iodide (DMPP, Fluka).

Concentrations of the drugs are given in molar concentration or in terms of their salts, as indicated in the text.

Whenever possible means ± S.E. of mean are given. In many experiments Ca^{2+} was reduced to less than 5×10^{-9} M by addition to Ca-deficient Krebs solution of 1 mM ethylene glycol bis-(amino-ethylether)-*N*,*N*′-tetra-acetic acid (EGTA), obtained from Fluka. This was used in preference to EDTA, because the binding constant for Mg by EGTA is much less than by EDTA (Sillen & Martell, 1964). The Ca^{2+} in Krebs solution without added $CaCl_2$ was determined by flame emission spectroscopy and was found to be 0·009 mM. This is probably due to impurity in the salts used (NaCl, $MgCl_2$). The distilled water used was de-ionized. Longitudinal muscle strips were not kept longer than 10 min in the same Krebs solution; the solution was changed every 10 min. Therefore Ca^{2+} leakage from the muscle does not contribute significantly to the Ca^{2+} concentration measured in the Krebs solution. Addition of 1 mM-EGTA to Ca-free Krebs solution containing traces of Ca from impurities reduces Ca^{2+}, depending on pH. Using stability constant of $7·6 \times 10^6$ for pH 7·3

(Sillen & Martell, 1964) the 0·009 mM-Ca^{2+} measured and 1 mM-EGTA in the Ca-free Krebs solution result in an estimated Ca^{2+} of $1{\cdot}2 \times 10^{-9}$ M.

RESULTS

Inhibition of ACh release by readmitting K to K-free solution

When K ions were omitted from the Krebs solution, the ACh release during the resting state was enhanced from 189·9 to 912·5 p-mole g^{-1} min^{-1} (Fig. 1). On readmission of K ions, the release of ACh reduced to 38·8 p-mole g^{-1} min^{-1}. The effect of K-free solution on resting release of ACh

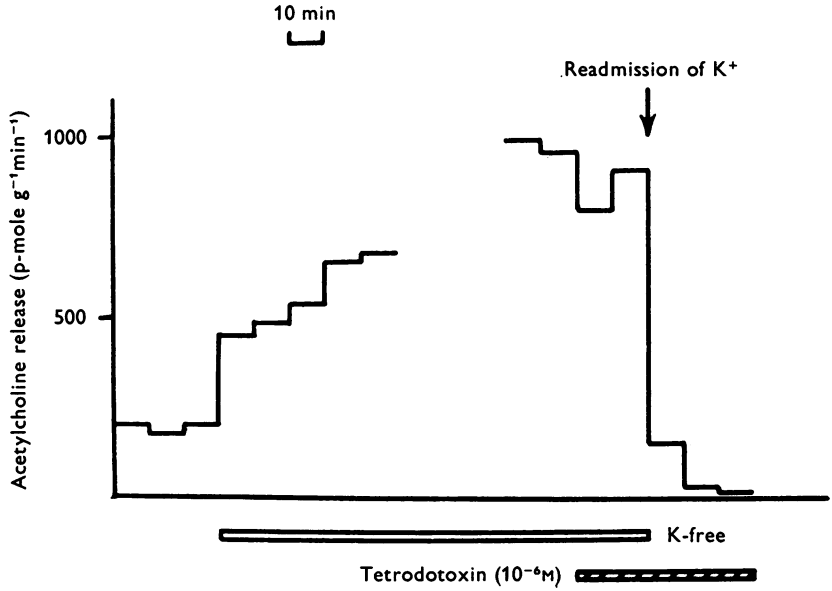

Fig. 1. ACh releasing effect of K removal and reduction of the release by the readmission of K^+. Longitudinal muscle strip with attached Auerbach's plexus. The values of ACh release are the means of two experiments with identical schedules. Ordinate: rate of ACh release, p-mole g^{-1} min^{-1}. Abscissa: time (min) as indicated. Note the fast fall of ACh release even below the control resting release on readmission of K^+ to the bathing Krebs solution after the tissue had been kept for 90 min in K-free solution.

was not affected by tetrodotoxin (10^{-6} M) indicating that no axonal conduction is involved in the release mechanism of K-free solution. The control resting release was reduced in the presence of tetrodotoxin from 189·5 to 100·6 p-mole g^{-1} min^{-1}, a result already reported by Paton *et al.* (1971).

Upon removing K from Krebs solution, the ACh output in response

to stimulation with 1 Hz was also enhanced from 892·2 ± 67·7 to 1112·2 ± 271·8 p-mole g^{-1} min^{-1} (Fig. 2). When K ions (5·9 mM) were readmitted to the tissues which had been suspended for 120 min in K-free solutions the release of ACh was promptly reduced to 130·0 ± 12·4 p-mole g^{-1} min^{-1}.

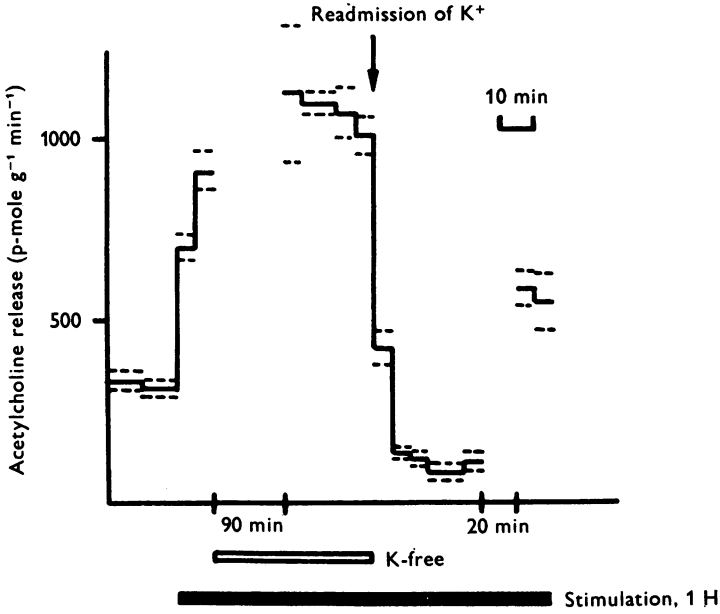

Fig. 2. Effect of removal of K^+ on the ACh release induced by axonal (field) stimulation with 1 Hz. When K^+ is readmitted the stimulation induced release of ACh is inhibited. Longitudinal muscle strip with attached Auerbach's plexus. The values of acetylcholine are the means of four identical experiments; dashed lines indicate S.E. Note the almost complete inhibition of ACh release on readmission of K^+. A partial recovery from this inhibition occurs within 30–50 min. Ordinate: rate of ACh release, p-mole g^{-1}. Abscissa: time (min) as indicated.

The reduction did not last longer than 25–50 min and depended on the exposure time to K-free solution. In one experiment during which the tissue had been exposed to K-free Krebs solution for 25 min the output produced by 1 Hz stimulation in response to readmission of K was reduced from 1025·6 to 342·5 p-mole g^{-1} min^{-1}, a reduction much less than those observed in tissue which had been suspended for 120 min in K-free solution.

The extent of reduction of ACh release depended on the exposure time to K-free solution; the longer the exposure time the greater was the reduction of ACh release in response to readmission of K^+ ions (Fig. 3). The recovery in ACh release was reached after 120 min exposure to

K-free solution, a results in good agreement with the findings of Rang & Ritchie (1968*a*) on C-fibres. Further increase of exposure time failed to produce a further reduction in ACh release.

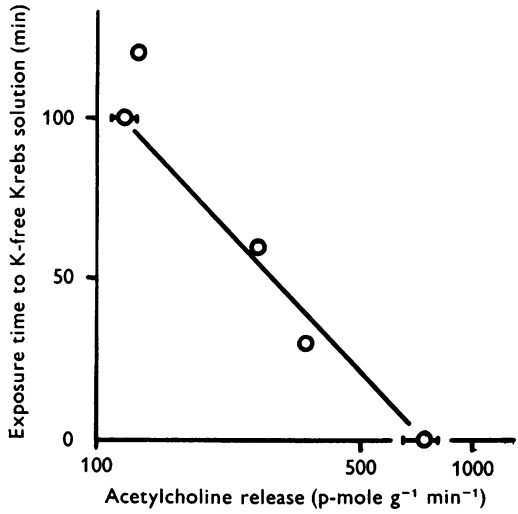

Fig. 3. The effect of the duration of K deprivation on the reduction of ACh release in response to readmission of 5·9 mM-K to tissue which had been kept in K-free Krebs solution. Guinea-pig ileal longitudinal muscle strip with attached Auerbach's plexus. Note the semilogarithmic scale. Ordinate: ACh release in p-mole g^{-1} min^{-1}. Abscissa: exposure time (min) to K-free Krebs solution. Each point represents the mean of four experiments. Horizontal lines indicate S.E., where they are greater or less than the size of the symbol. Note that 100 min exposure time to K-free Krebs solution produced the greatest reduction in release of ACh on readmission of K.

In three experiments when not 5·9 but 47·3 mM was readmitted to tissue in K-free solution only a slight reduction in ACh release was observed: at 1 Hz stimulation the release was reduced from $838{\cdot}6 \pm 41{\cdot}5$ to $680{\cdot}5 \pm 71{\cdot}6$ p-mole g^{-1} min^{-1}. Clearly this concentration of K^+ would generally be expected to cause an immediate increase of ACh release as was, in fact, observed when tissues were in medium containing 5·9 mM-K (Paton *et al.* 1971; Vizi, 1975*a*).

The removal of Ca^{2+} in the presence of EGTA reduced the release of ACh from $165{\cdot}7 \pm 32{\cdot}6$ to $47{\cdot}5 \pm 21{\cdot}2$ p-mole g^{-1} min^{-1} (Fig. 4*B*). When K^+ was also omitted from the external medium, the release started to increase, reaching a maximum within 50–60 min as shown in Fig. 4*B*. On switching the solution into normal Krebs there was a fast and transient increase in ACh release followed by a reduction to even below the control

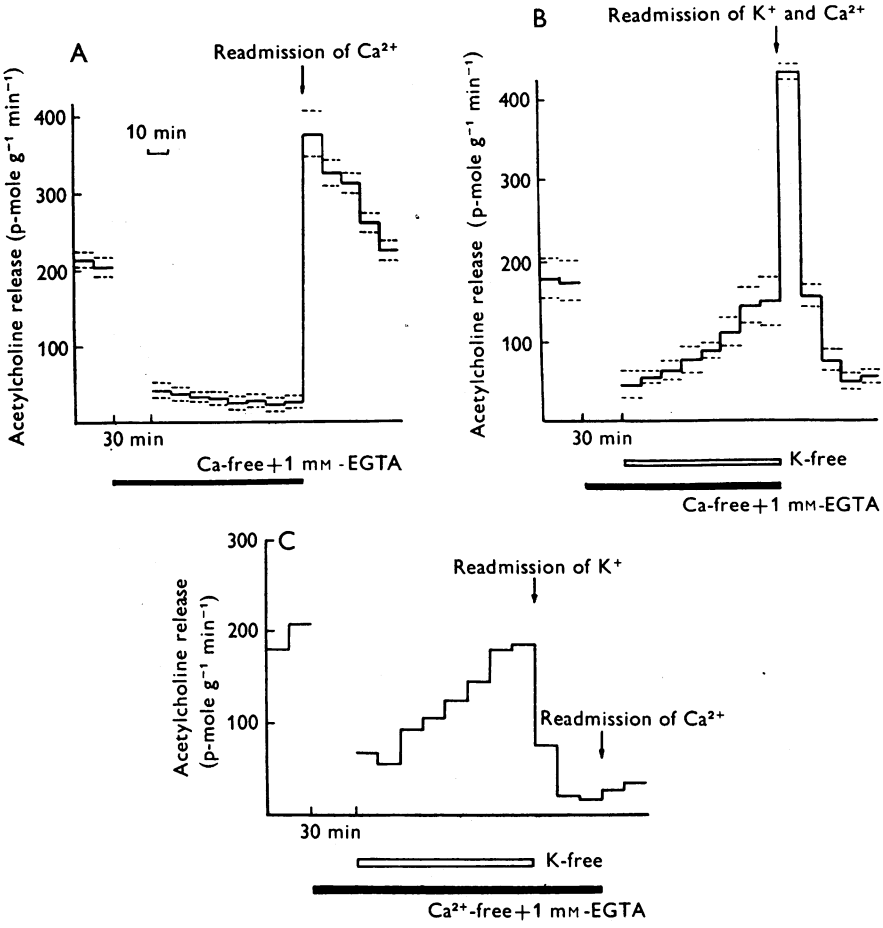

Fig. 4. Effect of Ca deprivation on the release of ACh in response to removal and readmission of K ions. Longitudinal muscle strip with attached Auerbach's plexus. Ordinate: rate of ACh release, p-mole g^{-1} min^{-1}. Abscissa: time (min) as indicated in Fig. 4*a*. Dashed lines indicate S.E. *A*, effect of removal and readmission of Ca on the resting release of ACh. The values of ACh release are the means of four identical experiments. Note that after 110 min incubation with Ca-free Krebs solution containing 1 mM-EGTA the readmission of Ca^{2+} produced an 'overshoot' in the release) the output ($378{\cdot}8 \pm 31{\cdot}2$ p-mole g^{-1} min^{-1} being significantly higher than during the control period ($215{\cdot}7 \pm 13{\cdot}6$ p-mole g^{-1} min^{-1}). *B*, effects of K and Ca deprivation and readmission on the resting release of ACh. Three identical experiments. Note the transient increase followed by a reduction of ACh release in response to the readmission of Ca and K. In Ca-free Krebs solution 1 mM-EGTA was also present. *C*, effect of K deprivation on ACh release from Auerbach's plexus which had been exposed to 1 mM-EGTA in Ca-free Krebs solution and of successive readmission of K and Ca. Note that when readmission of Ca had been preceded by the readmission of K there was no transient increase in the release of ACh. Average of two identical experiments. On the abscissa the period during which the release was not measured is indicated in minutes.

TABLE 1. The inhibitory effect of membrane ATPase stimulation on acetylcholine release induced by electrical stimulation with different frequencies. Longitudinal muscle strip of guinea-pig ileum with attached Auerbach's plexus

	ACh output (p-mole g^{-1} min^{-1} (S.E. of mean))			
	Control	K^+-free (120 min) (inhibition of membrane ATPase)	*Readmission of K^+ (stimulation of membrane ATPase)	% Reduction
Resting† (6)	217·9 ± 38·1	637·2 ± 151·6	53·8 ± 6·6	75·3
Stimulation:				
0·1 Hz‡ (5)	465·9 ± 24·2 (40·3 p-mole g^{-1} $volley^{-1}$)	873·2 ± 88·9 (33·3 p-mole g^{-1} $volley^{-1}$)	63·0 ± 4·0 (1·6 p-mole g^{-1} $volley^{-1}$)	96·1
1·0 Hz§ (5)	739·9 ± 82·0 (8·5 p-mole g^{-1} $volley^{-1}$)	1147·6 ± 255·7 (7·9 p-mole g^{-1} $volley^{-1}$)	115·7 ± 11·3 (1·0 p-mole g^{-1} $volley^{-1}$)	88·2
10 Hz¶ (4)	2280·2 ± 1187·5 (3·54 p-mole g^{-1} $volley^{-1}$)	4690·5 ± 310·6 (5·5 p-mole g^{-1} $volley^{-1}$)	649·8 ± 67·4 (1·0 p-mole g^{-1} $volley^{-1}$)	71·7

* After the tissue had been kept in K-free Krebs solution for 2 hr, K^+ was readmitted. 10 min after the K^+ had been readmitted the release to stimulation was measured.

† Collection period, 10 min.

‡ Collection period, 10 min.

§ Collection period, 5 min.

¶ Collection period, 1 min.

Krebs solution, eserine sulphate, 2 μg/ml. Supramaximal field stimulation (12 V cm^{-1}). The output per volley is indicated in brackets and calculated as described by Paton & Vizi (1969): the resting release measured in the same experiment is deducted from the total output released during the period of stimulation and the result is divided by the number of stimuli.

value. The transient increase in ACh release in response to readmission of K^+ and Ca^{2+} might be due to a fast Ca^{2+} influx. This, in fact, is evidenced by the finding that the readmission of K^+ in the absence of Ca^{2+} did not result in a transient increase of ACh release, there was even a reduction: the output of ACh was reduced from 160·5 to 47·0 p-mole g^{-1} min^{-1} (Fig. 4*C*). Readmitting the Ca^{2+} now, when the effect of readmission of K^+ had completely developed, caused no increase in ACh release, an effect otherwise seen on Ca readmission (Fig. 4*A*).

In the foregoing experiments it was shown that the removal of K enhances the release of ACh and the readmission of K results in a reduction of the release. It was then decided to determine whether or not the inhibitory effect of the Na pump stimulation on ACh release was frequency dependent. Table 1 shows the data obtained. Ten min after the K had been readmitted to the tissue which had been immersed in a K-free Krebs solution, the output per stimulus of ACh was measured. This procedure markedly reduced the output during sustained stimulation both at low (0·1 Hz) and high (10 Hz) frequency. Although the output per stimulus was smaller the higher the frequency of stimulation applied (Table 1), the readmission of K was still able to reduce the release at high frequency stimulation. At 0·1 Hz stimulation the reduction was 96·1 % while at 10 Hz it was of 71·7 %.

Since it has been shown (Vizi, 1973) that in Auerbach's plexus the stimulation of ganglion cells leads to a significant increase of ACh release, we also studied how the membrane ATPase stimulation influences the release of ACh induced by DMPP, as opposed to field stimulation. It was observed (Table 2) that DMPP, a well-known ganglion stimulant, enhanced the resting release of ACh. When the longitudinal muscle strip with attached Auerbach's plexus had been kept in

TABLE 2. The inhibitory effect of membrane ATPase stimulation on ACh release induced by ganglionic stimulation in Auerbach's plexus. Longitudinal muscle strip of guinea-pig ileum with attached Auerbach's plexus

	ACh release (p-mole g^{-1} min^{-1} (S.E. of mean))		
	Control	K^+-free (inhibition of membrane ATPase)	*Readmission of K^+ (stimulation of membrane ATPase)
—	260·5 ± 10·8 (3)	585·4 ± 46·3 (3)	46·3 ± 5·6 (3)
DMPP, 10^{-5} M	686·8 ± 96·5 (3)	1215·6 ± 210·5 (3)	41·3 ± 8·3 (3)

* After the longitudinal strip had been kept in K-free Krebs solution for 2 hr, K^+ was readmitted. The tissue was exposed to DMPP for 2 min, 15 min after K^+ had been readmitted. Krebs solution, eserine sulphate, 2 μg/ml. The number of experiments is indicated in brackets.

K-free Krebs solution, DMPP was still able to release ACh. However, when the tissue had been kept in K-free solution for 120 min and K was readmitted, DMPP then failed to release ACh, under conditions when the Na pump was stimulated. 40–50 min after K^+ ions had been readmitted DMPP was again capable of releasing ACh.

Effects of K substitution with Rb or Cs ions on ACh release

The reactions of Rb^+ are essentially similar to those of K^+. When the K^+ was replaced by RbCl (5·9 mM) the release of ACh was not changed. Furthermore, the readmission of K^+ to the tissue which had been exposed to RbCl (5·9 mM) failed to reduce the ACh release (Fig. 5*A*). However, when Rb was added to the tissue which had been incubated in K-free Krebs solution, the release of ACh was reduced from 509·5 to 40·2 p-mole g^{-1} min^{-1} (Fig. 5*B*). The action of Cs^+ however is quite different from that of Rb^+ and K^+. Cs^+ is not able to replace K^+ ions in stimulating (Na^+–K^+)-activated ATPase as shown by Skou (1965). If this is true and there is really a correlation between the stimulation of membrane ATPase and inhibition of ACh release, the administration of Cs^+ ions to tissue which had been exposed to K-free solution should not result in an inhibition of the release. Fig. 5*C* shows this to be the case. The admission of Cs^+ failed to produce a reduction of ACh release: there was even a further increase (Fig. 5*C*). The effect of replacing extracellular K^+ by Cs^+ on the ACh release is illustrated in Fig. 6. Initially the resting output of ACh was $187·9 \pm 37·3$ p-mole g^{-1} min^{-1} in a solution containing 5·9 mM-K^+. About 5 min after replacement of the original Krebs solution with one containing 5·9 mM CsCl instead of KCl and KH_2CO_3 the resting output of ACh had increased twofold. The ACh release in response to stimulation with 1 Hz was enhanced by this procedure from $791·9 \pm 188·2$ to $4276·5 \pm 1052·0$ p-mole g^{-1} min^{-1} ($P < 0·01$; $n = 3$). The calculated output per volley was enhanced from 11·2 p-mole g^{-1} $volley^{-1}$ to 50·0 p-mole g^{-1} $volley^{-1}$. In those experiments in which the release of ACh was augmented in K^+-free Cs^+-containing solution, return to the control Krebs solution produced an immediate decline toward the control value (Fig. 6).

Effect of Na^+–K^+-activated ATPase inhibition and stimulation on the release of labelled NA from isolated rat iris

After the rat irides had been incubated with [^{3}H]NA, the tritiated NA and its metabolites (Langer, 1970) released spontaneously were determined by scintillation counting. The resting output collected in 2·5 min became fairly stable in 30–40 min: the release amounted to

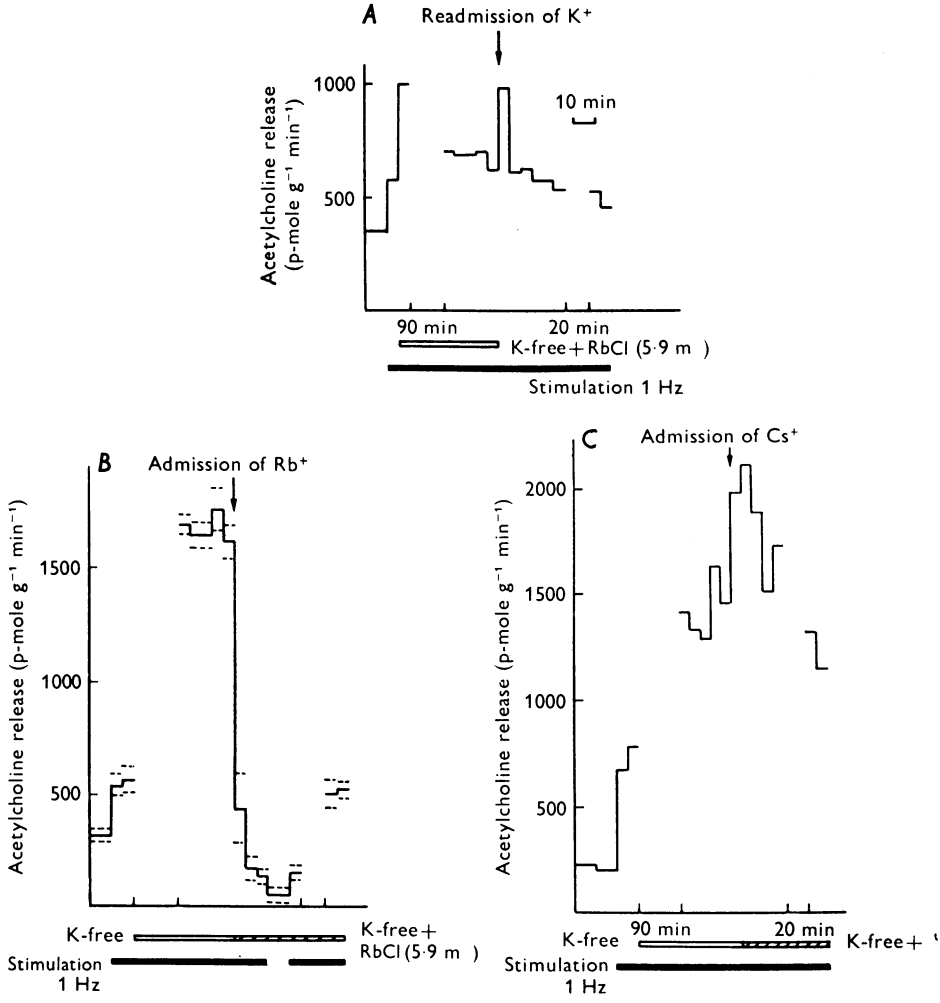

Fig. 5. Effect of K substitution by Rb or Cs on the release of ACh in response to stimulation with 1 Hz. Guinea-pig ileal longitudinal muscle strip with attached Auerbach's plexus. Ordinate: rate of ACh release, p-mole g^{-1} min^{-1}. Abscissa: time (min) as indicated in Fig. 5*A*. Changes in Krebs composition are indicated by horizontal bars. *A*, the effect of Rb (5·9 mM) substitution for K on the release of ACh. Two identical experiments. Note that Rb effectively replaced K. *B*, the effect of the admission of Rb to tissue which had been immersed in K-free Krebs solution for 115 min. The values of ACh release are the means of two experiments with identical schedules. Note the effect of Rb: it is like K (see Fig. 2) and reduces the release of ACh when it is added after bathing the tissue in K-free solution. *C*, effect of Cs admission to tissue which had been kept in K-free Krebs solution for 115 min. The values of ACh are the means of two identical experiments. Note that Cs cannot replace K.

$2{\cdot}23 \times 10^3$ d.p.m./2·5 min even in the absence of K. After 50–70 min in K-free solution the release started to increase, reaching a value of $5{\cdot}9 \times 10^3$ d.p.m./2·5 min. However, when K^+ was readmitted the release was reduced even below the control value (Fig. 7).

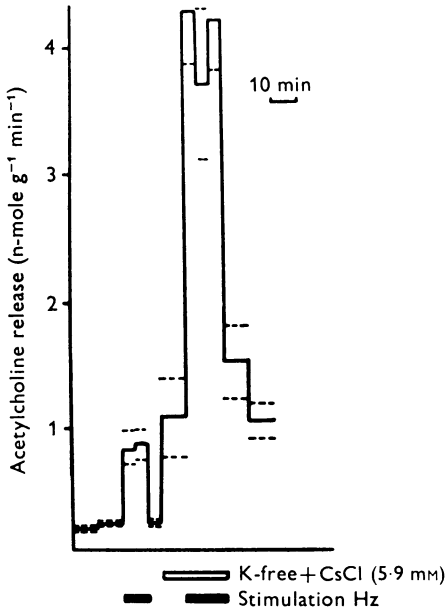

Fig. 6. The effect of replacement of K by Cs on the release of ACh induced by stimulation with 1 Hz. Guinea-pig ileal longitudinal muscle strip with attached Auerbach's plexus. The values of ACh release are the means of four identical experiments; dashed lines indicate S.E. Ordinate: rate of ACh release, n-mole g^{-1} min^{-1}. Abscissa: time (min) as indicated. Note that after 10 min loading in K-free Krebs solution adding CsCl caused a (5·9 mM) rapid rise in ACh release. Readmission of K failed to reduce the release of ACh.

DISCUSSION

In preceding papers (Paton *et al.* 1971; Vizi, 1972, 1974, 1975*a*, *b*) it has been shown that a variety of experimental conditions known to lead to inhibition of $(Na^+ - K^+)$-activated ATPase are able to trigger ACh release from Auerbach's plexus and cortical slices even in the absence of Ca^{2+}. A similar observation was made by Baker & Crawford (1975) on motor nerve terminals, where ouabain and the removal of K enhanced the m.e.p.p. frequency in Ca-free Ringer solution. Baker & Crawford suggest that their results could be explained if the rise in $[Na]_i$ or fall in $[K]_i$, that occur as a result of Na-pump inhibition, either cause release

of Ca from internal stores or an increased effectiveness of internal Ca^{2+} ions in releasing transmitter. The other explanation that has been put forward (Paton *et al.* 1971; Vizi, 1972, 1975*a*) is that the Na pump *per se* is directly involved in the release mechanism of ACh, inhibition of the

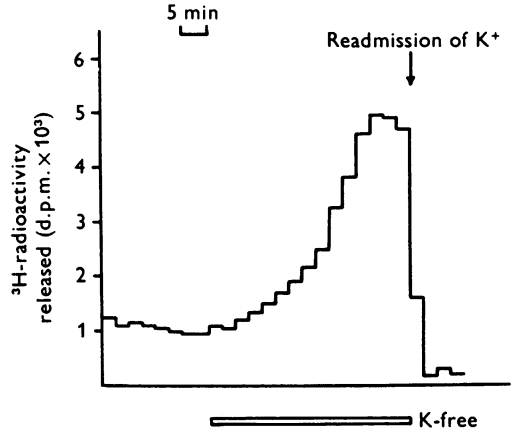

Fig. 7. NA release from isolated rat iris induced by K deprivation and fast reduction in the release when K was readmitted to the tissue which had been kept in K-free Krebs solution for 85 min. The radioactivity released in 2·5 min was measured (Farnebo & Hamberger, 1971). Ordinate: radioactivity released, d.p.m./2·5 min. No attempt was made to separate [^{3}H]NA and tritiated metabolites also released. The values are the means of two identical experiments. Rats used were 120 and 135 g in weight. Note the increase in the release of NA in response to the deprivation of K and fast decrease in the release when K was readmitted.

pump provoking ACh release. This paper supports the latter suggestion by providing evidence that the stimulation of the Na pump leads to a total inhibition of the release of ACh, either induced by nerve stimulation, or during rest. It is also shown that a similar mechanism operates on NA release.

Stimulation of Na pump and inhibition of ACh and NA release

Stimulation of the Na pump was induced by readmitting K^+ ions to preparations previously exposed to K^+-free solutions. The procedure has been shown to be effective in mammalian non-myelinated nerve by Rang & Ritchie (1968*a*) and in the smooth muscle of guinea-pig ileum by Bolton (1973), and depends on the fact that the Na-pump is switched off in K^+-free solution, allowing accumulation of Na in the cell, and greatly increased Na-pump activity on reintroduction of K^+.

This procedure is thus only effective with preparations that allow easy access of the bathing solution to all the cells, so that K^+ concentration immediately adjacent to the cells falls low enough in K^+-free solutions to inhibit the pump. The guinea-pig ileum is quite suitable, as the preparation is very accessible. In the striated muscle neuromuscular junction leakage of K^+ from the cells makes it difficult to inhibit the ATPase in K^+-free solution in a predictable manner (Keynes, 1954; Birks & Cohen, 1968) unless special superfusion techniques are used. With such techniques Baker & Crawford (1975) succeeded in showing the stimulating effect of K^+ removal on the resting release of ACh from motor nerve terminals (shown by an increase in m.e.p.p. frequency).

In our experiments, when K^+ was omitted from the Krebs solution, the ACh release during the resting state and in response to stimulation was increased. The ACh output per stimulus was enhanced at both low and high frequency of stimulation. On readmission of K^+ ions, there was an immediate reduction in the rate of ACh release. Rb^+ but not Cs^+ was capable of substituting K^+ in ACh release. Therefore, it seems that the nerve terminals cannot discriminate between K^+- and Rb^+-ions, a fact also true for membrane ATPase (Skou, 1965) and for activation of Na pump in mammalian non-myelinated nerve fibre (Rang & Ritchie, 1968*a*).

This reduction of ACh release on readmitting K^+ ions cannot be caused by a direct effect of K^+ ions on the nerve terminals as predicted by the Nernst equation, since this should produce depolarization and increase in transmitter release. It is almost certainly due to the greatly increased activity of the membrane ATPase which will occur when K is readmitted.

Garcia & Kirpekar (1973, 1975) reported that there is a marked release of noradrenaline from cat spleen slices in Na-free solution which is not dependent on extracellular Ca. Lastoweczka & Trifaró (1974) perfusing bovine adrenal glands with Na-free Locke solution observed that there is a sharp increase in the release of catecholamines either in the presence or absence of extracellular Ca^{2+}. It was also shown that ouabain, a rather selective membrane ATP ase inhibitor, promotes the release of NA from isolated rat vas deferens even in the absence of Ca and presence of tetrodotoxin (Vizi, 1975*b*), under conditions which exclude the enhanced Ca-influx and the spontaneous firing that are otherwise expected to occur and could account for the enhanced release.

In a discussion of the reduction of ACh and NA release by readmission of K^+ ions the following mechanisms might be taken into account.

Hyperpolarization. It can be suggested that resumption of membrane ATPase activity on readmission of K^+-ions results in hyperpolarization which may account for inhibition of ACh release. Hyperpolarization was, in fact, observed on smooth muscle (Tomita & Yamamoto, 1971) and C-fibres (Rang & Ritchie, 1968*a*). It is unlikely that the readmission of K^+ ions reduced the release of ACh by changing the membrane potential

by hyperpolarizing the nerve terminals, since it has been shown that hyperpolarization increases the quantal content of the e.p.p. (Hubbard & Willis, 1968) but not the m.e.p.p. frequency (Liley, 1956*a*). On the other hand, if the nerve endings are depolarized, the m.e.p.p. frequency is enhanced (Liley, 1956*b*) but the quantal content reduced (Hubbard & Willis, 1968; Takeuchi & Takeuchi, 1962). However, in our experiments the output per stimulus was reduced when the hyperpolarization was expected to occur.

Accumulation of $[K^+]_i$. Although stimulation of the membrane ATPase may lead to accumulation of $[K^+]_i$ in the terminals this is also unlikely to reduce the rate of ACh release. By activating the membrane ATPase with Rb^+ instead of K^+, the raised Na^+ inside the terminals present after the period in K-free Krebs solution should fall, but there should be no corresponding increase in the intracellular K^+ concentration. However, the release of ACh was reduced to the same extent by the readmission of Rb^+ as by the readmission of K^+ (Fig. 5*B*). Baker & Crawford (1975) suggested that the fall in the intracellular K^+ concentration might in some way underlie the rise in m.e.p.p. frequency observed in K-free Ringer solution, but they also observed that the m.e.p.p. frequency recovered completely after the admission of Rb^+ which suggests that intracellular K^+ can be replaced by Rb.

Loss of $[Na^+]_i$. Activation of membrane ATPase by the readmission of K^+ results in a rapid reduction in $[Na^+]_i$ as well as gain in K^+. Table 3 shows the data obtained in vagus C-fibres which are, in fact, a good model for the non-myelinated nerve terminals. In normal Krebs solution the values (m-mole kg^{-1} fibre water) were: Na, 70·4; Ca 11·3. In K-free Krebs solution the corresponding values for Na and Ca were 109·7 and 12·0 m-mole kg^{-1}. These values for the internal Na content are not different from those reported by Rang & Ritchie (1968*b*). They also reported that the cell K falls in K^+-free solution from 165·5 to 121·7 m-mole kg^{-1}. Exposure of the tissue kept in K^+-free solution to a Krebs solution containing 5·9 mM-K resulted in a rapid ouabain-sensitive loss of Na^+. The cell Na content (44·9 m-mole kg^{-1}) falls even below the control value (70·4 m-mole kg^{-1}). The release of ACh was inhibited in this condition (see Figs. 1 and 2). Although Rang & Ritchie (1968*b*) concluded that only a small fraction of the total extrusion of Na takes place by an electrogenic mechanism, the rapid reduction of Na^+ obtained in our experiments in response to K-readmission indicates that the Na pump also plays a role in maintaining the $[Na^+]_i$ in C-fibres. There is growing evidence that accumulation of $[Ca^{2+}]_i$ by mitochondria is dependent on the $[Na^+]_i$. In this way it would be easy to explain any increase of ACh release in response to Na pump inhibition as a consequence of accumulation of $[Na^+]_i$. In Na-free

Krebs solution, however, when the ($Na^+ - K^+$)-activated ATPase is also inhibited, there was a significant increase in transmitter release (Vizi, 1972; Garcia & Kirpekar, 1973) even in the absence of Ca. Under this condition although the intracellular Na content fell to 26·0 m-mole kg^{-1} (Table 3) the release of ACh was increased (Paton *et al.* 1971; Vizi, 1972), even in those cases when Ca-influx was excluded, and the intracellular Ca level was also decreased (Table 3). This fact indicates that the reduction of the intracellular Na^+ concentration does not control the release rate of ACh.

TABLE 3. Changes in ion content of rabbit desheathed vagus nerves in response to removal and readmission of K^+ ions

	Intracellular content (m-mole kg^{-1} wet wt.) (S.E. of mean)		
	Na^+	Ca^{2+}	Mg^{2+}
Control* (1)†	31·5	3·2	25·1
Control, 120 min loading (5)	70·4 ± 4·8	11·3 ± 2·8	25·6 ± 2·1
K-free, 120 min loading (4)	109·7 ± 9·7	12·0 ± 1·35	24·2 ± 1·3
K^+-free, 120 min loading and readmission of K^+ (5·9 mM) for 10 min (7)	57·3 ± 10·1	49·7 ± 9·9	25·1 ± 6·4
K^+-free, 120 min loading and readmission of K^+ (5·9 mM) for 30 min (4)	44·9 ± 1·0	12·0 ± 2·6	18·9 ± 1·7
K^+-free, 120 min loading and readmission of K^+ (5·9 mM) + ouabain (2×10^{-5} M) for 30 min (3)	76·0 ± 3·6	50·3 ± 14·3	20·5 ± 2·1
K^+-free + Ca^{2+}-free + EGTA 1 mM, 120 min (5)	101·7 ± 3·6	0·99 ± 0·3	22·6 ± 0·8
K^+-free + Ca^{2+}-free + EGTA 1 mM, 120 min loading and readmission of K^+ (5·9 mM) in Ca-free Krebs soln. (EGTA 1 mM) for 30 min (5)	51·3 ± 9·1	1·13 ± 0·3	21·2 ± 0·9
Na^+-free,‡ 120 min loading (5)	26·0 ± 2·8	22·6 ± 1·1	23·6 ± 1·6
Na^+-free + Ca-free + EGTA 1 mM, 120 min loading (5)	31·5 ± 3·2	2·8 ± 0·4	21·5 ± 1·8

* Ionic content has been measured immediately after the vagus had been dissected (3 min).

† Number of experiments is in brackets.

‡ In Na-free solutions NaCl was replaced by sucrose to maintain osmolality and $NaHCO_3$ was substituted by $KHCO_3$.

Change in Ca fluxes. It has been shown that the admission of Ca^{2+} to tissue which had been kept in Ca-free Krebs solution induced a fast and prolonged rise in the release of ACh. A similar but transient increase in release was also seen when K^+ and Ca^{2+} were together readmitted to tissues. If K^+ was readmitted before Ca^{2+}, the addition of Ca^{2+} no longer stimulated the release of ACh. This suggests that an increase of Ca influx can in some way trigger ACh release. However, during stimulation of the Na pump, when K^+ was readmitted to vagus C-fibres which had been loaded in K-free Krebs solution, an increase in Ca-influx was detected (Table 3). The Ca content rose from $12{\cdot}0 \pm 1{\cdot}4$ to $49{\cdot}1 \pm 9{\cdot}9$ m-mole kg^{-1}, and yet the release of ACh was inhibited. An increase in Ca influx is however not necessary for inducing ACh release, since the removal of K^+ in the Krebs solution could cause release of ACh in the absence of extracellular Ca^{2+} (EGTA being present), although the amount released was less than in the presence of Ca^{2+}. In Ca-free solution the influx is excluded, although (Baker, 1972; Baker & Glitsch, 1973) there is still a possibility that the Ca efflux, e.g. in exchange for Na^+, is enhanced, and Ca^{2+} passing the membrane from inside to outside might cause release of ACh when K^+ is removed.

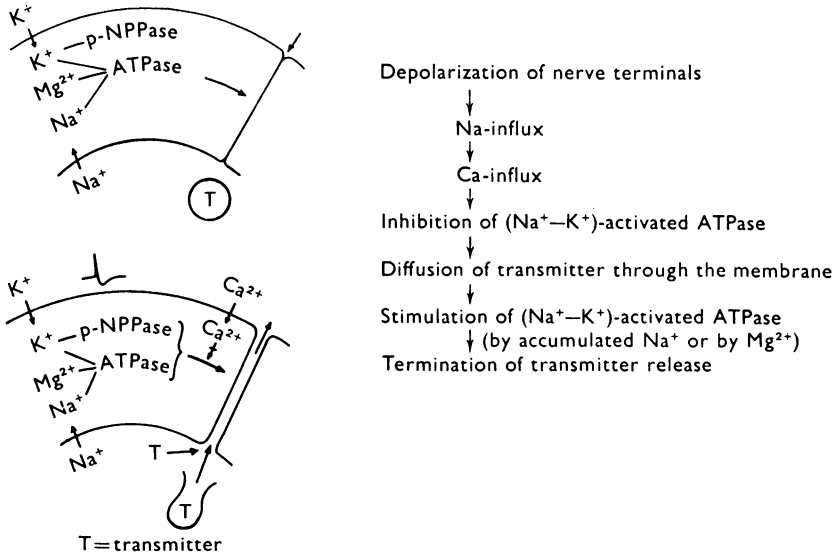

Fig. 8. Model of the proposed mechanism by which (Na^+–K^+-activated ATPase acts as a trigger of ACh release. It is proposed that Ca entry into the membrane as a result of the change in Na^+ concentration during action potential (Baker & Blaustein, 1968; Baker, Blaustein, Hodgkin & Steinhardt, 1969; Baker, 1972) and the subsequent transient inhibition of (Na^+–K^+)-activated ATPase is the underlying mechanism in the physiological release of transmitters.

Since it is very unlikely that either hyperpolarization or change in Na^+ or K^+ concentration can account for the inhibition of ACh or NA release, it is suggested that the stimulation of membrane ATPase *per se* is responsible for the reduction or inhibition of transmitter release.

The influx of Ca^{2+} could then cause transmitter release by inhibiting the ATPase (Fig. 8). During stimulation of the membrane ATPase, although Ca might enter the cell, its inhibitory effect on the enzyme might be prevented and transmitter release terminated. It has been shown that Mg^{2+} enters axons during electrical activity, perhaps via the late Ca conductance pathway (Baker & Crawford, 1972), and this influx of Mg may limit the extent of inhibition of $(Na^-{}^+K^+)$-activated ATPase by Ca^{2+}.

One possible objection to this hypothesis is that the turnover time of the Na pump is rather long compared to the time taken to mobilize transmitter in response to nervous stimulation. It seems quite possible that inhibition of the pump and subsequent initiation of transmitter release could occur in less than the time required for operation of a complete pump cycle.

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