Biochemical Pharmacology, Vol. 36, No. 6, pp. 803–807, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

INHIBITION BY *P*-NITROPHENYLPHOSPHATE OF ACETYLCHOLINE RELEASE INDUCED BY Na^+-DEPRIVATION

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(*Received* 24 *June* 1986, *accepted* 8 *September* 1986)

Abstract—The effect of *p*-nitrophenylphosphate (*p*-NPP) on the release of acetylcholine evoked by drugs and ionic environments known to inhibit Na^+,K^+-ATPase was studied in isolated cortical slices of rat brain and longitudinal muscle strip of guinea-pig ileum. *p*-NPP inhibited the release of acetylcholine induced by sodium deprivation provided that the circumstances were in favour of the function of the K^+-activated part of ATPase. However, it failed to antagonize the increase in the acetylcholine release elicited by omission of K^+ or by administration of ouabain. Therefore it is concluded that the K^+-stimulated phosphatase moiety of the Na^+, K^+-ATPase might be involved in the release of acetylcholine.

That the release of acetylcholine is connected with the activity of Na^+, K^+-activated ATPase was first proposed in 1971 by Paton *et al.* [1]. Since then a great body of evidence has accumulated that the membrane ATP-ase ("sodium pump") has a substantial role in the regulation of the resting release of transmitters both in the peripheral and in the central nervous system [2–6]. It has been shown that whilst inhibition of Na^+, K^+-ATPase results in an increase in transmitter release, stimulation of the enzyme has the opposite effect [7]. It is generally accepted that the operation of ATPase consists of three sequential steps: Na^+-dependent phosphorylation of ATPase which requires the presence of Mg^{2+}, a second step requiring Mg^{2+}, and third by hydrolysis of the phosphate group by a K^+-stimulated phosphatase moiety [8]. A simplified scheme depicted by the upper part of the Fig. 1 shows the above mentioned mechanism and its interaction with the most commonly used inhibitors of the enzyme. Purified membrane preparations isolated from various tissues exhibit an ouabain sensitive neutral phosphatase activity [9–18] which is inhibited by Na^+ and stimulated by K^+ (Fig. 1, lower part). The similarities and the differences between the phosphatase moiety of Na^+, K^+-ATPase [18–23] have been discussed by several workers. It is well established that the K^+-*p*-NPPase is involved in the terminal step of the reaction sequence catalyzed by Na^+, K^+-ATPase [9, 10, 24–26]. *p*-NPP is supposed to phosphorylate Na^+, K^+-ATPase at the "phosphatase" site even at very low concentrations and in the absence of external sodium, to be hydrolysed in a K^+-stimulated step [19, 27]. This reaction provides an opportunity to study the role of the K^+-activated part of the membrane ATPase in the regulation of the release of acetylcholine.

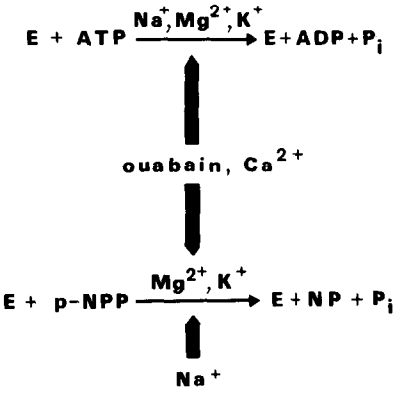

Fig. 1. Simplified scheme for the normal activity of Na^+, K^+-ATPase and K^+, Mg^{2+}-*p*-nitrophenylphosphatase (*p*-NPP). E, enzyme. Vertical arrows indicate inhibitory effects of ouabain, Ca^{2+} and Na^+. The Na^+, K^+-activated ATPase system, which mediates the active flux of Na^+ and K^+ across the cell membrane, consists of two main reaction steps, i.e. a Na^+-dependent phosphorylation and a K^+-dependent dephosphorylation of the phosphorylated compound formed in the first reaction, with an intermediate exchange of cations [10]. The K^+, Mg^{2+}-*p*-nitrophenylphosphatase might well represent the dephosphorylation step of Na^+, K^+-activated ATPase [14].

MATERIALS AND METHODS

Two kinds of *in vitro* preparation have been used to study the release of acetylcholine.

Slices of cerebral cortex. Albino rats of both sexes weighing 120–140 g were used. The animal was stunned by a blow on the neck and immediately decapitated. The brain was removed as rapidly as possible (10 sec) and placed in a moist atmosphere. Slices of cerebral cortex were cut with a blade. Each with a thickness of 0.3–0.8 mm and weighing between 30 and 50 mg. Two or three slices were incubated in 4 ml of Krebs solution at 37° gassed with a mixture of 95% O_2 and 5% CO_2.

Longitudinal muscle strip with Auerbach plexus attached. Guinea pigs of both sexes weighing 300–400 g were used. The longitudinal muscle strips were set up in an organ bath of 3.5 ml capacity in Krebs

solution at 37°, gassed with 5% CO_2 and 95% oxygen [28].

The composition of the Krebs solution was as follows (mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, glucose 11.5, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2. A sodium-free solution was prepared by the isosmotic substitution of the sodium salts with sucrose and the substitution of potassium salts with equimolar concentration of $KHCO_3$. In the experiment serving as a control to the effect of *p*-NPP in Na^+-free Krebs solution, 6 mM NaCl was included in the medium. K^+-free Krebs was prepared by omitting potassium salts.

Eserine sulphate, 2×10^{-6} g/ml, was present throughout the experiments in order to inhibit cholinesterase. Before collecting the first samples for the assay of released acetylcholine, the slices were allowed to equilibrate under resting conditions for 60 min.

Assay of acetylcholine and identification of the spasmogenic substance as acetylcholine. Acetylcholine was assayed biologically according to the method described by Paton and Vizi [28]. Gel filtration (Sephadex G-10 column of 4 ml) was used [29] to separate the substances obtained from isolated tissues and prior to bioassay using guinea-pig ileum. Eluate fractions each of 0.5 ml were tested on guinea-pig ileum and the radioactivity of those fractions having the same elution volume as [^{14}C]-ACh. The elution profile of [^{14}C]-labeled ACh and the spasmogenic substance provided to be identical. This finding and the observation that atropine sulphate (2×10^{-4} M) showed exactly the same time course for inhibiting contractions caused by both ACh and the spasmogenic substance indicate that the spasmogenic substance was ACh. The ACh extraction procedure and the calculation of the rate of acetylcholine synthesis have been described in our earlier paper [30].

Drugs used were: para-nitrophenylphosphate disodium salt (Na_2-*p*NPP, Reanal), ouabain and acetylcholine iodide (The British Drug Houses Ltd.), physostigmine sulphate (Burroughs Wellcome), morphine hydrochloride (Alkaloida, Hungary). The concentration of all drugs is expressed as mol l^{-1}. The materials were dissolved in distilled water and administered in volumes less than one tenth of that in the organ bath.

The statistical significance of two experiments made in the presence and absence of pNPP were assessed by Student's *t*-test for paired data. Data are presented as mean ±SEM.

RESULTS

Effect of p-*NPP on the release of ACh induced by the inhibition of* Na^+, K^+-*ATPase*

After replacing normal Krebs solution with Na^+-deficient Krebs solution, the resting release of ACh from cortical slices promptly increased from 31.5 ± 1.5 (N = 8) to 57.1 ± 3.7 (N = 8) pmol g^{-1} min^{-1} (Fig. 2a). This increase was significant ($P < 0.01$) but when *p*-NPP at a concentration of 3 mM was present in the solution, Na^+-deprivation failed to enhance the rate of ACh. When the tissue which had been kept in Na-deficient solution was exposed to K^+-excess the release was transiently increased (Fig. 2a). In the presence of p-NPP, high K^+ (25.0 mmol l^{-1}) did not release ACh (Fig. 2b). The removal of extracellular Na^+ led to an inhibition of membrane ATPase and thereby to an increase of ACh release [2].

Since it has been shown that ouabain releases acetylcholine by inhibiting Na^+, K^+-ATPase, we have studied whether or not *p*-NPP is able to prevent this effect. Figure 3 a and b show the data obtained in cortex slices of rat brain. *p*-NPP did not influence the effect of ouabain, the maximal output induced by ouabain in the absence and presence of *p*-NPP being 118.1 ± 14.4 (N = 4) and 99.7 ± 4.1 pmol g^{-1} min^{-1} (N = 3), respectively. This difference is not significant, ($P > 0.5$).

When cortical slices were kept for 60 min in K^+-free Krebs solution the release of ACh did not significantly increase: amounting to 34.6 ± 2.9 pmol g^{-1} min^{-1} in normal and 37.9 ± 5.4 pmol g^{-1} min^{-1} in K^+-free Krebs solution (three experiments). This is a very unexpected result in the light of the fact that K^+-deprivation produces a rapid inhibition of membrane ATPase [9]. However, it has been shown by other authors [31, 32] that Na^+ transport may occur when the K^+ is removed from the bathing solution. This has been attributed to the leakage of K^+ out of the tissue thereby sustaining ATPase activity. This could be the case with cortical slices also where the removal of potassium from the extracellular space around the nerve terminals may be insufficient to prevent accumulation of potassium leaking from the tissue whereas the longitudinal muscle strip with Auerbach plexus attached is extremely accessible to extracellular solutions and has been shown [2, 33] to be very sensitive to omission of extracellular K^+ and to release ACh in response to K^+ deprivation. The effect of *p*-NPP on release induced by K^+-deprivation was therefore investigated using the latter preparation. Figure 4 shows that p-NPP did not affect the release of ACh resulting from the omission of K^+.

The effect of p-*NPP on the synthesis rate of ACh in rat cortical slices*

The effect of *p*-NPP on the rate of the synthesis of ACh has been studied in three experiments. Table 1 shows that the tissue exposed to 3 mM p-NPP synthetizes ACh at the same rate as control tissue. While the release of ACh induced by Na^+-deprivation was completely prevented by the addition of *p*-NPP, the inhibition of the synthesis which resulted from Na^+-deprivation [1] was not influenced. Although excess K^+ enhanced the rate of synthesis, in Na^+-deficient solution it failed to increase it. This may be the reason for the transient ACh release in response to excess K^+, which is otherwise maintained until K^+ is removed.

DISCUSSION

It has been suggested [3, 34] that the actual activity of Na^+, K^+-ATPase influences membrane permeability, and thereby transmitter release. In the present study we have attempted to examine more closely the connection between the release of ace-

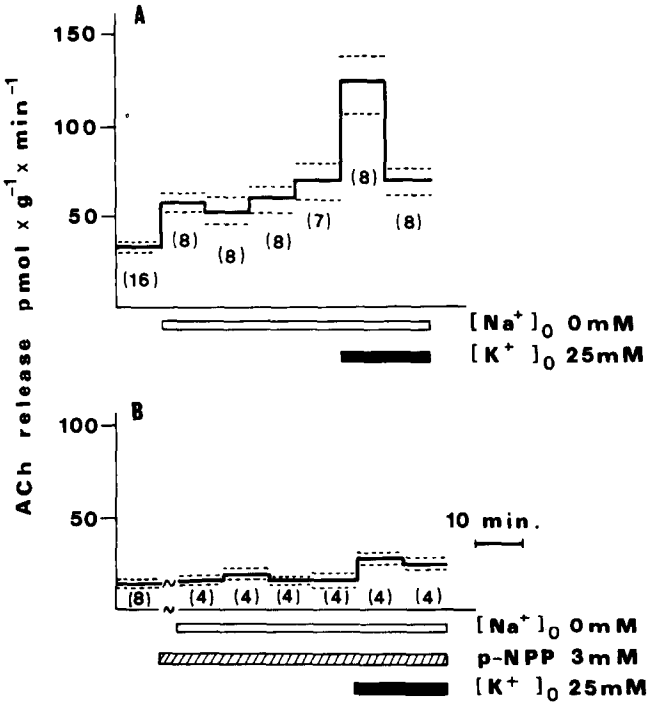

Fig. 2. Effect of *p*-nitrophenylphosphate (*p*-NPP) on the release of ACh (B) induced by Na^+-deprivation and by K^+-excess (A) in rat cortical slices. Experimental procedures are as described in Materials and Methods. Horizontal bars represent the mean, broken lines represent SEM and the number of experiments is indicated in parentheses.

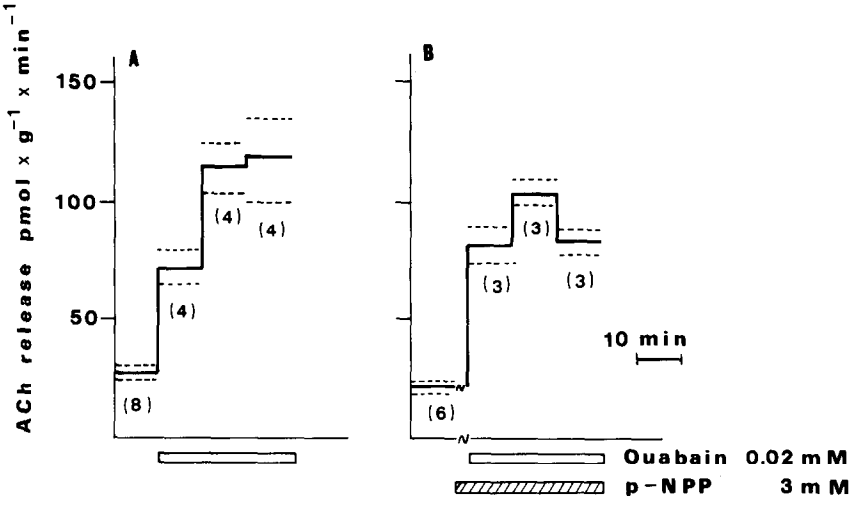

Fig. 3. Effect of pNPP (B) on the release of ACh induced by ouabain (A) in rat cortical slices. Experimental procedures are as described in Materials and Methods. Horizontal bars represent the means ± SEM (broken lines) and the number of experiments is indicated in parentheses.

tylcholine and Na^+, K^+-ATPase. The sodium pump can be considered to exhibit a number of partial reactions which we have attempted to relate to increases in ACh release. Na^+-withdrawal affects the first reaction step of the enzyme. In these circumstances the phosphatase part of the ATPase is still capable of splitting some simple phosphates (e.g. *p*-NPP) which have the ability to phosphorylate the "phosphatase" site of the enzyme directly [19]. Other manipulations (K^+-deprivation, ouabain administration) exert their inhibitory effect on the last, K^+-activated part of ATPase [8, 9, 35, 36]. The effect of inhibitors on the second step as well as the effect of stimulation of ATPase (norepinephrine administration, K^+-readmission following K^+-withdrawal) on the release of acetylcholine and other transmitters has been described previously [7, 37–41]. It has been shown that there is close parallelism between the

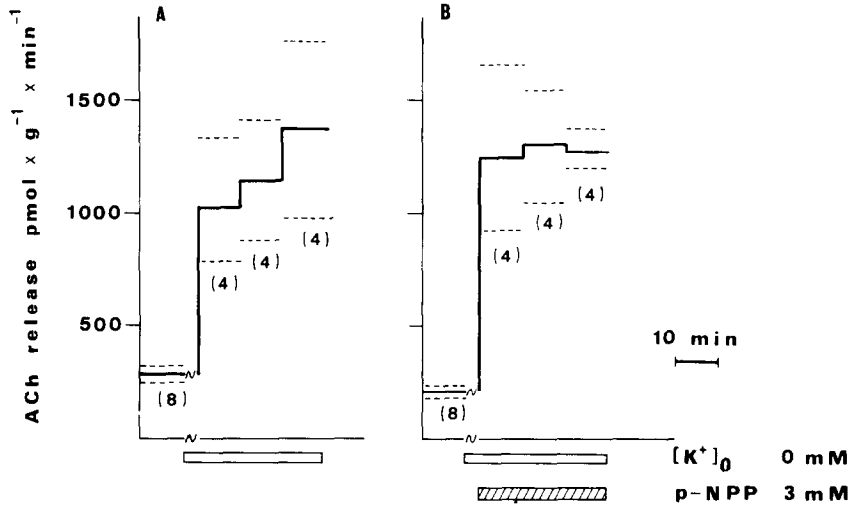

Fig. 4. Effect of pNPP on the release of ACh (B) induced by omission of K^+ (A) in guinea-pig ileum myenteric plexus. Experimental procedures are as described in Materials and Methods. Horizontal bars represent the means ± SEM (broken lines). The number of experiments is indicated in parentheses.

Na^+, K^+-ATPase activity and the release of acetylcholine. It can be stated that all circumstances in which the enzyme is inhibited increase the Ca^{2+}-independent release [37, 42, 43] of transmitters, whereas those which stimulate it have the effect of inhibiting release [34]. The withdrawal of sodium or potassium, several drugs and ions (cardiac glycosides, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), Ca^{2+}, Ba^{2+}) have been described as inhibiting the Na^+, K^{2+}-ATPase [36, 44–49] and releasing acetylcholine [2, 34]. On the other hand, norepinephrine [18], magnesium ions [35, 36] and readmission of K^+ following K^+-withdrawal stimulate the Na^+, K^+-ATPase (or *p*-NPPase) and decrease the transmitter release [34].

As reported in this paper, *p*-NPP prevents the increase in the release of acetylcholine elicited by Na^+-withdrawal provided that potassium is present to activate the *p*-NPPase. However, *p*-NPP proved to be ineffective when the acetylcholine release was stimulated by K^+-deprivation or ouabain, i.e. factors inhibiting the potassium-stimulated reaction step of the enzyme. Therefore it is concluded that the K^+-activated phosphatase moiety of the ATPase may be the important factor in the regulation of the release of acetylcholine, and possibly of other transmitters.

There are several possible explanations of the mechanism by which *p*-NPP prevents the release of acetylcholine. The energy liberated by the splitting of *p*-NPP is comparable to that of ATP [50]. If we assume that the decrease of membrane permeability and suppression of acetylcholine release is an active process requiring energy in a form that is normally supplied by ATP-hydrolysis, we may also assume that, in circumstances where the hydrolysis of ATP is impaired, its substitution by a suitable exogenous substrate could maintain this active suppression. It has been found that the presence of *p*-NPP in Na^+-free media prevents Ca^{2+}-influx into the C-fiber preparation (E. S. Vizi and T. Torok, unpublished). This possibility that inhibition of Ca^{2+} influx may account for the inhibition of ACh release is unlikely because the release of ACh by Na^+-deprivation is mainly independent of Ca^{2+} [2, 51]. This is true also for noradrenaline release [38]. It was of interest that *p*-NPP also prevented the release of ACh in response to excess K ions when Na ions were removed. This effect of *p*-NPP can be explained by its interference

Table 1. Effect of *p*-nitrophenylphosphate (*p*-NPP) on the synthesis of acetylcholine. Cortical slice of the rat

Treatment	Rate of ACh synthesis* (ACH nmol g^{-1} hr^{-1})	Significance** P
1. —	1.76 ± 0.21 (4)	
2. *p*-NPP (3 mM)	1.91 ± 0.30 (4)	2:1 >0.5
3. Na-deprivation	0.34 ± 0.03 (4)	3:1 <0.01
4. Na-deprivation + *p*-NPP (3 mM)	0.39 ± 0.08 (4)	4:1 <0.01
5. K-excess (25 mM)	4.48 ± 0.61 (4)	5:1 <0.01
		6:3 >0.5
6. Na-deprivation + K-excess (25 mM)	4.46 ± 0.11 (4)	6:5 <0.01
7. Na-deprivation + K-excess (25 mM) + *p*-NPP (3 mM)	0.43 ± 0.08 (4)	7:6 >0.5

* Exposure time, 1 hr. The content of ACh was measured just before the experiments and after 3 times 20 min collection period.

** Student *t* test. Number of experiments in parentheses.

with Ca^{2+} influx since the release of ACh evoked by excess K^+ depends on $[Ca^{2+}]_o$.

In summary, *p*-NPP inhibited the release of ACh evoked by sodium deprivation provided that the circumstances favoured the continued function of the K^+-activated part of ATPase. It is concluded that the K^+-stimulated phosphatase moiety of Na^+, K^+-ATPase might play an important role in the mechanism of ACh release.

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