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EFFECT OF PHALLOIDIN ON Mg^{2+}-ATPase, (K^+-Na^+)-ATPase AND K^+-DEPENDENT *p*-NITROPHENYL PHOSPHATASE ACTIVITY OF PLASMA MEMBRANES ISOLATED FROM RAT LIVER

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Abstract—In the perfused isolated rat liver phalloidin induces a rapid release of potassium. A similar effect is produced by a high concentration of ouabain in the perfusion medium. Therefore the effect of phalloidin on plasma membrane preparations of rat liver has been tested. In fresh membrane preparations phalloidin does not affect the specific activity of Mg^{2+}-dependent ATPase, (Na^+-K^+)-ATPase and K^+-dependent *p*-nitrophenyl phosphatase. In membrane preparations dialysed against distilled water treatment with phalloidin causes an nonspecific inhibition of the enzymes which can not always be reproduced. It is suggested, that phalloidin acts by the formation of a potassium channel and not by inhibiting the cation pump.

THE MAIN toxic activity of the mushroom *Amanita phalloides* is due to the cyclopeptic poisons phalloidin and alpha-amanitin.[1–3] Phalloidin acts rapidly in mouse, rat and man producing degenerative destruction which is exclusively located in the liver, whereas alpha-amanitin shows a more delayed action.[1] Recently it has been demonstrated in our laboratory that in the isolated perfused rat liver the primary response to phalloidin, detectable by biochemical methods, is a rapid loss of potassium. Simultaneously with the release of potassium, sodium is being introduced into the perfused liver in equimolar amounts.[4, 5] Thus it is probable that the primary response to phalloidin consists in the increased permeability to potassium. It would be of great interest to determine the effect of phalloidin on (K^+-Na^+)-ATPase activity of purified rat-liver plasma membranes. In the present paper the effect of phalloidin on Mg^{2+}-ATPase, (Na^+-K^+)-activated Mg^{2+}-ATPase and K^+-dependent phosphohydrolysis of *p*-nitrophenyl phosphate is reported.

EXPERIMENTS

Experiments with *perfused rat liver* have been carried out as previously described by Frimmer *et al.*[4]

Plasma membranes have been isolated from the liver of female wistar rats, weighing 160–200 g. Isolation has been performed by two methods. Using isotonic medium with sucrose the procedure described by Coleman *et al.*[6] was followed; the preparation in hypotonic medium (1 mM $NaHCO_3$) has been effected according to the method of Neville.[7] However the original technique of Neville hasbeen slightly modified. The

membranes have been centrifuged in the second gradient for 1 hr at 10,000 *g* (r max); the gradient was discontinuous and consisted of 50, 37, 30, 25, 20, 15, 10, 7 and 3% sucrose. The membrane pellets have been tested for enzyme activities and the amount of DNA. Purity has also been checked by electron microscopy.

Enzymes. The activity of the specific plasma membrane enzymes has been tested by 5'nucleotidase (EC 3.1.3.5.),[8] Mg^{2+}-ATPase (EC 3.6.1.4),[9] (Na^{+}-K^{+})-ATPase, and neutral and alkaline *p*-nitrophenyl phosphatase.[10] The absence of microsomes, lysosomes and mitochondria has been proved by tests using the enzyme activities of glucose -6-phophatase (EC 3.1.3.9),[11] acid phosphatase (EC 3.1.3.2)[12] and succinate-tetrazolium reductase (EC 1.3.99.1).[13]

The total ATPase activity has been assesed in a medium containing 5 mM $MgCl_2$, 20 mM KCl, 100 mM NaCl, 3 mM ATP and 150 mM Tris pH 7·4. Mg^{2+}-ATPase was determined using the same medium containing 150 mM Tris instead of KCl and NaCl. Tests were performed in volumes of 1·3 ml medium containing 0·02–0·1 mg membrane protein. These mixtures had been incubated for 15 min at 37°. The activity was measured by the amount of P_i liberated during incubation. (Na^{+}-K^{+})-ATPase activity has been defined as the difference between total ATPase and Mg^{2+}-ATPase. Enzyme activities are given in μmoles of P_i released per mg membrane protein per hr. The effect of phalloidin* on ATPase activity has been tested in concentrations of 20·0–400·0 μg/mg membrane protein. In controls the effect of 10^{-4} molar ouabain (Fluka ag., Buchs-Switzerland). Before assessing the activity of the ATPase one part each membrane membrane preparation has been dialysed against distilled water overnight (12–14 hr) at 4°.

The *p*-nitrophenyl phosphatase activity was measured in a medium containing 5 mM $MgCl_2$, 10 mM KCl, 2·5 mM Tris (pH 8·9 or 7·4) and 5 mM Na_2-*p*-nitrophenyl phosphate. The test tubes (0·4 ml) have been incubated for 5 min at 37°. *p*-nitrophenyl phosphatase activity has been determined using 0·05–0·1 mg membrane protein in each tube. Specific activity is expressed as μmole *p*-nitrophenol liberated per mg membrane protein per hr. In some experiments membranes had been preincubated with 75 μg phalloidin/mg membrane protein for 0, 5, 10 and 30 min at 37°.

Chemical determination. Protein was measured by the method of Lowry *et al.*[14] The amount of DNA in the membrane preparations was analysed as described by Burton,[15] method of Bruce and Dubin [16] has been used for the determination of P_i.

For examination by *electron microscopy* the membrane pellets were fixed with 5% glutaraldehyde in 0·1 M cadodyl buffer (pH 7·2). The postfixation was made in 1% buffered OsO_4 for 1 hr at 4°.

For this experiment the membranes were prepared using the method of Neville.[7]

RESULTS AND DISCUSSION

Results of a typical experiment on isolated perfused rat liver are shown in Fig. 1.† If ouabain is present in the perfusion medium potassium is released from the liver into the medium. Addition of phalloidin in a small dose caused a further release of potassium. This observation supported our previous hypothesis that phalloidin acts by specific inhibition of the (Na^{+}-K^{+})-ATPase, as it is known for ouabain.

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†Preliminary results of this study have been presented at the Congress of Pharmacology 1968, abstracts in: *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.* **260**, 125 (1968).

The purity of plasma membrane fractions has been indirectly demonstrated by tests using various enzymes known to be associated with particular cell fractions. The data obtained in our experiments are comparable to those reported by Coleman *et al.*[6] and Emmelot and Bos.[10] Electron micrographs of the plasma membrane fraction indicated that the preparation was not contaminated with mitochondria and nuclei (Fig. 2a).

The specific activities of the Mg^{2+}-ATPase and (Na^+-K^+)-ATPase, the effect of different concentrations of phalloidin and a standard concentration of ouabain are listed in Tables 1 and 2. In Table 1 plasma membrane preparation is carried out by the

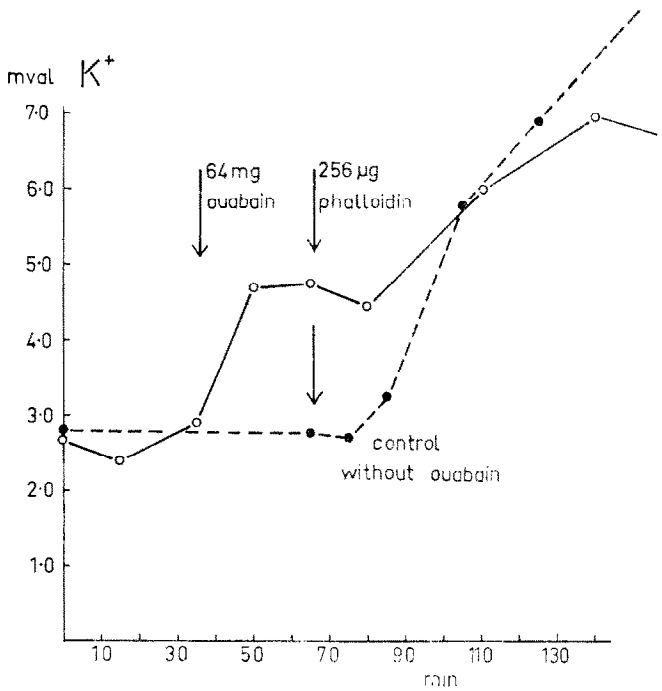

FIG. 1. Release of K^+ in the perfused isolated rat liver. K^+ (mval) content of the perfusion medium is plotted against perfusion time in min. Perfused liver 1 ○———○: The arrows point to the addition of ouabain (64 mg/200 ml perfusion medium) and phalloidin 256 µg/200 ml medium. Perfused liver 2 ●———●: Addition of phalloidin (arrow) without previous addition of ouabain (control).

method of Neville[7] and in Table 2 by the method of Coleman *et al.*[6] Using fresh membrane preparations phalloidin up to 400 µg/mg membrane protein has only a slight effect on Mg^{2+}-ATPase and (Na^+-K^+)-ATPase (0–20 per cent change). On the contrary ouabain (10^{-4} M/l.) inhibited the (Na^+-K^+)-ATPase activity in all experiments by up to 47–54 per cent and after dialysis of the membranes by 39 per cent (Table 1).

It is well known that in liver-cell membranes the effect of inhibitors of ATPase depends on the membrane structure. For other membrane systems it has been reported that (Na^+-K^+)-ATPase is activated by dialysis.[17] However the (Na^+-K^+)-ATPase activity of the liver plasma membranes decreased by dialysis.[9] After dialysis we found a loss of membrane protein in the range of 6–36 per cent. Electron micrographs indicate fragmentation and structural change of dialysed plasma membranes (Fig. 2b). After

TABLE 1. EFFECT OF PHALLOIDIN AND OUABAIN ON ATPASE ACTIVITY IN PLASMA MEMBRANE PREPARATIONS OF RAT LIVER CELLS (PREPARED BY METHOD OF NEVILLE[7])

No of Prep. (n)	treatment	Mg^{2+}-ATPase μmoles P_i/mg protein rel. per hr	μg phalloidin/mg membrane protein				Ouabain	$(K^+$-$Na^+)$-ATPase μmoles P_i/mg Protein rel. per hr	μg phalloidin/mg membrane protein				Ouabain 10^{-4}moles
			20·0	100·0	200.0	400·0			20·0	100·0	200·0	400·0	
5	fresh*	54·72 ± 26·71	− 1·0%‡		− 0·3%	+ 1·1%	− 2·8%	10·60 ± 3·13	− 0·3%		+ 6·6%	+ 0·9%	− 54·5%
	dialysis†	49·31 ± 29·08	− 6·8%		− 2·0%	− 1·0%	− 8·1%	5·34 ± 1·20	+ 10·2%		− 2·2%	+ 3·1%	− 38·7%
1	fresh	161·06		− 3·4%			− 2·8%	17·61		− 9·9%			− 46·8%
	dialysis	85·13		− 9·6%			± 0%	7·09		− 83·0%			− 39·2%

* fresh plasma membrane preparations (measurement immediately after preparation).
† membrane preparation dialysed against distilled water for 12–14 hr.
‡ average per cent inhibition (−) or activation (+) of ATPase activity.

TABLE 2. EFFECT OF PHALLOIDIN ON ATPASE ACTIVITY IN PLASMA MEMBRANE PREPARATIONS OF RAT LIVER CELLS (PREPARED BY THE METHOD OF COLEMAN *et al.*[6])

No of Prep. (n)	treatment	Mg^{2+}-ATPase μ moles P_i/mg protein rel. per hr	μg phalloidin/mg membrane protein			$(Na^+$-$K^+)$-ATPase μmoles P_i/mg protein rel. per hr	μg phalloidin/mg membrane protein		
			20·0	200·0	400·0		20·0	200·0	200·0
5	fresh*	32·39 ± 14·45	− 1·5‡	+ 5·3	− 2·2	4·76 ± 2·92	+ 2·03	+ 20·2	− 14·8
4	dialysed†	23·25 ± 7·36	− 2·7	− 11·6	− 4·9	2·89 ± 2·30	+ 6·0	− 47·2	− 53·9

* plasma membrane preparations (freezed 12 hr at − 20°).
† membrane preparations dialysed against distilled water for 12–14 hr.
‡ average per cent inhibition (−) or activation (+) of ATPase activity.

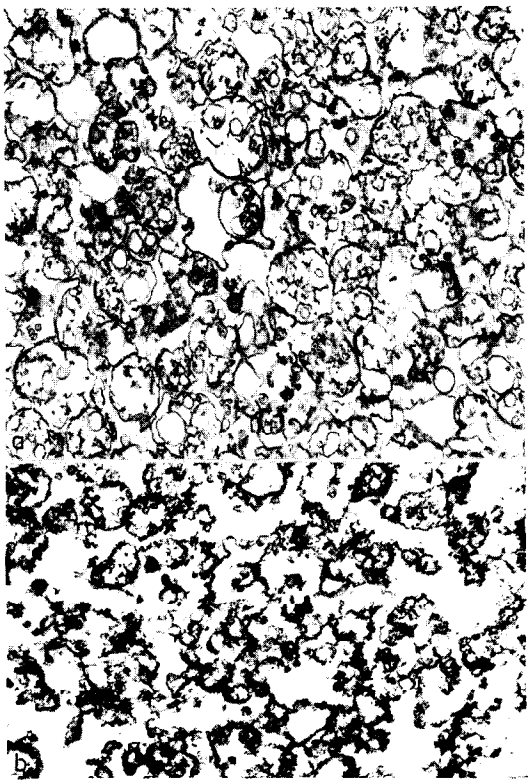

FIG. 2. Electron micrograph of isolated rat liver membranes (× 10,500) following the method of Neville;[7] (a) fresh membrane-preparation, (b) dialysed membranes (12–14 hr against distilled water).

TABLE 3. EFFECT OF PHALLOIDIN ON NEUTRAL AND ALKALINE NPPASE IN PLASMA MEMBRANE PREPARATIONS

No of Prep. (n)	treatment	alk. Mg^{2+}-NPPase mμ moles nitrophenol rel./mg prot./min	45–235 μg phalloidin/ mg membrane protein	alk. K^+-Mg^{2+}NPPase mμ moles nitrophenol rel./mg prot./min	45–235 μg phalloidin/ mg membrane protein
5	fresh	*3·11 ± 0·61	*3·03 ± 0·64	*3·32 ± 1·36	* 2·75 ± 1·31
		P = > 0·5		P = > 0·5	
		neutr. Mg^{2+}-NPPase	45–175 μg phalloidin/ mg membrane protein	neutr. K^+Mg^{2+}NPPase	45–175 μg phalloidin/ mg membrane protein
2	fresh	9·16*	8·53*	1·16*	1·23*

* Average of two measurements for each membrane preparation.

the procedure of dialysis various concentrations of phalloidin had a different effect on the (Na^+-K^+)-ATPase activity (Tables 1 and 2). Using membrane fractions prepared by the method of Neville, inhibition of the (Na^+-K^+)-ATPase has been observed in one out of six experiments (Table 1). Thus the inhibitory effect was rarely observed. With membrane fractions prepared by the method of Coleman and treated by dialyses more reproducible results have been obtained. Treatment with phalloidin in high concentrations (200 and 400 μg/mg membrane protein) results in inhibition of (Na^+-K^+)-ATPase activity by 47–53 per cent. From this observation it may by concluded that phalloidin alters the structure of rat liver plasma membranes unspecifically. However this is only true with dialysed membranes prepared by the method of Coleman *et al.*[6]

In isolated rat liver membranes Emmelot and Bos[9, 10] have recently demonstrated neutral and alkaline Mg^{2+}-dependent and K^+-Mg^{2+}-dependent nitrophenyl phosphatase activity (NPPase). These authors discussed the possibility that K^+ activated NPPase may be involved in the transport of K^+ ions across the membranes. Stimulated by these findings we have tested the effect of phalloidin on NPPase in plasma membrane preparations at neutral and alkaline pH. Table 3 lists the results. There was no significant inhibition of the enzymes by phalloidin. Also in further experiments preincubation of the membranes with 75 μg phalloidin per mg membrane protein for 5, 10 and 30 min at 37° did not inhibit NPPases.

The results of our work described in this paper did not support our initial working hypothesis. In fresh rat liver plasma membrane preparations treated with phalloidin. ATPase and NPPase were not affected by specific inhibition of the enzyme. Ouabain which appears to interact with a K^+ receptor inhibited the (Na^+-K^+)-ATPase reproducibly. The effect of phalloidin on dialysed membranes seemed to be nonspecific and could not always be reproduced in our experiments. Recent work in our laboratory showed that this poison acts by creating a potassium channel rather than by inhibiting a cation pump. Frimmer and Weil[18] have pointed out that in isolated perfused rat liver tetraethylammonium ions, which specifically block the potassium channel,[19] inhibited competitively the release of potassium by phalloidin.

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