Anti-phalloidine and anti-α-amanitine action of silybin in comparison with compounds similar to structural parts of silybin

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Summary. Silybin significantly antagonises the lethal poisoning of mice with α-amanitine or phalloidine. In the same test, taxifolin, coniferyl alcohol, fisetin and (+)-catechin were not effective.

Silybin and the natural combination of its isomers, silymarin, are isolated from the fruits of the milk thistle (*Silybum marianum* L. Gaertn.)[1]. The protective action of both substances against the toxins phalloidine and α-amanitine has been repeatedly demonstrated during the last years[2-7]. Since silybin possesses a new type of structure, in that a phenylchromanone basic frame of the taxifolin type is bound with one coniferyl alcohol molecule[4], the question arises whether the anti-phalloidine and anti-α-amanitine effect is a result of this particular total structure of silybin, or whether compounds with structures similar only to parts of the silybin structure have the same effect. For these experiments, the phenylchromone fisetin or the phenylchroman (+)-catechin were available as well as the phenylchromanone taxifolin.

Materials and methods. Silybin, taxifolin (Madaus, Cologne), coniferyl alcohol, fisetin, and (+)-catechin (Roth, Karlsruhe) were converted into the watersoluble salts with N-methylglucamine in an equimolar ratio, and the aqueous solution was stabilized by the addition of polyvinylpyrrolidone (PVP). The experimental animals were male and female mice (NMRI strain; Süddeutsche Versuchstierzuchtanstalt, Tuttlingen) weighing 25-30 g. The animals were kept under equal conditions, i.e. constant room temperature, standard feeding with 'ssniff' (Intermast, Soest) and drinking water ad libitum. They were damaged by i.p. injection of 3.0 mg/kg phalloidine or 0.5 mg/kg α-amanitine. 1 h before the intoxication, the test substances (as N-methylglucamine salts) were injected i.v. in doses of 100 mg/kg or 200 mg/kg dissolved in 20 ml saline with 4% PVP (mol.wt 10,000) as an additive. The dose of 37.3 or 74.6 mg coniferyl alcohol corresponds to the portion of this substance found in 100 or 200 mg silybin respectively. The controls received an equimolar injection

Table 1. Action of different substances on phalloidine poisoning in female mice

Substance	mg/kg i.v.	Number of animals	Death rate (%)	χ^2	$\chi^2_{10.05}$	p
Control	-	20	95			
Silybin	100	20	35	13.3	3.841	<0.05
Silybin	200	20	0	32.5	3.841	<0.05
Control	-	20	85			
Coniferyl alcohol	37.3	20	95			0.698
Coniferyl alcohol	74.6	20	70			0.775
Control	-	20	100			
Taxifolin	100	20	85			0.885
Taxifolin	200	20	80			0.947
Control	-	20	85			
Fisetin	100	20	75			0.653
Fisetin	200	20	85			0.331
Control	-	20	80			
(+)-Catechin	100	20	95			0.829
(+)-Catechin	200	20	85			0.500

The test substances were injected i.v. as the N-methylglucamine salt 1 h before poisoning with 3.0 mg/kg phalloidine i.p.

Table 2. Action of different substances on α-amanitine poisoning in male mice

Substance	mg/kg i.v.	Number of animals	Death rate (%)	χ^2	$\chi^2_{10.05}$	p
Control	-	20	100			
Silybin	100	20	35	16.4	3.841	<0.05
Silybin	200	20	20	23.4	3.841	<0.05
Control	-	20	75			
Coniferyl alcohol	37.3	20	90			0.796
Coniferyl alcohol	74.6	20	70	0.0	3.841	>0.05
Control	-	20	95			
Taxifolin	100	20	75			0.909
Taxifolin	200	20	90			0.500
Control	-	20	75			
Fisetin	100	20	75	0.133	3.841	>0.05
Fisetin	200	20	70	0.000	3.841	>0.05
Control	-	20	85			
(+)-Catechin	100	20	80			0.500
(+)-Catechin	200	20	80			0.500

The test substances were injected i.v. as the N-methylglucamine salt 1 h before poisoning with 0.5 mg/kg α-amanitine i.p.

of N-methylglucamine solution in 0.9% NaCl with 4% PVP. Each group consisted of 20 mice. The death rate after the poisoning was measured. All animals were observed over a period of 7 or 14 days. The results were calculated over a program on the Olivetti P 652 with the 'Vierfelder χ^2 test', corrected by Yates. If the expected values were 5, then the exact p-values were calculated by Fisher's test.

Results. The toxic doses of α-amanitine and phalloidine were chosen so that 75–100% of the controls will die. The treatment with silybin 1 h before the intoxication reduced the death rate in both experiments significantly. Under the same conditions taxifolin, coniferyl alcohol, fisetin and (+)-catechin were ineffective[8].

Discussion. The toxins used from the death-cup toadstool (Amanita phalloides) show different modes of action. The site of action of phalloidine is the plasma mebrane of the liver cell[9]. Death in small rodents follows the i.p. application of a lethal dose within 2 or 3 h. The liver is swollen to its maximum, light-microscopically, one is impressed by massive vacuolization of the liver cells, and some of the vacuoles contain erythrocytes[10].

α-Amanitine blocks the RNA-polymerase B in the eukaryotic cells[11]. The absence of the synthesis of ribonucleic acids can explain the long time-effect sequence between poisoning and the manifestation of the damage in the parenchyma. Mice die not before 2–4 days after the intoxication. From experiments carried out with phalloidine and silybin, on the isolated perfused liver or on isolated hepatocytes, it has been shown that silybin protects the liver cell by interacting with the outer cell membrane[6,12]. The prophylactic and therapeutic use of silybin against the toxic action of phalloidine and α-amanitine underlie, in vivo, the same temporal conditions. Therefore, it may be assumed that silybin hinders α-amanitine from penetrating into the cell.

The identification of the molecular structure of silybin a few years ago[1] has shown that this compound cannot be classified into any of the known groups of natural compounds, but rather is a new and peculiar chemical structure. As the presented experimental results show, silybin occupies a special position, also in a pharmacological view.

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Current(I)-voltage(V) relationships of the neuromembrane of an identifiable giant neurone of an African giant snail (*Achatina fulica* Férussac) in the presence of an inhibitory tripeptide, L-Lys-L-Phe-L-Tyr

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Summary. An inhibitory tripeptide, L-Lys-L-Phe-L-Tyr, caused membrane hyperpolarization of the TAN (tonically autoactive neurone) resulting in an elevated firing level. The tripeptide, however, did not markedly affect either the TAN I-V curve or the firing pattern obtained by transmembrane triangular current injection.

In a previous paper[2], we reported that a tripeptide, L-Lys-L-Phe-L-Tyr, obtained as a fragment of physalaemin (a hypotensive endecapeptide[3,4]) had an inhibitory effect on the excitability of a giant neurone (the TAN, tonically autoactive neurone) identified in suboesophageal ganglia of the African giant snail, *Achatina fulica* Férussac. A dipeptide, L-Phe-L-Tyr, also had the same effect on the same neurone[5], although the inhibition was less than that obtained with L-Lys-L-Phe-L-Tyr. None of the amino acids involved, however, had any effect on the TAN excitability when tested individually[6].

In the present study, we used a transmembrane triangular current injection method to measure current(I)-voltage(V) relationships (I-V curve) of the TAN neuromembrane. This system was then used to study the effect of the application of L-Lys-L-Phe-L-Tyr. We studied also the effect of this tripeptide on both the TAN firing level and the firing pattern produced by a depolarizing current injection.

Material and methods. The pharmacological characteristics and the localization of the neurone examined (the TAN) in the suboesophageal ganglia have been described in previous papers[7-9]. The electrophysiological methods adopted in the present study have also been described in detail in these papers. 2 glass microelectrodes were implanted in the TAN soma: one to record the intracellular potential and one to inject a current into the soma. The I-V curve, the firing level and the firing pattern of the neurone were measured simultaneously by the injection of a transmembrane triangular current (hyperpolarizing, depolarizing and hyperpolarizing, 120 sec/cycle)[10]. L-Lys-L-Phe-L-Tyr (donated by Dr A. Inoue of Daiichi Pharmaceutical Co., Tokyo) was dissolved in the snail's physiological solution[11] and applied directly to the dissected ganglia (the bath application).

Results and discussion. Figure 1, A and B, show I-V curves of the TAN neuromembrane obtained under the following conditions: A, in the physiological state; B, 3 min after the application of L-Lys-L-Phe-L-Tyr in a concentration of 2×10^{-4} kg/l. There was no distortion of the current intensity recording, indicating that electrical rectification was not a problem with the current injecting microelectrode used. In the physiological state (A), spontaneous spike