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MODE OF INHIBITORY ACTION OF MELITTIN ON Na^+-K^+-ATPase ACTIVITY OF THE RAT SYNAPTIC MEMBRANE

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Abstract—The effects of melittin from bee venom, cardiotoxin from Formosan cobra venom, and ouabain on Na^+-K^+-ATPase activity of the synaptic membrane isolated from rat cerebral cortex were studied. Melittin was the most potent in inhibiting Na^+-K^+-ATPase activity. Mg^{2+}-ATPase was less susceptible than Na^+-K^+-ATPase to the inhibitory action of toxins. High K^+ (30 mM) reversed the inhibitory action of melittin on Na^+-K^+-ATPase but did not affect that of cardiotoxin. A comparison between the effects of ouabain and melittin was studied, using double-reciprocal plots of Na^+-K^+-ATPase activity against K^+. It was shown that both were competitive with K^+ for binding to the K^+ site. Moreover, a median-effect plot revealed that ouabain and melittin antagonized each other when inhibiting Na^+-K^+-ATPase. Phosphatidylcholine (PC) was the only one of the phospholipids tested capable of protecting Na^+-K^+-ATPase from the inhibitory action of melittin but not that of ouabain. However, the inhibitory action of cardiotoxin on this enzyme was decreased by phosphatidylserine and sphingomyelin, in addition to PC. All of these findings suggest that melittin polypeptide potently inhibits Na^+-K^+-ATPase, possibly by binding to the K^+ site.

Na^+-K^+-ATPase is the biochemical manifestation of the sodium pump. It regulates the active transport of Na^+ and K^+ across the cell membrane [1]. The sodium pump is involved in several physiological processes [2–4], so that a change in Na^+-K^+-ATPase activity will affect these processes. For instance, there is evidence for increased plasma levels of a sodium-pump inhibitor in some animal models of hypertension and essential hypertension [5, 6]. It is, therefore, considered that further exploration of either endogenous or exogenous Na^+-K^+-ATPase inhibitors will be useful in both clinical and basic research.

Melittin and cardiotoxin, which are single polypeptides isolated from bee venom [7] and from Formosan cobra venom [8], respectively, have been found to be capable of inhibiting ATPase activity in various tissues [9–14]. However, their action on the isolated synaptic membrane still remains to be elucidated. In this paper, the effects of melittin, cardiotoxin and ouabain on Na^+-K^+-ATPase and Mg^{2+}-ATPase activities of the synaptic membrane isolated from the rat cerebral cortex were comparatively studied. Their modes of action on ATPases were investigated by changing the concentration of K^+ and Ca^{2+}, by treatment of the synaptic membrane with Triton X-100, phospholipase A_2 and various kinds of phospholipids, since a direct interaction of melittin or cardiotoxin with phospholipids has been reported [15–21].

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MATERIALS AND METHODS

Toxins. Melittin, isolated from bee venom, was purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A.

Cardiotoxin and phospholipase A_2 were isolated from the venom of *Naja naja atra* by CM-Sephadex C-50 column chromatography [8]. The trace amount of phospholipase A_2 in the cardiotoxin fraction was eliminated by rechromatography twice in a CM-cellulose column. Homogeneity was verified by disc gel electrophoresis [22].

Preparation of synaptic membranes. Long–Evans rats of either sex weighing 120–200 g were used. The synaptic membrane of the cerebral cortex was prepared with a slight modification of the method described by De Lores Arnaiz *et al.* [23]. The crude mitochondrial fraction containing synaptosomes was osmotically shocked in distilled water (10 ml/g). The synaptic membranes were sedimented by centrifugation at 20,000 *g* for 30 min and then purified by ultracentrifugation on a discontinuous sucrose gradient (0.32, 0.8, 0.9, 1.0 and 1.2 M) at 50,000 *g* for 2 hr. The synaptic membranes that sedimented in the 1.0 M sucrose layer were collected and diluted to 0.32 M with redistilled water. The membranes were concentrated by centrifugation at 17,000 *g* for 20 min, suspended in 0.32 M sucrose–5 mM histidine, pH 7.4, and stored at −30° until used for ATPase assay.

Assay of ATPase activity. The activities of Na^+-K^+-Mg^{2+}-ATPase and Mg^{2+}-ATPase were assayed as described previously [14]. When melittin, cardiotoxin, Triton X-100, phospholipids, 30 mM K^+ or

Ca^{2+} was present, it was added to synaptic membrane protein (0.2 to 0.3 mg/ml) suspended in 0.32 M sucrose–5 mM histidine, pH 7.4, and preincubated at 37° for 20 min. The enzymic activity was then initiated by adding the reaction mixture containing 100 mM NaCl, 20 mM KCl, 0.5 mM ethyleneglycolbis(amino-ethylether)tetra-acetate (EGTA), 30 mM histidine-imidazole buffer, pH 7.2, 5 mM $MgCl_2$ and 3 mM ATP with or without 1 mM ouabain. Further incubation at 37° for 20 min was carried out, and the reaction was stopped by 5% trichloroacetic acid. P_i released was determined colorimetrically as described by Lebel *et al.* [24]. Phospholipid [phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and phosphatidylserine (PS) which were dried under N_2 gas to get rid of solvent, $CHCl_3$ and methanol] vesicles were prepared by homogenization in a Potter–Elvehjem homogenizer fitted with a Teflon pestle with redistilled water. Protein was determined by the method of Lowry *et al.* [25] with bovine serum albumin as standard.

RESULTS

Characterization of Na^+-K^+-*ATPase and* Mg^{2+}-*ATPase of the synaptic membrane.* The rat synaptic membranes contained higher Na^+-K^+-ATPase activity than Mg^{2+}-ATPase activity (Table 1). Na^+-K^+-ATPase was distinct from Mg^{2+}-ATPase in the following aspects: Na^+-K^+-ATPase had a lower K_m value and a higher V_{max} value than Mg^{2+}-ATPase (Table 2). Pretreatment of the synaptic membrane with Triton X-100 increased Na^+-K^+-ATPase but did not affect Mg^{2+}-ATPase activity (Table 1). K^+ (30 mM) and PE did not affect Na^+-K^+-ATPase activity, but higher concentrations of K^+ (75 mM), Ca^{2+} (0.2 to 5.0 mM) or SM (0.25 mg/ml and 0.4 mg/ml) caused reductions. PS at a low concentration of 0.016 mg/ml did not affect, but at higher concentrations (0.03 mg/ml and 0.125 mg/ml) reduced, Na^+-K^+-ATPase activity. High K^+ (30 and 75 mM), Ca^{2+} (0.2 to 5.0 mM), PC and SM (0.25 mg/ml) enhanced Mg^{2+}-ATPase activity whereas PE, SM (0.4 mg/ml) and PS (0.016 mg/ml) did not alter it significantly. PS at 0.03 mg/ml and 0.125 mg/ml also reduced Mg^{2+}-ATPase activity (Table 1). In the following experiments on protecting ATPase from toxin inhibition, a concentration of phospholipids was chosen that had no effect or only slightly affected ATPase activity.

Effects of melittin and cardiotoxin on ATPase activities. As shown in Fig. 1, Na^+-K^+-ATPase was more susceptible than Mg^{2+}-ATPase to the inhibitory actions of melittin or cardiotoxin. Melittin was more potent than cardiotoxin in inhibiting ATPases, as indicated by IC_{30} values (Table 2). Triton X-100 did not affect the inhibitory action of these two toxins on ATPases except that it potentiated the inhibition of Mg^{2+}-ATPase induced by high concentrations of cardiotoxin. Kinetic studies on the inhibitory actions of melittin and cardiotoxin revealed that melittin inhibited these enzymes in a mixed manner while cardiotoxin inhibited Na^+-K^+-ATPase in a mixed manner and inhibited Mg^{2+}-ATPase uncompetitively (Fig. 2). V_{max} values of Na^+-K^+-ATPase and Mg^{2+}-ATPase were reduced by these two toxins (Table 2). K_m values of Na^+-K^+-ATPase were also reduced by them but that of Mg^{2+}-ATPase was not increased significantly by melittin (Table 2).

Effects of K^+ *and* Ca^{2+} *on the inhibitory actions of melittin and cardiotoxin.* As shown in Fig. 3, 30 mM K^+ exerted different effects on the actions of melittin and cardiotoxin. It decreased the inhibitory action of melittin on Na^+-K^+-ATPase but enhanced that on Mg^{2+}-ATPase. By contrast, it did not affect the inhibitory actions of cardiotoxin on Na^+-K^+-ATPase and Mg^{2+}-ATPase. A comparison between ouabain and melittin is shown in Fig. 4. The double-reciprocal plot of Na^+-K^+-ATPase activity against K^+ shows that both of them were competitive with K^+. V_{max} values for control, melittin and ouabain were the

Table 1. Effects of detergent, ions and phospholipids on Na^+-K^+-ATPase and Mg^{2+}-ATPase activities of the synaptic membrane*

Preincubation	Concentration in preincubation	Specific activity (μmoles P_i/mg protein/min)	
		Na^+-K^+-ATPase	Mg^{2+}-ATPase
Control		0.270 ± 0.006 (83)	0.126 ± 0.003 (93)
Triton X-100	80 μM	0.326 ± 0.008† (26)	0.120 ± 0.006 (26)
K^+	30 mM	0.272 ± 0.005 (44)	0.174 ± 0.004† (54)
	75 mM	0.204 ± 0.016† (7)	0.235 ± 0.005† (9)
Ca^{2+}	0.2 mM	0.237 ± 0.006† (42)	0.134 ± 0.002† (44)
	0.5 mM	0.235 ± 0.005† (18)	0.141 ± 0.003† (21)
	2.0 mM	0.166 ± 0.013† (21)	0.150 ± 0.002† (19)
	5.0 mM	0.114 ± 0.006† (26)	0.144 ± 0.002† (25)
Phosphatidylcholine	1.0 mg/ml	0.293 ± 0.005† (24)	0.134 ± 0.002† (22)
Phosphatidylethanolamine	0.375 mg/ml	0.268 ± 0.006 (21)	0.121 ± 0.002 (24)
Sphingomyelin	0.25 mg/ml	0.239 ± 0.006† (33)	0.136 ± 0.003† (32)
	0.4 mg/ml	0.190 ± 0.022† (2)	0.116 ± 0.016 (2)
Phosphatidylserine	0.016 mg/ml	0.259 ± 0.009 (12)	0.122 ± 0.02 (15)
	0.03 mg/ml	0.156 ± 0.021† (3)	0.108 ± 0.006† (3)
	0.125 mg/ml	0.113 ± 0.019† (6)	0.094 ± 0.003† (11)

* Data are presented as mean ± S.E. Numbers in parentheses represent the total number of experiments.

† $P < 0.05$, compared with control.

Table 2. Effects of cardiotoxin and melittin on Na^+-K^+-ATPase and Mg^{2+}-ATPase*

	K_m (mM)	V_{max} (μmoles P_i/mg protein/min)	IC_{30} (μM) Without Triton X-100	IC_{30} (μM) With Triton X-100
Na^+-K^+-ATPase				
Control	0.816 ± 0.038 (4)	0.435 ± 0.005 (4)		
Melittin	0.482 ± 0.015† (4)	0.147 ± 0.001† (4)	0.13 ± 0.01 (3)	0.14 ± 0.02 (3)
Cardiotoxin	0.563 ± 0.010† (4)	0.217 ± 0.002† (4)	1.20 ± 0.10 (3)	0.91 ± 0.17 (3)
Mg^{2+}-ATPase				
Control	1.212 ± 0.043 (4)	0.204 ± 0.003 (4)		
Melittin	1.333 ± 0.094 (4)	0.167 ± 0.007† (4)	0.35 ± 0.01 (3)	0.33 ± 0.02 (3)
Cardiotoxin	0.755 ± 0.030† (4)	0.132 ± 0.002† (4)	20.00 ± 0.50 (3)	2.00 ± 0.13 (3)

* IC_{30} is the concentration of toxins required to inhibit 30% of ATPase activity. The final concentrations of cardiotoxin and melittin used in the determination of K_m and V_{max} were 1.5 and 0.35 μM respectively. Values are means ± S.E. Numbers in parentheses represent the total number of experiments.

† $P < 0.05$, compared with control.

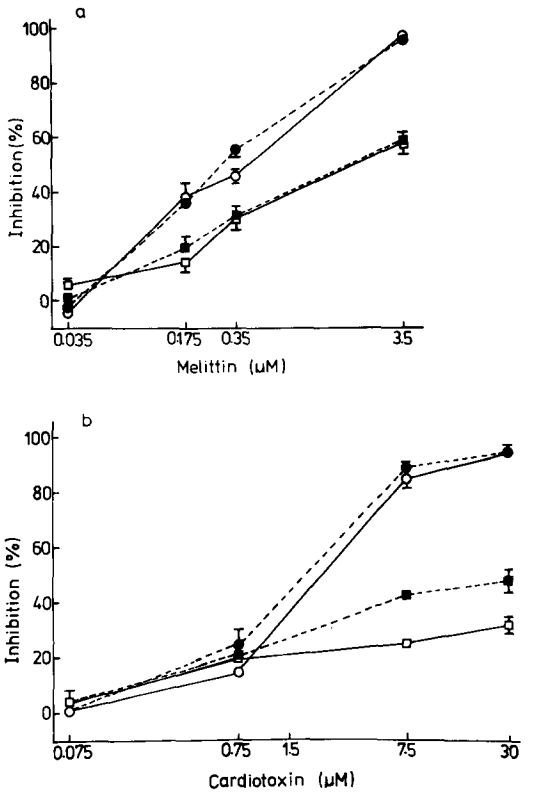

Fig. 1. Inhibition of Na^+-K^+-ATPase and Mg^{2+}-ATPase of the rat synaptic membrane by melittin (a) and cardiotoxin (b). The results of more than six experiments are expressed as mean ± S.E. (bars). Some standard errors that were smaller than the symbols are not shown. Key: (○——○) Na^+-K^+-ATPase; (●·····●) Na^+-K^+-ATPase with 80 μM Triton X-100; (□——□) Mg^{2+}-ATPase; and (■·····■) Mg^{2+}-ATPase with 80 μM Triton X-100.

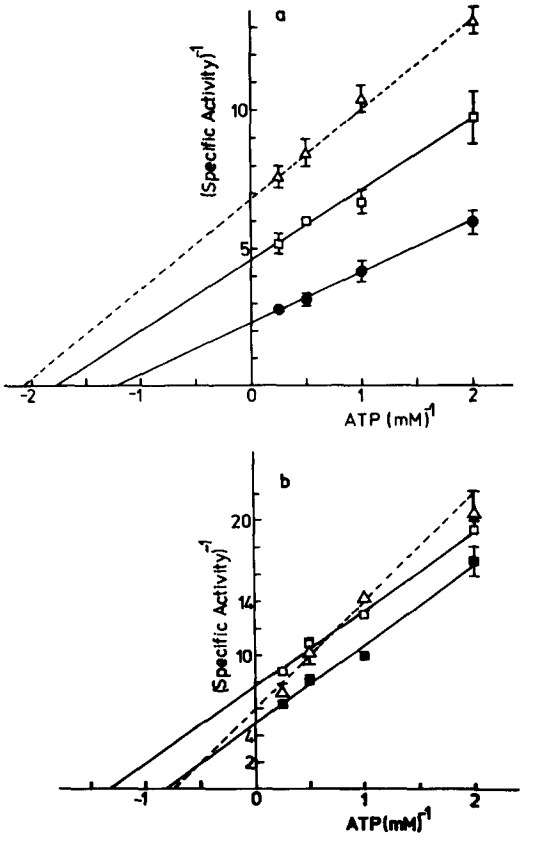

Fig. 2. Lineweaver-Burk plot of ATPase activity of the rat synaptic membrane. The results of more than six experiments are expressed as mean ± S.E. (bars). Some standard errors that were smaller than the symbols are not shown. The unit of specific activity is μmoles P_i/mg protein/min. Key: (a) Na^+-K^+-ATPase; (b) Mg^{2+}-ATPase; (●——● and ■——■) control; (△·····△) 0.35 μM melittin; and (□——□) 1.5 μM cardiotoxin.

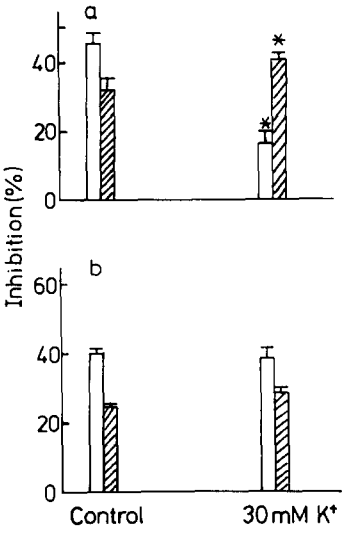

Fig. 3. Effect of K^+ on the inhibitory action of melittin and cardiotoxin on ATPase of the rat synaptic membrane. The results of more than six experiments are expressed as mean ± S.E. (bars). Key: (a) 0.35 μM melittin; (b) 1.5 μM cardiotoxin; (□) Na^+-K^+-ATPase; (▨) Mg^{2+}-ATPase; and (*) $P < 0.05$, compared with respective control.

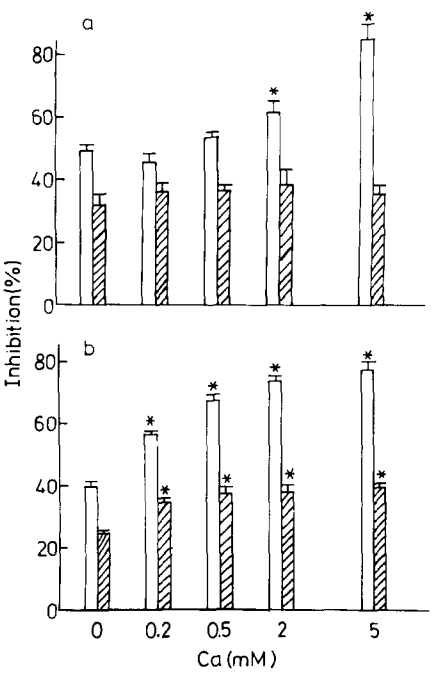

Fig. 5. Effect of Ca^{2+} on the inhibitory action of melittin and cardiotoxin on ATPase of the rat synaptic membrane. The results of more than six experiments are expressed as means ± S.E. (bars). Key: (a) 0.35 μM melittin; (b) 1.5 μM cardiotoxin; (□) Na^+-K^+-ATPase; (▨) Mg^{2+}-ATPase; and (*) $P < 0.05$, compared with the respective control.

same, 0.278 μmoles P_i/mg protein/min, and K_m values were 1.015, 6.667 and 9.091 mM respectively.

Low concentrations of Ca^{2+} (0.2 and 0.5 mM) did not affect the action of melittin on Na^+-K^+-ATPase, but higher concentrations (2 and 5 mM) increased it. None of the concentrations of Ca^{2+} affected the action of melittin on Mg^{2+}-ATPase (Fig. 5a). On the other hand, various concentrations of Ca^{2+} (0.2, 0.5, 2 and 5 mM) increased the inhibitory action of cardiotoxin on Na^+-K^+-ATPase in a dose-dependent manner (Fig. 5b). Ca^{2+} also increased the inhibitory action of cardiotoxin on Mg^{2+}-ATPase.

Protection of ATPases by phospholipids from the inhibitory action of melittin and cardiotoxin. As shown in Fig. 6a, PC could, but PE, SM and PS could not, protect Na^+-K^+-ATPase from the inhibitory action of melittin. By contrast, all of these phospholipids except PE had a protective effect against the inhibitory action of cardiotoxin on Na^+-K^+-ATPase activity (Fig. 6b). On the other hand, all phospholipids except PE attenuated the inhibitory action of melittin on Mg^{2+}-ATPase activity but none including PE had an effect on that of cardiotoxin. These phospholipids appear to be able to incorporate into

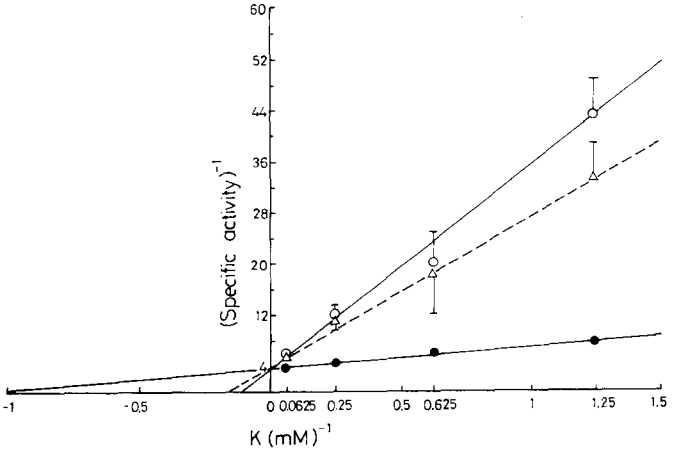

Fig. 4. Double-reciprocal plot of Na^+-K^+-ATPase activity against K^+ of the rat synaptic membrane. Some standard errors that were smaller than the symbols are not shown. The unit of specific activity is μmoles P_i/mg protein/min. Key: (●——●) control; (△·····△) 0.35 μM melittin; and (○——○) 5 μM ouabain.

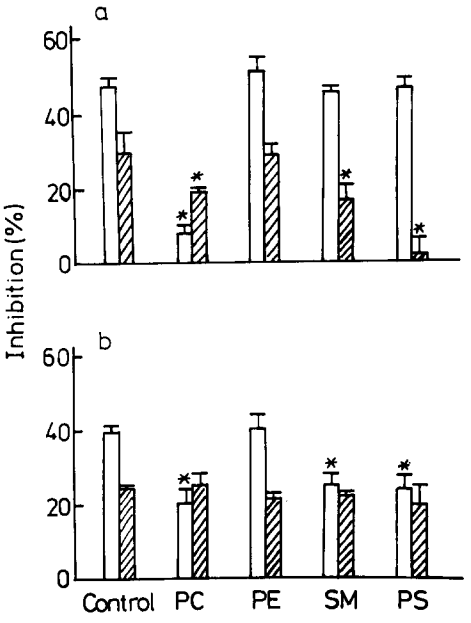

Fig. 6. Effects of phospholipids on the inhibitory action of melittin and cardiotoxin on Na^+-K^+-ATPase and Mg^{2+}-ATPase of the rat synaptic membrane. The results of more than six experiments are expressed as mean ± S.E. (bars). Key: (a) 0.35 μM melittin; (b) 1.5 μM cardiotoxin; (□) Na^+-K^+-ATPase; and (▨) Mg^{2+}-ATPase. PC, PE, SM and PS represent phosphatidylcholine (1 mg/ml), phosphatidylethanolamine (0.375 mg/ml), sphingomyelin (0.25 mg/ml) and phosphatidylserine (0.016 mg/ml) respectively. An asterisk (*) indicates $P < 0.05$ compared with the respective control.

the membrane, since after Na^+-K^+-ATPase had been inhibited by 77 nM phospholipase A_2, PC was able to restore the enzymic activity; PC reduced the percent inhibition induced by phospholipase A_2 from 63.8 ± 1.9 to 2.3 ± 4.4%.

Comparison between melittin and ouabain. As shown in Fig. 6a, PC could protect Na^+-K^+-ATPase activity from the inhibition induced by melittin. By contrast, PC did not protect Na^+-K^+-ATPase inhibited by ouabain. The percent inhibition of 5 μM ouabain on Na^+-K^+-ATPase in the presence of PC was 49.5 ± 4.4% (N = 11), which was not significantly different from that of ouabain alone, 56.6 ± 4.7% (N = 11). Pretreatment of the synaptic membrane with 23.1 nM phospholipase A_2 decreased Na^+-K^+-ATPase by 39.1 ± 5.1%. Addition of melittin to the phospholipase A_2 pretreated preparation failed to exert its usual inhibitory action (Table 3). However, ouabain inhibited Na^+-K^+-ATPase activity of the membrane preparation treated with phospholipase A_2 as well as that of the untreated one (Table 3). From the median-effect plot (Fig. 7), *m* values (analogous to Hill coefficient) [26] for the inhibitory action of melittin, ouabain and their mixture on Na^+-K^+-ATPase were 2.663, 0.600 and 2.471 respectively.

DISCUSSION

In this paper, we have found that melittin was the most potent polypeptide capable of inhibiting Na^+-K^+-ATPase activity of the synaptic membrane. The effective concentration of melittin to exert the inhibitory action was 3×10^{-7} g/ml, which is nontoxic to the mouse (LD_{50} of melittin in mouse is 5 μg/g). Cardiotoxin was less potent; Mg^{2+}-ATPase of the synaptic membrane, however, was less susceptible to the inhibitory action of toxins. The interaction of melittin or cardiotoxin with the Na^+-K^+-ATPase was different in high K^+ medium. The inhibition of melittin was reduced by high K^+, but that of cardiotoxin

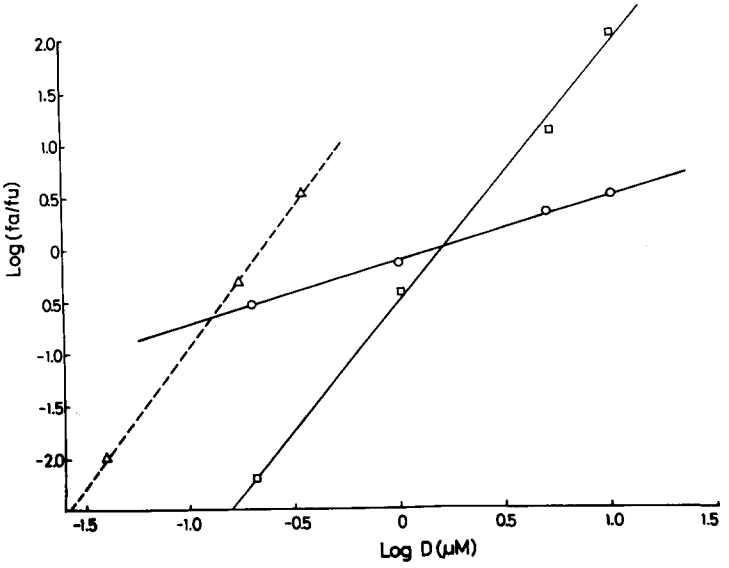

Fig. 7. Inhibition of Na^+-K^+-ATPase of the rat synaptic membrane by melittin and ouabain. The results are plotted by the median-effect plot, i.e. log (fa/fu) with respect to log (D). Key: (△---△) melittin; (○——○) ouabain; and (□——□) melittin and ouabain mixture. Abbreviations: fa = fraction of system affected by drug; fu = fraction of system unaffected by drug; and D = concentration of drug in micromolar.

Table 3. Effects of melittin and ouabain on Na^+-K^+-ATPase activity of the synaptic membrane pretreated with phospholipase A_2*

	Inhibition (%)	
	Without phospholipase A_2	With phospholipase A_2
Control	0	39.1 ± 5.1 (10)
Melittin	45.1 ± 8.3 (11)	53.6 ± 8.5 (12)
Ouabain	45.3 ± 4.1 (9)	82.3 ± 4.9† (8)

* The concentrations of phospholipase A_2, melittin and ouabain were 33.1 nM, 0.35 μM and 5 μM respectively. Values are means ± S.E. Numbers in parentheses represent the total number of experiments.
† $P < 0.05$, compared with ouabain alone.

was not affected. It is known that, in various tissues, the *in vitro* inhibition of Na^+-K^+-ATPase by cardiac glycosides can be antagonized by high KCl concentration [27–32]. The mode of antagonism by KCl of ouabain appears to be dependent on the concentration of KCl [29, 31, 32]. Akera *et al.* [32] indicated that ouabain inhibits Na^+-K^+-ATPase activity competitively at KCl concentrations below 25 mM and noncompetitively above 25 mM. A possible competition between the glycoside and K^+ for the K^+ site on the transport system has been reported by Glynn [33]. In this paper, a kinetic study of the interaction between melittin or ouabain and K^+ was performed at KCl concentrations between 0.8 and 16 mM as shown in Fig. 4. With these conditions, melittin and ouabain were competitive with K^+. The apparent dissociation constant for the potassium–enzyme complex was 1.015 mM, which is close to that (0.96 mM) reported by Akera *et al.* [32]. This finding suggests that melittin, probably like ouabain, acts at a site closely related to the K^+ binding site in the external surface of this enzyme. Akera and Brody [34] suggested that the mechanism of antagonism between KCl and ouabain seems to be an inhibition of the ouabain–enzyme binding process. The interactions of melittin and ouabain with Na^+-K^+-ATPase as analyzed by the median-effect plot [35] revealed that they antagonized each other. It is probable that both of them act at the K^+ site and that melittin inhibits the ouabain–enzyme binding process, or vice versa, as suggested by Akera and Brody [34] in antagonism between KCl and ouabain. The interaction between them merits further investigation.

Although melittin acts like ouabain in being antagonized by K^+, their actions on this enzyme were different in one respect. The inhibitory action of melittin on Na^+-K^+-ATPase was reversed by PC, but that of ouabain was not. Pretreating the synaptic membrane with phospholipase A_2 which partially inhibited Na^+-K^+-ATPase did not affect the inhibitory action of ouabain while that of melittin was greatly reduced. These results imply that the inhibition of Na^+-K^+-ATPase by melittin was related to phospholipid-PC but that that of ouabain was unrelated to phospholipid. This contention is in line with the report of Taniguchi and Iida [36] that phospholipid could not restore ATPase activity after inhibition by ouabain and that the binding of ouabain to Na^+-K^+-ATPase was not affected in spite of phospholipase A or C treatment. In the experiments of Chipperfield and Whittam [37] on reconstitution of the sodium pump from protein and phospholipid, evidence was obtained that ouabain binds to protein. Therefore, the binding of ouabain to Na^+-K^+-ATPase did not require a phospholipid moiety.

Sessa *et al.* [38] have demonstrated that melittin causes release of marker ions from PC liposomes, suggesting that in neutral liposomal membranes the target of melittin is phospholipids. Both electrostatic and hydrophobic forces have to be considered as important binding parameters [39]. The first step could be an ionic interaction between basic residues of melittin and negative groups of phospholipids, either phosphate or carboxylic. The second step would be the insertion of hydrophobic residues within the bilayer, this involving at least the Try residue, but probably all the hydrophobic part of the peptide [38]. Mollay *et al.* confirmed the hydrophobic nature of interaction of melittin with PC [40] and PE [41]. In our experiments, after treatment with phospholipase A_2, PC could restore Na^+-K^+-ATPase activity. This finding indicated that phospholipid was incorporated into the membrane. Among all phospholipids tested, only PC had a protective effect on the inhibition of Na^+-K^+-ATPase by melittin; other phospholipids had no effect. One possible reason for this result is that the concentrations of PE, PS and SM were not as high as that of PC because of their inhibitory actions on Na^+-K^+-ATPase. However, in a comparative study, the inhibitory action of cardiotoxin on Na^+-K^+-ATPase was decreased significantly by PS and SM, in addition to PC. Therefore, the specificity of the interaction of melittin or cardiotoxin with phospholipids is indicated.

Ca^{2+} changes the conformation of membrane macromolecules. It induces the membrane macromolecules to undergo a transition from hydrophilic to a more hydrophobic state, the increase in hydrophobicity being due to externalization of the hydrophobic group initially located inside the protein [42]. Only higher concentrations of Ca^{2+} enhanced the inhibition of melittin on Na^+-K^+-ATPase. This effect was not as profound as that of cardiotoxin, in which all concentrations of Ca^{2+} enhanced its inhibition on Na^+-K^+-ATPase. Probably, the hydrophobicity and

the high affinity of melittin toward the membrane were greater than those of cardiotoxin such that a full effect could be obtained even in the absence of external Ca^{2+}.

In conclusion, a potent inhibitory action of melittin on Na^+-K^+-ATPase of the synaptic membrane was demonstrated. The possible interaction of melittin with the K^+ site of the enzyme, similar to ouabain, was explored. PC was able to protect this enzyme from the inhibitory action of melittin but not from that of ouabain. The mode of inhibition by cardiotoxin appeared to differ from that of melittin since Triton X-100, high K^+, Ca^{2+} and phospholipids exerted differential influences on them.

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