Inhibitory effect of calmodulin inhibitors on palytoxin-induced K^+ release from rabbit erythrocytes

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Palytoxin (PTX) caused K^+ release from rabbit erythrocytes at a concentration as low as 10^{-11} M. The K^+ release due to PTX at a concentration below 10^{-9} M was dependent on Ca^{2+} in medium. The effect of Ca^{2+} was substituted fully by Sr^{2+} and partially by Ba^{2+}. W-7 (2×10^{-4} M), a known inhibitor of calmodulin, markedly inhibited the rate of K^+ release due to PTX. W-5 (2×10^{-4} M), an analog of W-7 with lower affinity to calmodulin than W-7, showed weaker inhibition. Other calmodulin antagonists, such as prenylamine, chlorpromazine and compound 48/80, also inhibited the PTX-induced K^+ release. These results suggest that the K^+ release induced by PTX involves the process(es) mediated by intracellular Ca^{2+} and calmodulin.

Palytoxin *Calmodulin inhibitor* *K^+ release* *Erythrocyte*

1. INTRODUCTION

Palytoxin (PTX; $C_{129}H_{223}N_3O_{54}$; M_r 2677), extracted from marine coelenterates of the genus *Palythoa*, is the most poisonous animal toxin known to date [1]. The stereochemistry of PTX has recently been determined [2,3]. PTX in low concentrations (10^{-11}–10^{-8} M) causes depolarization and/or contraction in various types of nerve and muscle tissues [4]. PTX also induces the release of K^+ from erythrocytes [5] and smooth muscle cells [6]. Recently, it has been shown that the K^+ loss induced by PTX in erythrocytes is enhanced by external Ca^{2+} [7]. Here, we have investigated the effects of several calmodulin inhibitors on the PTX-induced K^+ release from rabbit erythrocytes to determine if calmodulin plays a role in this action of PTX.

2. MATERIALS AND METHODS

Erythrocytes obtained from rabbit heparinized (30 IU/ml) blood were washed 3 times in 5 volumes of physiological salt solution (PSS) (NaCl, 136.9 mM; $CaCl_2$, 1.0 mM; $MgCl_2$, 1.0 mM; glucose, 5.5 mM; Hepes, 10 mM, pH 7.3) by centrifugation for 5 min at 3000 rpm. The buffy coat was removed by aspiration. Packed cells were resuspended in PSS (approximately 100000 cells/ml) and stored at 4°C. No measurable K^+ loss was observed during the cold storage up to 5 h. Determination of K^+ was carried out using either K^+-selective electrode (Philips, IS 561 K) or flame photometer (Hitachi, Type 208). The latter method was employed in the experiments with prenylamine, chlorpromazine and trifluoperazine, since these compounds change the sensitivity of K^+-selective electrode. In the experiments with the K^+-selective electrode, the loss of K^+ was measured continuously at 37°C. In the case of flame photometry, erythrocytes were incubated with PTX for 27 min at 37°C and the reaction was stopped by a 3 min centrifugation at 2200 rpm at 25°C. The amount of maximum releasable K^+ was determined by adding saponin (10 μg/ml) to the solution at the end of each experiment.

PTX isolated from *Palythoa tuberculosa* was

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kindly donated by Dr Y. Hirata (Meijō University, Nagoya). The toxin was diluted from a 10^{-4} M aqueous stock solution before use with a solution containing 0.5% bovine serum albumin and 2 mM Hepes (pH 7.0) [7]. Chlorpromazine hydrochloride, trifluoperazine dihydrochloride, compound 48/80 (all from Sigma) and verapamil hydrochloride (Eisai) were dissolved in distilled water. Prenylamine lactate (Hoechst, Japan) and R-24571 (calmidazolium) (Boehringer, Mannheim) were dissolved in dimethylsulfoxide. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and *N*-(6-aminohexyl)-1-naphthalenesulfonamide (W-5) (both from Rikaken) were dissolved in PSS. Various Ca^{2+} concentrations were prepared by adding appropriate amounts of EGTA to PSS using an apparent binding constant of EGTA for Ca^{2+} of 1.61×10^5 M^{-1} (pH 7.3) [8].

3. RESULTS AND DISCUSSION

PTX (10^{-10}–10^{-9} M) caused rapid release of K^+ from rabbit erythrocytes in the presence of 1 mM Ca^{2+}. When Ca^{2+} was omitted from PSS (nominally free Ca^{2+}), no measurable K^+ release was observed by PTX (10^{-10}–10^{-9} M). Extracellular Ca^{2+} over 10^{-5} M augmented the action of PTX (10^{-10}–10^{-9} M) (fig.1). PTX at 10^{-8} M induced marked K^+ release even in the absence of extracellular Ca^{2+} or at below 10^{-5} M Ca^{2+} (fig.1). Several species of divalent cations were tested for their ability to substitute the role of Ca^{2+} on K^+ release due to PTX (10^{-9} M). The effects of Ca^{2+} were substituted fully by Sr^{2+} (1 mM) and partially by Ba^{2+} (1 mM) (30%). Mn^{2+} (1 mM) and Co^{2+} (1 mM) were ineffective (not shown).

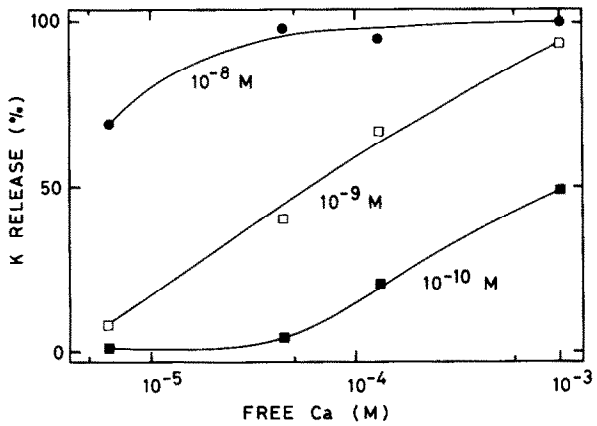

Fig.1. Ca^{2+} dependency of the K^+ release due to PTX. The amount of K^+ released during a 20 min PTX-incubation period was measured with a K^+-selective electrode. Ca^{2+} concentration in medium was changed 10 min before the application PTX by adding appropriate amounts of EGTA. Concentration of PTX: (●) 10^{-8} M; (□) 10^{-9} M; (■) 10^{-10} M. Abscissa, logarithm of free Ca^{2+} concentration (M). Ordinate, amount of K^+ released by PTX (%).

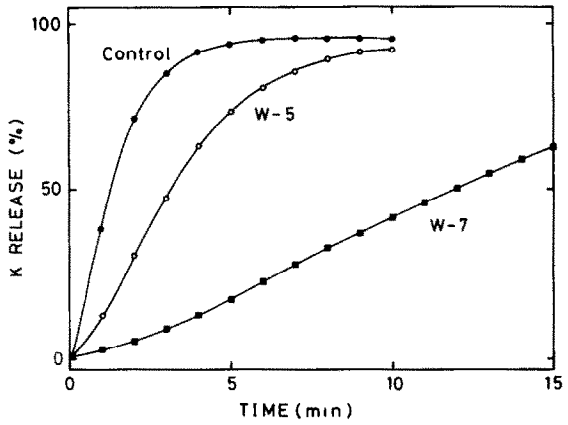

Fig.2. Effect of W-7 and W-5 on the time course of K^+ release induced by PTX. K^+ release was continuously measured with a K^+-selective electrode. Either W-7 or W-5 was added 10 min before the application of PTX (10^{-9} M). (●) Control; (■) W-7 (2×10^{-4} M); (○) W-5 (2×10^{-4} M). Abscissa, time (min). Ordinate, amount of K^+ released by PTX (%).

In the next experiment, the effects of calmodulin inhibitors on the K^+ release due to PTX were investigated. Fig.2 shows the effects of W-7 (2×10^{-4} M) and W-5 (2×10^{-4} M) on the K^+ release due to PTX (10^{-9} M). W-7 strongly inhibited the rate of the K^+ release induced by PTX. W-5, an analog of W-7 which has no chloride in the structure and possesses a lower affinity to calmodulin than W-7 [9], showed less inhibition. As shown in fig.3, chlorpromazine (10^{-4} M) and prenylamine (10^{-5} M) inhibited the K^+ release induced by PTX. Compound 48/80 (500 μg/ml) also significantly inhibited the PTX-induced K^+ release. Trifluoperazine (10^{-4} M) and R-24571 (calmidazolium) (10^{-6} M), caused hemolysis by themselves. Verapamil, a Ca^{2+} channel antagonist with no affinity to calmodulin, did not affect the K^+ release due to

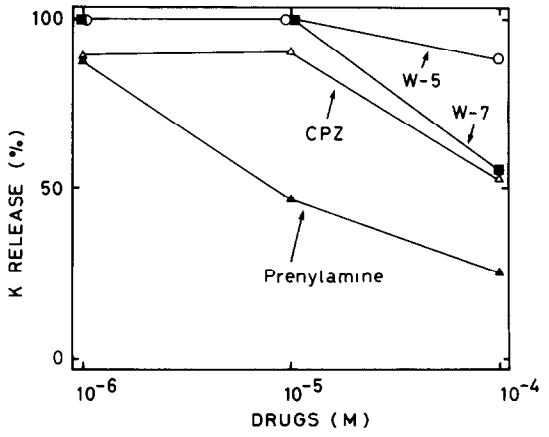

Fig.3. Inhibitory effects of various calmodulin inhibitors on PTX-induced K^+ release. The amount of K^+ released during a 30 min PTX-incubation period was measured by flame photometry. Erythrocytes were preincubated with calmodulin inhibitors for 10 min and PTX (2×10^{-10} M) was then added to induced K^+ release. (○) W-5; (■) W-7; (△) chlorpromazine; (▲) prenylamine. Abscissa, concentration of drugs (M). Ordinate, amount of K^+ released by PTX (%).

PTX even at a concentration of 10^{-4} M.

These results support the hypothesis that PTX increases membrane permeability to K^+ through process(es) mediated by Ca^{2+} and calmodulin. It has recently been suggested that PTX increases the ion permeability by binding to $(Na^+ + K^+)$-ATPase, since ouabain and other cardiac glycosides specifically inhibit the action of PTX in erythrocytes [10,11] and smooth muscle cells [12]. At present, the interrelationship between the PTX-binding to $(Na^+ + K^+)$-ATPase and the activation of calmodulin-mediated process(es) remains to be elucidated.

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