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Inhibition of the actions of palytoxin on the K^+ efflux from red cells and on the Na/K-ATPase activity by a monoclonal antibody

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Abstract

Palytoxin (PTX) binds to the Na/K pump, inhibits the (Na/K)-ATPase and forms Na and K permeable channels in human red cells. Here, we report that a monoclonal antibody raised against a derivative of PTX (Bignami, G.S. et al. (1992) Toxicon 30, 687–700) inhibits these effects. The observations are consistent with a model in which (a) the antibody binds and, thus reduces the concentration of free PTX available to react with the Na/K pump, and, (b) the PTX-antibody complex also binds to the PTX receptor on the Na/K pump but in such a way that a cation permeable channel is not formed, probably by reducing the concentration of free PTX. Using this model, we estimate that the apparent dissociation constant for the binding of PTX to antibody is 0.2 nM.

Key words: ATPase, Na^+/K^+-; Palytoxin; Potassium ion efflux; Red blood cell

1. Introduction

Palytoxin (PTX), one of the most potent non-protein marine toxins is produced by coelenterates of the genus *Palythoa* [2]. It has been reported that human consumption of contaminated coral reef fish or crabs produces severe abdominal muscle spasms and respiratory distress which can lead to death [3]. These symptoms are probably a reflection of the interaction of PTX with the ouabain binding site on the (Na + K)-activated ATPase leading to an increase in the permeability of cells to monovalent cations and inactivation of the Na/K pump (Ref. [4] and references therein, Refs. [5,6]). Recently, a monoclonal antibody raised against a derivative of palytoxin has been prepared and shown to be effective in the detection of picogram quantities of palytoxin and to slow the toxin-induced lysis of mouse erythrocytes [1,7]. Since the lytic effect of PTX is a consequence of the increased permeability of the cells to Na and K, we decided to measure directly the effect of the antibody on the capacity of PTX to induce the formation of a cation permeable pathway and to inhibit the Na/K pump. Our results show that the antibody reduces the potency of PTX to produce increased Na and K permeability of the red cell membrane. These results can be accounted for quantitatively by a model in which the antibody binds palytoxin thus lowering the concentration of free toxin available to react with the ouabain-binding site on the Na/K pump. The PTX-antibody complex thus formed can also bind to the same pump site (s), but in a way such that a cation permeable channel is not opened. We also show that the antibody reduces the capacity of PTX to inhibit the (Na + K)-activated ATPase activity in a preparation purified from eel electric organ.

2. Materials and methods

All salts used were reagent grade, purchased from Mallinckrodt (St. Louis, MO). The palytoxin used in this study was isolated from Okinawan *Palythoa tuberculosa* and was a generous gift from Profs. K. Hirata

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and D. Uemura (Nagoya University, Nagoya, Japan). Stock solutions (~ 1 mM) were prepared by dissolving the appropriate amount either with a 0.1% aqueous solution of albumin or with an 80% aqueous solution of ethanol and were stored at −50°C until needed for further dilution. There were no significant differences in the results obtained with either solution. The palytoxin-neutralizing monoclonal antibody, 73D3 (mAb) was produced by Hawaii Biotechnology Group (Aiea, HI) and was stored at 4°C in PBS (150 mM NaCl, 10 mM Na-phosphate (pH 7.0)) at a concentration of 1 mg/ml.

K-efflux from human red cells. This procedure was performed as previously described [4]. Briefly, human red blood cells were collected in heparinized tubes and washed five times in 150 mM choline chloride, 0.1% albumin, 1 mM $MgCl_2$, 0.1% albumin and 10 mM Tris-Mops (pH 7.4 at 4°C). After the last wash, the cells were packed and kept on ice. Prior to the determination of the K efflux into K-free medium, an aliquot of cells was washed five times in flux medium (140 mM NaCl, 1 mM $MgCl_2$, 0.5 mM Na-borate, 10 mM Tris-Mops, pH 7.4 at 37°C) and the packed cells added to 5 ml medium (with or without PTX-Ab complex) at 37°C to make a 3% cell suspension, under continuous stirring. The presence of borate has been shown to be necessary for the effect of PTX to be seen at low concentrations [4,8]. The external K was monitored continuously using a pH meter/datalogger mod. 6091 (Jenco Electronics, San Diego, CA), with which data points could be obtained once every 10 s. Preincubation of PTX and mAb was done at room temperature, by adding appropriate aliquots of each of them to 5 ml flux medium, to obtain the desired final concentrations. Reproducible results were obtained with an incubation period of 30 min. Control experiments included addition of PTX to cells as well as flux measurements using a flux medium in which PTX had been preincubated alone for 30 min, in the absence of borate, followed by addition of borate.

1. $R1 + PTX \overset{K_1}{\rightleftharpoons} R1PTX$
2. $R2 + PTX \overset{K_2}{\rightleftharpoons} R2PTX$
3. $Ab + PTX \overset{K_0}{\rightleftharpoons} PTXAb$
4. $R1 + PTXAb \overset{K_{11}}{\rightleftharpoons} R1AbPTX$
5. $R2 + PTXAb \overset{K_{12}}{\rightleftharpoons} R2AbPTX$

Fig. 1. Reactions involved in the overall effect of palytoxin on the K efflux from red cells. Reactions 1 and 2 give rise to the K efflux, a consequence of the binding of toxin (PTX) to two sites on the receptor (R_1 and R_2). Reaction 3 describes the binding of PTX to its antibody (Ab) to produce the complex PTXAb and reactions 4 and 5 deal with the interaction of this complex with the palytoxin receptor. The experimental points were then fitted using reactions 1 and 2 (in the absence of antibody) and the full set when Ab was present.

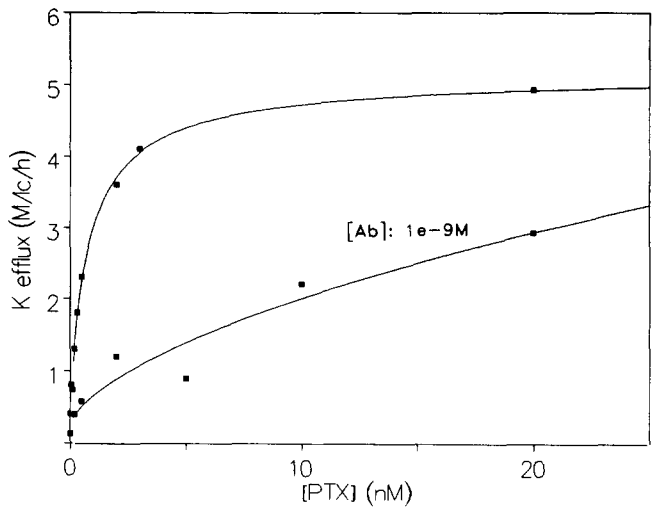

Fig. 2. K efflux induced by palytoxin: effect of antibody-palytoxin complex. The figure shows the effect of varying the PTX concentration on the K efflux. The curve labelled [Ab]:1e-9 M was obtained using the complex formed between the mAb and the concentration of PTX indicated on the abscissa. The solid curves are the result of the curve fitting described in the text, using the following kinetic constants: (a) PTX alone: $v_1 = 0.8$ M/lc/h; $K_1 = 0.02$ nM; $v_2 = 3$ M/lc/h; $K_2 = 0.9$ nM. (b) PTX preincubated with antibody (1 nM): the parameters above plus $K_{I1} = 0.4$ nM; $K_{I2} = 0.9$ nM; $K_{Ab} = 0.2$ nM. The hematocrit used was 3% for all the experimental points shown. The K efflux, in the absence of PTX and PTX-Ab was 3.2 mM/lc/h (three determinations). The points shown were obtained in four different experiments. The S.D. for each flux point was $\leq 5\%$ of the mean.

Na / K-ATPase activity. Highly purified Na/K-ATPase was obtained from the electric organ of the electric eel [9]. The enzyme activity was determined using the coupled enzymatic assay in which the hydrolysis of ATP to ADP is proportional to the oxidation of NADH [10]. The protocol followed in the experiments was as follows: 12 μl enzyme (0.16 mg protein/ml) was added to buffer (0.5 ml) (140 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 10 mM Tris-Mops, 0.5 mM Na-borate) containing 0.9 mg/ml ATP, 2.5 mM phospho*enol*pyruvate, 0.5 mM NADH and 10 U each pyruvate kinase and lactate dehydrogenase (Sigma) and the absorbance recorded continuously at 340 nm (Hitachi U2000 dual cuvette spectrometer). After approx. 5 min, 0.1 ml of buffer, 0.1 ml PTX stock solution or 0.1 ml preincubated PTX-Ab stock solution (1:1, molar ratio) was added to the sample cuvette, vortexed and the measurement resumed.

Curve fitting and determination of the apparent dissociation constant for the PTX-Ab complex. The data in Fig. 2 were fitted to the model described in the equations shown in Fig. 1. The curve fitting was done using the program 'Ligand' [11]. We also used this program for all the other models described in the next section.

3. Results and discussion

3.1. Effect of PTX-Ab on the K^+-efflux from human red cells

Previous work from our laboratory has shown that the increase in cation flux stimulated by PTX can be described by a model in which the toxin binds with two different affinities to the region of ouabain binding on the Na/K pump [5]. Recently, Bignami [7] has shown that the time to lyse murine red blood cells in the presence of PTX is longer when mAb is present. Since the lysis of red cells under these circumstances can be ascribed to a colloid osmotic mechanism due to the increase in the cation permeability, we decided to determine the effect that the complex PTX-Ab has on the permeability of red cells to cations, through measurements of the efflux of K from human red blood cells exposed to complexes made by incubation of a fixed concentration of mAb and varying concentrations of PTX.

Fig. 2 shows the K efflux induced by PTX when in the presence of toxin alone or after PTX had reacted with a constant concentration (1 nM) of antibody. It is evident that the presence of antibody reduces the apparent potency of PTX to increase the K permeability of the red cell membrane. The results could not be fitted by a model in which we assumed that the only action of the antibody was to bind free palytoxin. Rather, we found that the simplest model with which we could fit the present data was one in which the PTX-Ab complex acts as an inhibitor, preventing free PTX from binding either to the receptor or from adopting the appropriate conformation to produce an increased flux, or a combination of both. Fitting of the data was done assuming that PTX binds to one site on the antibody and that the kinetic constants for binding of free PTX to its receptor are not changed. With these assumptions, and a two-site model for the binding of the complex (PTX-Ab) to the PTX receptor the data could be fitted with the following values for the apparent dissociation constants for the palytoxin-antibody-receptor complex (cf. Eqs. (4) and (5), Fig. 1): $K_{I1} = 0.04$ nM, $K_{I2} = 0.4$ nM and an apparent dissociation constant for the palytoxin-antibody complex, $K_o = 0.2$ nM. The values obtained for the apparent affinity constants of the complex for the receptor are very similar to those obtained in the case of free PTX binding to its receptor ($K_1 = 0.02$ nM and $K_2 = 0.9$ nM). They also compare well with those obtained previously [5], except for K_2, which we find in this study to be almost 50-times lower than the previously found value. We think that this discrepancy can be attributed to a more complete set of data of flux vs. concentration available since the first study was published.

Addition of antibody to a suspension of cells already exposed to PTX failed to inhibit the PTX-induced K efflux. This result contrasts with the effect of ouabain which rapidly chases PTX from its binding site on the red cell membrane [5].

3.2. Effect of PTX-Ab on (Na + K)-activated ATPase

Another characteristic of the action of palytoxin is its ability to inhibit the (Na + K)-activated ATPase activity associated with the Na/K pump [4,5]. In order to determine if this action of PTX was affected when the toxin reacted with the monoclonal antibody, we studied the effects of mAb on the enzymatic activity of an isolated, purified preparation from electric eel in the absence an presence of PTX and its antibody. The results shown in Fig. 3 indicate that upon addition of 0.8 μM PTX (final concentration), the enzyme activity is inhibited 60% (line labelled 'PTX'). Further shown in the figure is that this inhibition is partially relieved (enzyme inhibited 40%) if PTX is preincubated with its antibody at a 1 : 1 molar ratio (cf. line 'PTX + Ab', Fig. 3). Higher molar ratios (Ab/PTX) could not be attained due to the concentration at which the antibody was present. Nonetheless, this result indicates that the complex of PTX to its antibody leads to a reversal of

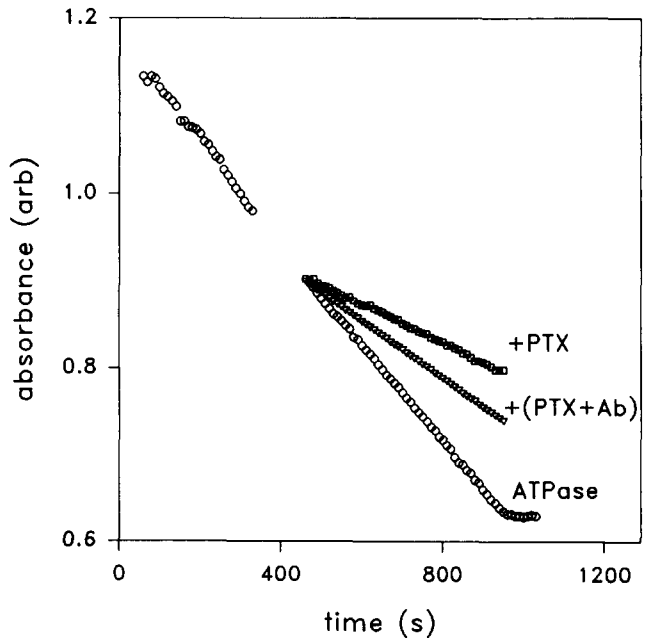

Fig. 3. Na/K-ATPase: effect of PTX and PTX-Ab on its enzymatic activity. The figure is a superposition of three different experiments. In all three cases, the enzyme activity (proportional to the slope of the absorbance vs. time line) was determined for approx. 5 min (beginning of the gap in the figure). This was followed by the addition of: 0.1 ml buffer ('ATPase'), 0.1 ml PTX stock solution in buffer ('PTX') or 0.1 ml preincubated PTX-Ab stock solution ('PTX + Ab') to the sample cuvette. This protocol was followed in three different experiments.

the inhibition of the enzyme which is brought about by PTX [5,6]. The remaining toxic activity is probably due to the free PTX still present in the PTX-Ab complex, at the molar ratio used.

The observations reported in this paper show that a monoclonal antibody raised against a derivative of palytoxin partially reverses the increase in K permeability of human red cell membranes and inhibition of the (Na + K)-activated ATPase from eel electric organ produced by PTX. The action of the PTX-Ab complex on the increase in the cationic permeability of human red cells can be quantitatively accounted for by two consequences of the binding of PTX to antibody: (1) reduction in the concentration of free PTX available to bind to the ouabain-binding region of the Na/K pump, and (2) binding of the PTX-Ab complex to the same site (s) but in a way that does not induce channel formation. The estimated value for the apparent association constant of palytoxin-antipalytoxin antibody yields an apparent affinity for the monoclonal antibody which is 5-times higher than the one previously reported using a polyclonal antibody [12].

References

[1] Bignami, G.S., Raybould, T.J.G., Sachinvala, N.D., Grothaus, P.G., Simpson, S.B., Lazo, C.B., Byrnes, J.B., Moore, R.E. and Vann, D.C. (1992) Toxicon 30, 687–700.
[2] Moore, R.E. and Scheuer, P.J. (1971) Science 172, 495–498.
[3] Alcala, A.C., Alcala, L.C., Garth, J.S., Yasamura, D. and Yasumoto, T. (1988) Toxicon 26, 105–107.
[4] Habermann, E. (1989) Toxicon 27, 1171–1187.
[5] Tosteson, M.T., Halperin, J.A., Kishi, Y. and Tosteson, D.C. (1991) J. Gen. Physiol. 98, 969–985.
[6] Grell, E., Lewitzi, E. and Uemura, D. (1988) Prog. Clin. Biol. Res. 268B, 393–400.
[7] Bignami, G.S. (1993) Toxicon 31, 817–820.
[8] Ahnert-Hilger, G., Chhatwal, G.S., Hessler, H.-J. and Habermann, E. (1982) Biochim. Biophys. Acta 688, 486–494.
[9] Liang, S.-M. and Winter, C.G. (1976) Biochim. Biophys. Acta 452, 552–565.
[10] Scharschmidt, B.F., Keeffe, E.B., Blankenship, M.N. and Ockner, R.K. (1979) J. Lab. Clin. Med. 93, 790–799.
[11] Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220–239.
[12] Levine, L., Fujiki, H., Gjika, H.B. and Van Vunakis, H. (1988) Toxicon 26, 1115–1121.