Cobra polypeptide cytotoxin I and marine worm polypeptide cytotoxin A-IV are potent and selective inhibitors of phospholipid-sensitive Ca^{2+}-dependent protein kinase

J.F. Kuo, Robert L. Raynor, Gonzalo J. Mazzei, Randall C. Schatzman, R. Scott Turner and William R. Kem

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322 and *Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, FL 32610, USA*

Received 12 January 1983

The effects of a number of polypeptide cytotoxins and neurotoxins on various protein kinases were examined. It was found that cobra cytotoxin I and marine worm cytotoxin A-IV effectively and specifically inhibited phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK) relative to myosin light chain kinase and cyclic nucleotide-dependent protein kinases. Inhibition of PL-Ca-PK by these cytotoxins could be overcome by phosphatidylserine. Neurotoxins, in comparison, were much less effective inhibitors. The present findings indicated that these polypeptide cytotoxins, unlike other agents reported to date, were selective inhibitors of PL-Ca-PK and could be used to differentiate Ca^{2+}-dependent events regulated by phospholipid or calmodulin.

Ca^{2+} *Phospholipid* *Protein phosphorylation* *Neurotoxin*

1. INTRODUCTION

Diverse groups of animals are known to produce polypeptide toxins. The neurotoxins act by binding to specific receptors which regulate ion channels [1]. Cytotoxins, on the other hand, are surface-active amphipathic agents which usually interact with membrane lipid bilayer components (such as phospholipids), producing a generalized toxic response in cardiovascular and other tissues [2–4]. The ubiquitous occurrence of PL-Ca-PK has been established [5,6]. This enzyme is distinct from other species of Ca^{2+}-dependent protein kinases requiring calmodulin as a cofactor, as exemplified by MLCK [7]. PL-Ca-PK, present in cells as soluble and membrane-bound forms [8], has been purified and characterized [9–12]. In a continuing effort to elucidate the regulatory mechanism and functional roles of PL-Ca-PK, we examined the effects of certain purified polypeptide cytotoxins and neurotoxins on PL-Ca-PK relative to MLCK. We found that certain cytotoxins were potent and, unlike other agents reported to date, were relatively selective inhibitors of PL-Ca-PK.

Abbreviations: PL-Ca-PK, phospholipid-sensitive Ca^{2+}-dependent protein kinase; MLCK, myosin light chain kinase; A-PK and G-PK, cyclic AMP- and cyclic GMP-dependent protein kinases, respectively

2. EXPERIMENTAL

2.1. *Toxins and other reagents*

Pure cytotoxin I and neurotoxin I (both from cobra *Naja naja oxiana*) were purchased from Calbiochem-Behring (San Diego CA). Melittin and apamin (both from bee venom) and phosphatidylserine (from bovine brain) were from Sigma (St Louis MO); polymyxin B sulfate was from Pfizer (New York NY); calmodulin was from Sciogen (Detroit MI). Cytotoxin A-IV [4] and neurotoxin B-IV [13], both from marine worm *Cerebratulus lactaeus*, were purified as described. Phosphatidylserine was microdispersed to clarity by sonication.

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

2.2. *Enzymes*

PL-Ca-PK was purified to 80–95% homogeneous from bovine heart [9], to homogeneous from pig spleen [11], and to about 80% homogeneous from pig brain as in [9] (unpublished). Homogeneous cardiac MLCK [7] and partially purified cardiac A-PK [14] and G-PK [15] were prepared as reported.

PL-Ca-PK was incubated in 0.2 ml 50 mM Pipes (pH 6.5) and activated by 2 μg phosphatidylserine and 0.5 mM $CaCl_2$ [9–11]. MLCK was incubated in 0.2 ml 25 mM Tris–HCl (pH 7.5) and activated by 0.5 μg calmodulin and 0.1 mM $CaCl_2$ [16]. The amounts of phosphatidylserine and calmodulin chosen were such that they supported the Ca^{2+}-stimulated activities of the enzyme 50–60% of that maximally stimulated (12–35-fold) by Ca^{2+}. A-PK and G-PK were incubated in 0.2 ml 25 mM potassium phosphate (pH 7.5) and activated 10–15-fold by 0.5 μM cyclic AMP and cyclic GMP, respectively [14,15]. The activities of the enzymes were linear with respect to the amount of enzyme and the time of incubation. All experiments were performed 2 or 3 times and the average activity values stimulated by Ca^{2+}, cyclic AMP or cyclic GMP were reported or used for calculation.

3. RESULTS AND DISCUSSION

Marine worm cytotoxin A-IV and cobra cytotoxin I (cardiotoxin I) were more effective than cobra neurotoxin I in inhibiting various protein kinases purified from bovine hearts (fig.1). The inhibition by toxins, in a decreasing order, was PL-Ca-PK > MLCK ⩾ A-PK and G-PK. Cytotoxin A-IV also more specifically inhibited PL-Ca-PK compared to a cardiac phosphodiesterase stimulated by calmodulin/Ca^{2+} (not shown).

Inhibition of PL-Ca-PK by cytotoxin A-IV and cytotoxin I was progressively overcome by increasing concentrations of phosphatidylserine (fig.2). Detailed kinetic analysis indicated that both cytotoxins A-IV and I inhibited the enzyme competitively with respect to phosphatidylserine, with K_i-values of 0.3 and 0.8 μM, respectively. The results suggested that these cytotoxins may interact with the phosphatidylserine, or with a hydrophobic region on the enzyme to which the phospholipid cofactor binds. We reported [15] that melittin, a polypeptide cytotoxin from bee venom, inhibited PL-Ca-PK and MLCK competitively with respect to their respective cofactor.

The relative activity of various toxins on cardiac PL-Ca-PK MLCK were compared (table 1). It ap-

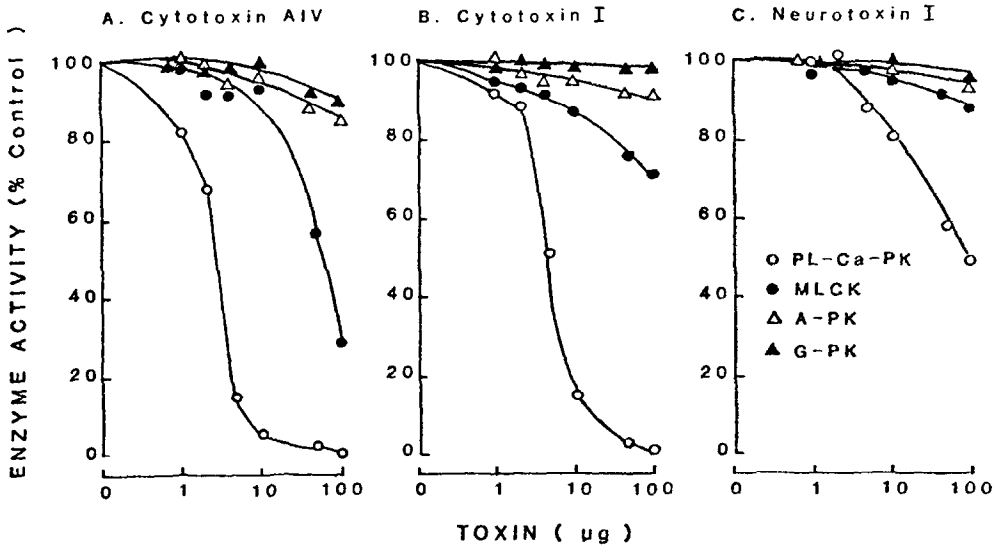

Fig.1. Comparative inhibition of various protein kinases by polypeptide toxins. The enzymes used were purified from bovine heart. Results similar to those shown above for the cardiac PL-Ca-PK were also obtained for the enzyme from pig spleen and brain.

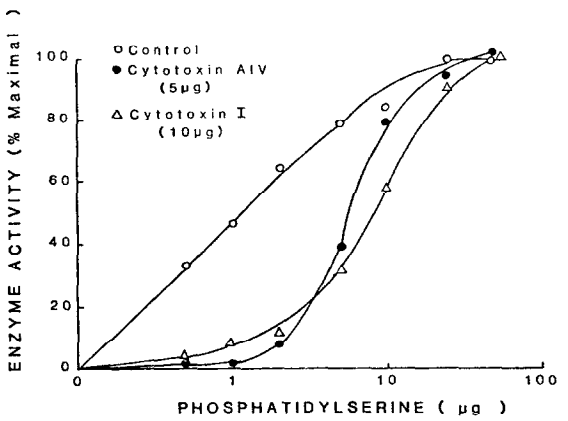

Fig.2. Reversal by phosphatidylserine of cytotoxin inhibition of cardiac PL-Ca-PK. Results similar to those shown above for the cardiac enzyme were also noted for the spleen enzyme.

peared that cytotoxins (cytotoxin A-IV, cytotoxin I, and melittin) were generally more effective inhibitors than neurotoxins (neurotoxin B-IV, neurotoxin I, and apamin) for PL-Ca-PK. It was also noted that cytotoxins A-IV and I as well as neurotoxin I were generally more specific inhibitors for PL-Ca-PK than for MLCK, as indicated by their ratios of IC_{50}-values (concentrations causing a 50% inhibition) for the two enzymes of about 0.03–0.05. In contrast, neurotoxin B-IV was a more specific inhibitor for MLCK, as indicated by an IC_{50} ratio of 10. Little specificity, however, was noted for the two bee venom toxins (melittin and apamin), with IC_{50} ratios of 0.58 and 0.46, respectively. Interestingly, polymyxin B (a membrane-active polypeptide antibiotic) was as effective as all cytotoxins tested in inhibiting PL-Ca-PK; furthermore, like cytotoxins A-IV and I but unlike melittin, it exhibited a specificity for PL-Ca-PK inhibition as indicated by an IC_{50} ratio of 0.02. It is worth noting that, of the 3 neurotoxins studied, apamin was more effective than neurotoxins B-IV and I in inhibiting PL-Ca-PK. Apamin is one of the smallest neurotoxin polypeptides known [3]. The ability of apamin, but not other larger neurotoxins, to inhibit PL-Ca-PK may be related to its smaller size.

Since we found that cytotoxin I was more active than neurotoxin I in inhibiting PL-Ca-PK (presumably in part via interaction between the cytotoxin and phosphatidylserine), we suspected

Table 1

Comparative potency of polypeptide toxins to inhibit cardiac PL-Ca-PK and MLCK

Source and toxin	IC_{50} (µg/ml)		IC_{50} ratio of PL-Ca-PK:MLCK
	PL-Ca-PK	MLCK	
Marine worm			
Cytotoxin A-IV	13 (1.3)[a]	360 (34.6)	0.04
Neurotoxin B-IV	750 (125)	75 (12.5)	10.0
Cobra			
Cytotoxin I (cardiotoxin I)	26 (3.7)	750 (107.1)	0.03
Neurotoxin I	475 (67.9)	>10000 (1428.5)	<0.05
Honey bee			
Melittin (cytotoxin)[b]	7 (2.5)	12 (4.3)	0.58
Apamin (neurotoxin)	60 (30.0)	130 (65.0)	0.46
Bacterium			
Polymyxin B (antibiotic)	2 (2.0)	105 (105.0)	0.02

[a] The values in parentheses are in µM, using the following M_r-values: cytotoxin A-IV, 10400; neurotoxin B-IV, 6000; cytotoxin I and neurotoxin I, both 7000; melittin, 2800; apamin, 2000; polymyxin B, 1000

[b] The same results were obtained using either a commercial (Sigma) preparation or a pure preparation of melittin, an original gift of Dr G. Kreil (Institute of Molecular Biology, Austrian Academy of Science) [16]

that the phospholipid could more effectively protect mice from a lethal dose of cytotoxin I than that of neurotoxin I. We found that this was indeed the case. The time required to kill male mice (25 g body wt, CF-1 strain, Charles River Breeding Labs, Wilmington MA) by a subcutaneous injection (in 0.26 ml saline) of cytotoxin I (1500 μg) was 15.8 ± 3.3 min (mean ± standard error, $n = 6$). If phosphatidylserine (400 μg) was mixed with cytotoxin I before injection, the killing time was significantly increased to 32.5 ± 8.2 min ($n = 8$, $p < 0.01$). The killing time seen for neurotoxin I (100 μg) was 20.2 ± 4.0 min ($n = 6$); mixing of phosphatidylserine (400 μg) with neurotoxin I, however, did not increase the killing time (24.4 ± 4.9 min, $n = 6$). The lethal doses of toxins used have been predetermined to be the minimum amounts that kill mice in 15–20 min after the injection. There was no noticeable effect when the phospholipid was injected alone.

The present studies indicated that cytotoxin A-IV, cytotoxin I as well as polymyxin B were effective and relatively selective inhibitors for PL-Ca-PK compared to MLCK. These findings are of some potential importance, because we noted previously that many inhibitors studied, such as phenothiazine antipsychotic drugs [17], palmitoylcarnitine [18], and melittin [16] are nearly equipotent in inhibiting the phospholipid/Ca^{2+}- and calmodulin/Ca^{2+}-stimulated protein phosphorylation systems or, in some cases, inhibit the latter system even slightly more effectively. It appears that cytotoxins A-IV and I and polymyxin B could be used as agents to differentiate Ca^{2+}-dependent reactions or processes regulated by either phospholipid or calmodulin. It remains unclear whether an inhibition by these cytotoxins of protein phosphorylation is directly or indirectly related to their well-recognized toxic effects on cells.

ACKNOWLEDGEMENTS

This work was supported by USPHS grants HL-15696, NS-17608, GM-25849 and T32-GM-07594, and by NSF grant BNS-76-06266.

REFERENCES

[1] Albuquerque, E.X., Eldefrawi, A.T. and Eldefrawi, M.E. (1981) in: Handbook of Experimental Pharmacology, Snake Venoms (Lee, C.Y. ed) vol.52, pp.377–402, Springer Verlag, Berlin, New York.
[2] Lee, C.Y. (1979) in: Advances in Cytopharmacology (Ceccarelli, B. and Clement, F. eds) vol.3, pp.1–16, Raven, New York.
[3] Habermann, E. (1972) Science 177, 314–322.
[4] Kem, W.R. and Blementhal, K.M. (1978) J. Biol. Chem. 253, 5752–5757.
[5] Takai, Y., Kishimot, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695.
[6] Kuo, J.F., Andersson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Natl. Acad. Sci. USA 77, 7039–7043.
[7] Walsh, M.P., Vallet, B., Autric, F. and Demaille, J.G. (1979) J. Biol. Chem. 254, 12136–12144.
[8] Katoh, N. and Kuo, J.F. (1982) Biochem. Biophys. Res. Commun. 106, 590–595.
[9] Wise, B.C., Raynor, R.L. and Kuo, J.F. (1982) J. Biol. Chem. 257, 8481–8488.
[10] Wise, B.C., Glass, D.B., Chou, C.-H.J., Raynor, R.L., Katoh, N., Schatzman, R.C., Turner, R.S., Kibler, R.F. and Kuo, J.F. (1982) J. Biol. Chem. 257, 8489–8495.
[11] Schatzman, R.C., Raynor, R.L., Fritz, R.B. and Kuo, J.F. (1983) Biochem. J., in press.
[12] Kikkawa, U., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341–13348.
[13] Kem, W.R. (1976) J. Biol. Chem. 251, 4184–4192.
[14] Kuo, J.F., Krueger, B.K., Sanes, J.R. and Greengard, P. (1970) Biochim. Biophys. Acta 212, 79–91.
[15] Shoji, M., Patrick, J.G., Davis, C.W. and Kuo, J.F. (1977) Biochem. J. 161, 213–221.
[16] Katoh, N., Raynor, R.L., Wise, B.C., Schatzman, R.C., Turner, R.S., Helfman, D.M., Fain, J.N. and Kuo, J.F. (1982) Biochem. J. 202, 217–224.
[17] Wrenn, R.W., Katoh, N., Schatzman, R.C. and Kuo, J.F. (1981) Life Sci. 29, 725–733.
[18] Katoh, N., Wrenn, R.W., Wise, B.C., Shoji, M. and Kuo, J.F. (1981) Proc. Natl. Acad. Sci. USA 78, 4813–4817.