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INHIBITION OF METABOLIC RESPONSE OF POLYMORPHONUCLEAR LEUKOCYTE BY BISCOCLAURINE ALKALOIDS

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Abstract—Effects of biscoclaurine alkaloids on the various stimulus-responses of PMN, especially on the $O_2^{\cdot-}$ generation of PMN, were investigated. Results obtained were: cepharanthine inhibited various metabolic responses of PMN, its biological action probably being due to its membrane modifying action. Inhibition of $O_2^{\cdot-}$ generation by cepharanthine was stronger than any other inhibition of metabolic responses of PMN. The inhibitory effect of various biscoclaurine alkaloids on the $O_2^{\cdot-}$ generation of PMN was the descending order of tri-, di- and mono-ether type; the coclaurine type showed only a weak effect.

Cepharanthine, a biscoclaurine alkaloid, has been used for a long time as a drug for chronic inflammation and recently as one which protects leukocytopenia. Biochemically it is quickly incorporated into the biological membrane [1], resulting in membrane stabilizing action such as inhibition of lysolecithine-induced hemolysis [2], inhibition of membrane hydrolysis with phospholipase A_2 [3] and inhibition of membrane lipid peroxidation [4, 5].

In connection with these inhibitory effects of cepharanthine, it has recently been shown that it exhibits a very strong inhibitory action on several metabolic responses of polymorphonuclear leukocyte (PMN) [6, 7] and platelets [8] due to their membrane modification. However, little is known about the role of its inhibitory action on the stimulus-response mechanism of leukocytes, and which part of the chemical structure of cepharanthine participates in the above actions is still obscure.

In this study, the effects of various biscoclaurine alkaloids on the various stimulus-responses of leukocyte were examined in an attempt to understand the mechanism of inhibitory action of cepharanthine on the stimulus-induced responses of PMN, especially on the superoxide generation.

MATERIALS AND METHODS

Chemicals. Ferricytochrome *c* (cyt.*c*), chlorotetracycline (CTC) and formylmethionyl-leucyl-phenylalanine (FMLP) were purchased from Sigma Chemical Co. (St. Louis, MO). 3,3′-Dipropylthiodicarbocyanine iodide (diS-C_3-(5)], a cyanine dye, was obtained from Kankoshikiso Research Institute (Okayama, Japan) and 2-[(2-amino-5-methylphenoxy) methyl]-6-methoxy-8-aminoquinoline-*N,N,N′,N′*-tetraacetoxymethyl ester (Quin 2/AM) was from Dojindo Laboratory (Kumamoto, Japan). Biscoclaurine alkaloids were donated by Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). Quin 2/AM was dissolved in dimethyl sulfoxide (DMSO) to obtain a concentration of 60 mM; the final concentration of DMSO used was lower than 30 μM. All the other chemicals used were of analytical grade and obtained from Nakarai Chemical Co. (Kyoto, Japan).

Preparation of PMN. PMN were obtained from male guinea pigs weighing 300–400 g after an intraperitoneal injection of 2% casein as described previously [6]. The cells were washed three times by centrifugation, then suspended in calcium-free Krebs–Ringer phosphate buffer solution (KRP).

Analytical procedures. Superoxide ($O_2^{\cdot-}$) generation by PMN was monitored continuously by the cyt. *c* reduction method of Nakagawara *et al.* [9] with dual-wavelength spectrophotometer (Shimazu UV-300). PMN suspended in KRP (1×10^6 cells/ml) were incubated in a thermostatically controlled cuvette equipped with a magnetic stirrer. Cyt. *c* was added to the suspension to give a final concentration of 25 μM. The reduction rate of cyt. *c* at 37° was measured at 550 nm with a reference wavelength of 540 nm. The $O_2^{\cdot-}$ generation was calculated from the absorbance change by using the molar extinction coefficient of 19,100.

The intracelluar free calcium ion [$(Ca^{2+})_i$] was calculated from changes in the fluorescence intensity of Quin 2-loaded PMN as described by Tsien *et al.* [10]. The fluorescence intensity of the Quin 2-loaded PMN was measured with a fluorospectrophotometer (Shimazu RF-510) equipped with a thermostatically controlled cuvette holder and magnetic stirrer. The wavelength for excitation was 339 nm and for emission 490 nm.

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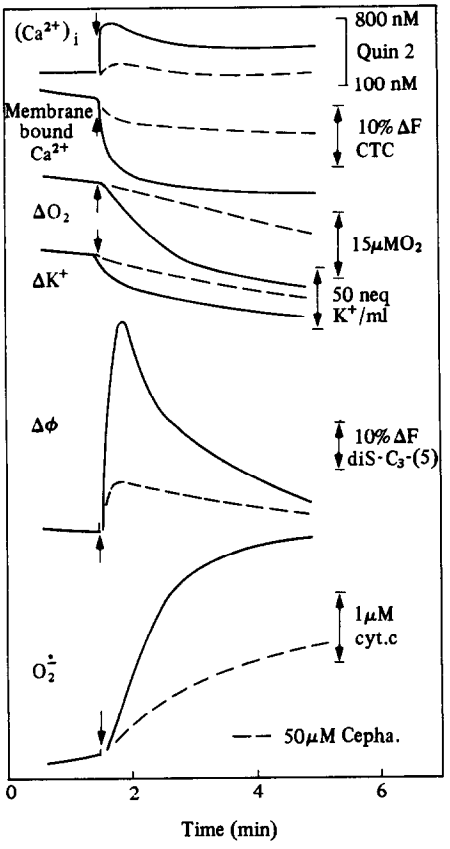

Fig. 1. Effect of cepharanthine on the various FMLP-induced responses in PMN. Arrows mark the addition of FMLP (5×10^{-8} M), in the absence of cepharanthine (———) and in the presence of cepharanthine (---) (5×10^{-5} M). For the measurement of K^+ release, guinea-pig PMNs were incubated in the medium of 0.15 M choline chloride–10 mM Tris–HCl buffer solution. For the other measurements, PMNs were incubated in KRP at 37°. $(Ca^{2+})_i$: changes in the concentration of intracellular free calcium ion monitored by Quin 2 fluorescence. Membrane bound Ca^{2+}: membrane bound calcium monitored by CTC fluorescence. O_2: oxygen consumption measured by oxygen electrode. K^+: K^+ release from the cells measured by K^+ selective electrode. ϕ: changes in membrane potential monitored by diS-C_3-(5) fluorescence. O_2^-: superoxide generation measured by the reduction of cyt. *c*.

The membrane potential of PMN was measured by the cyanine dye method in the medium of KRP at 37° as described in an earlier paper [6]. The final concentration of cyanine dye was 2 μM, and the wavelength for excitation was 622 nm and for emission 670 nm, respectively.

For the measurement of other metabolic responses of PMN to stimuli, the following methods were employed: membrane bound Ca^{2+} by the fluorescence change of CTC [11], oxygen consumption by Clark type oxygen electrode and K^+ efflux by K^+ selective electrode as described in previous papers [12, 13], and the release of lactate dehydrogenase (LDH) from cells by decrease in NADH at 340 nm [14].

RESULTS AND DISCUSSION

Metabolic responses of PMN and the effect of cepharanthine

PMNs show several metabolic changes in response to various stimuli. In Fig 1, various response reactions examined in this study were demonstrated. They are K^+ efflux measured directly as a metabolic response to the stimulation by FMLP and parallel membrane potential change, decrease in membrane bound Ca^{2+}, increase in $(Ca^{2+})_i$ and O_2^- generation. Among these responses, there probably is a close mutual relation though details are still obscure as to the causal relation.

From the reaction rate, however, ion permeability and membrane potential change were first induced and then followed by oxygen consumption and O_2^- generation. On each of these reactions, not on any specific reaction, cepharanthine acts inhibitively. Consequently, an obstruction to a certain membrane modifying action in which all the above response reactions participate seems to be an inhibitory mechanism of cepharanthine. To know whether these alkaloids are inhibiting above response reactions by toxic effect, the release of LDH from cells was examined. Release of LDH was not induced either by cepharanthine (50 μM) or by FMLP (6×10^{-8} M), this indicates that these alkaloids have no accel-

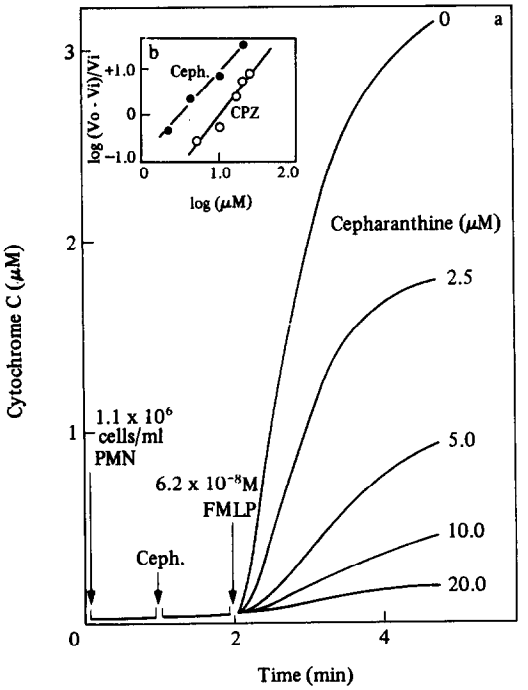

Fig. 2. FMLP-induced superoxide generation in PMN and its inhibition by cepharanthine. (a) PMN (1.1×10^6 cells/ml) were suspended in KRP containing 1 mM $CaCl_2$ at 37°. FMLP was added to the suspension to give a final concentration of 6.2×10^{-6} M in the presence of various concentrations of cepharanthine. The superoxide-dependent reduction of cyt. *c* was measured as in Fig. 1. Cepharanthine was added 1 min before the stimulation of PMN by FMLP. (b) Hill plots from data on the dose–response curves for the inhibition of superoxide generation by cepharanthine or CPZ.

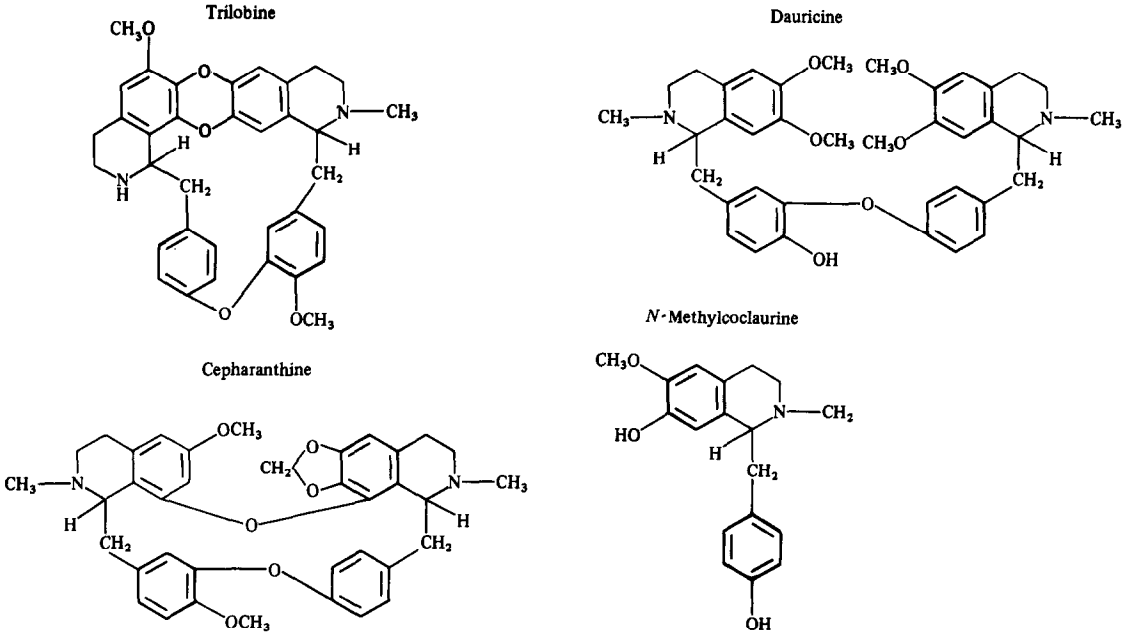

Fig. 3. Structures of the biscoclaurine alkaloids tested.

erative effect on membrane permeability due to cell damage.

Effect of cepharanthine on the $O_2^{\cdot-}$ generation of PMN by FMLP and its concentration dependency

As shown in Fig. 2a, cepharanthine inhibited FMLP-induced $O_2^{\cdot-}$ generation of PMN in a concentration dependent manner. Fifty per cent inhibition by 2.5–5.0 μM cepharanthine was considerable as compared with chlorpromazine (CPZ) [15] which inhibits $O_2^{\cdot-}$ generation by its membrane modifying action. The Hill plot shows that this inhibition appears as a cooperative phenomenon of the membrane, Hill constants of cepharanthine and CPZ being 1.99 and 1.92 respectively (Fig. 2b). A similar inhibitory effect of cepharanthine was observed also in the case of $O_2^{\cdot-}$ generation induced by other stimulants such as A23187, a Ca^{2+} ionophore, Con A, digitonin and myristic acid.

Effect of various biscoclaurine alkaloids on FMLP-induced $O_2^{\cdot-}$ generation of PMN

The biscoclaurine alkaloids used are grouped into three types: mono-, di- and tri-ether types as shown in Fig. 3. Most of biscoclaurine alkaloids represented

Table 1. Effects of various biscoclaurine alkaloids on the superoxide generation by PMN

Alkaloids			% Inhibition
Biscoclaurine	Tri-ether	Trilobine	98.8
		Isotrilobine	98.3
	Di-ether	Cepharanthine	96.3
		Tetrandrine	92.2
		Fangchinoline	82.4
		Thalrugosine	77.6
		Homoaromoline	67.0
		Isotetrandrine	65.9
		Oxyacanthine	63.1
		Berbamine	56.4
		Cepharanoline	56.0
		Cycleanine	36.2
		Hypoepistephanine	34.1
	Mono-ether	Neferine	63.6
		Isoliensinine	59.6
		Dauricine	50.8
Coclaurine		*N*-methylcoclaurine	64.6
		N,O,O-trimethylcoclaurine	29.3

Experimental conditions were the same as in Fig. 2 except that the concentration of added alkaloids was 5×10^{-5} M. Data represent the percent inhibition against control.

by cepharanthine have a strong inhibitory effect on the FMLP-induced $O_2^{\dot{-}}$ generation of PMN. Comparing the inhibitory activity of various alkaloids (50 μM) on the $O_2^{\dot{-}}$ generation, as shown in Table 1, the tri-ether type was the strongest, and the di-ether type and then the mono-ether type succeeded. On the contrary, coclaurine type alkaloids which have no ether linkage in their molecules showed only low inhibitory activity. From these results, it was made clear that a rigid stereostructure of the biscoclaurine alkaloids has a stronger inhibitory activity, i.e. the relation between molecular structure and inhibitory activity of these compounds was represented.

Inhibitory action of biscoclaurine alkaloids used in this experiment on the metabolic responses of leukocytes parallels those of platelets [3], lysolecithine-induced hemolysis [2] and lipid peroxidation of biological membranes [4, 5]. These results suggest that their biological actions are ascribed to the membrane modifying action of these alkaloids. In addition, their inhibitory activity on the $O_2^{\dot{-}}$ generation was stronger than that on any other response reactions of PMN. The intensity of the inhibitory action of cepharanthine was almost equal to that of collagen on the platelet aggregation [8].

As to chemical structure, their inhibitory activity was in the descending order of tri-ether, di-ether and mono-ether. Coclaurine alkaloids have only low activity. Our preliminary experiment indicated that these kinds of alkaloids inhibit metabolic responses selectively in the case of the stimulation of PMN by membrane perturbers such as digitonin and fatty acids [16]. Further investigations are necessary on the action mechanism of these alkaloids.

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