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粉防己碱对大鼠嗜中性白细胞胞浆游离钙的影响[1]

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Effects of tetrandrine on cytoplasmic free calcium in rat neutrophils

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ABSTRACT Neutrophils (NP) cytoplasmic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) under several conditions were measured with Quin 2 in rats. The process of loading Quin 2-AM into the cells followed by its hydrolysis was assured by monitoring the shift of fluorescent emission spectrum of Quin 2-AM to that of Quin 2. The resting $[Ca^{2+}]_i$ of NP in Ca^{2+}-containing and Ca^{2+}-free solutions were 186±45 and 46±16 nmol/L, respectively, indicating extracellular calcium concentration ($[Ca^{2+}]_o$) plays an important role on $[Ca^{2+}]_i$. Among agents tested, prostaglandin E_2 (PGE_2) 100 nmol/L did not change $[Ca^{2+}]_i$ significantly. Calcimycin 25 μmol/L, leukotriene B_4 (LTB_4) 60 nmol/L and platelet activating factor (PAF) 10 nmol/L increased $[Ca^{2+}]_i$ of NP in Ca^{2+}-containing solution from 185±54, 175±36 and 188±54 to 814±67, 577±229 and 540±174 nmol/L, respectively. But, compound 48/80 3.2 μg/ml, LTB_4 300 nmol/L, PAF 10 nmol/L and PAF 5 nmol/L plus LTB_4 150 nmol/L did not change significantly $[Ca^{2+}]_i$ of NP in Ca^{2+}-free solution, indicating that these agonists can not release intracellularly stored Ca^{2+} or no stored Ca^{2+} in NP is available. The rises of $[Ca^{2+}]_i$ produced by calcimycin, PAF and LTB_4 were markedly inhibited by tetrandrine (Tet) 65 μmol/L. These results, as a whole, show that Tet inhibits the rises of $[Ca^{2+}]_i$ of NP induced by calcimycin, PAF and LTB_4 via decreasing Ca^{2+} influx. The Ca^{2+} antagonism in this case may be related to its anti-allergic actions.

KEY WORDS tetrandrine; neutrophils; calcium channel blockers; A-23187 (calcimycin); platelet activating factor; leukotrienes B; prostaglandins E; aminoquinolines

提要 用 Quin 2 法测得大鼠嗜中性白细胞静息胞浆游离钙($[Ca^{2+}]_i$)为 186±45 nmol/L，此$[Ca^{2+}]_i$明显依赖于细胞外钙。PAF，LTB_4 和 A-23187 能促进 Ca^{2+} 内流而使$[Ca^{2+}]_i$升高，而 PGE_2 对$[Ca^{2+}]_i$无影响。粉防己碱对静息$[Ca^{2+}]_i$无影响，但能对抗 PAF，LTB_4 和 A-23187 所致的$[Ca^{2+}]_i$的升高，因此是一作用广泛的钙拮抗剂，这很可能是粉防己碱抗过敏作用的机理。

关键词 粉防己碱；嗜中性白细胞；钙通道阻滞剂；A-23187(卡西霉素)；血小板激活因子；白细胞三烯 B；前列腺素 E；氨基喹啉

嗜中性白细胞(neutrophils, NP)在机体的正常防御机理和许多炎症、过敏性疾病中均可激活，表现出趋化、吞噬、溶酶体酶释放、超氧阴离子产生和磷脂衍生物类过敏介质的释放。胞浆游离钙浓度$[Ca^{2+}]_i$是介导 NP 上述功能的重要偶联因子。粉防己碱(tetrandrine, Tet)是从防己科植物粉防己根中提取的有效成份，是过敏介质的拮抗剂和阻释剂[1]，但作用

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机理尚不完全明确。本文引用 Quin 2 {2-[(2-bis-[carboxymethyl]-amino-5-methyl-pheoxy) methyl]-6-methoxy-8-bis [carboxy-methyl] aminoquinoline}法[3]测定了大鼠 NP 的$[Ca^{2+}]_i$及其在不同情况下的变化，观察了 Tet 对$[Ca^{2+}]_i$的影响，分析了 Tet 的作用机理。

MATERIALS AND METHODS

Tet 系杭州第一制药厂产品，Quin 2-AM {2-[(2-bis[carboxymethyl]-amino-5-methyl-phenoxy)-methyl]-6-methoxy-8-bis[carboxy-methyl]-aminoquinolinetetrakis-[acethoxy-methyl]ester}，血小板激活因子(platelet activating factor, PAF)、化合物 48/80、前列腺素 E_2 (prostaglandin E_2, PGE_2)购自 Sigma 公司，白细胞三烯 B_4 (leukotriene B_4, LTB_4)系加拿大 Merck Frost 实验室提供，卡西霉素(calcimycin, A-23187)购自 Calbiochem Beohring 公司，角叉菜胶系辽宁省药物研究所提供，荧光分光光度计系日立 MPF-4。

大鼠 NP 的分离[2] 取 Sprague-Dawley 大鼠，体重 180±SD 15 g，乙醚麻醉后向右胸腔注入 1%角叉菜胶 0.2 ml，4 h 后杀死，向左、右胸腔各注入磷酸缓冲液约 5 ml，收集胸腔炎症渗出液，400×g 离心 7 min，去上清，加入少量蒸馏水破坏污染的红细胞，30 s 后加入足量的 Hanks 液，离心去上清，以适量 Hanks 液混匀 NP，Wright 染色，NP>95%。

Quin 2-AM 导入 NP[3] 取上述 NP 悬液适量，加 Quin 2-AM 液 30 μmol/L(以 DMSO 溶解)，对照组加等量 DMSO。37℃水浴 1 h 后，根据需要以含 Ca^{2+} 的 Hanks 液或含 EGTA 0.3 mmol/L 但不含 Ca^{2+} 的 Hanks 液洗 NP 两次，并稀释至 2×10^7 NP/ml。台盼蓝染色，NP 活性>95%。

荧光强度的测定及$[Ca^{2+}]_i$的计算[3-5] 取上述有 Quin 2 负荷的 NP 悬液 0.2 ml 置于吸收池(37℃恒温)，需要时加入一定浓度的 Tet，静置 5 min 后测定荧光强度。激发波长 336 nm，激发狭缝 2 nm，发射波长 492 nm，发射狭缝 15 nm，当 NP 悬浮于含 Ca^{2+} 的 Hanks 液中时，首先测定 NP 静息时和受到不同刺激物攻击后的荧光强度并记为 L，然后加洋地黄皂甙 50 μg/ml 以破坏细胞膜，使 Quin 2 被细胞外高浓度的 Ca^{2+} 所饱和，此荧光强度为 L_{max}，最后加 $MnCl_2$ 0.5 mmol/L，熄灭 Quin 2 的荧光，此时的荧光强度为 L_{min}。$[Ca^{2+}]_i$ 的计算公式为：$[Ca^{2+}]_i = K_d(I-0.16I_{tot})/(I_{tot}-I)$。其中：$K_d$=115 nmol/L，为 Quin 2 与 Ca^{2+} 之间的解离常数；$I=L-L_{min}$；$I_{tot}=L_{max}-L_{min}$。当 NP 悬浮于无 Ca^{2+} 的 Hanks 液中时，首先测定 NP 在静息时和加入不同刺激物后的荧光强度并记为 F，然后加洋地黄皂甙 50 μg/ml 破坏细胞膜，此时因 Ca^{2+} 被 EGTA 络合，故测得的荧光强度为 F_{min}，最后加 $CaCl_2$ 1.8 mmol/L，使 EGTA 被过量的 Ca^{2+} 所饱和，并有足量的 Ca^{2+} 与 Quin 2 结合，此时测得的荧光强度为 F_{max}。$[Ca^{2+}]_i$ 的计算公式为：$[Ca^{2+}]_i = K_d(F-F_{min})/(F_{max}-F)$，其中 K_d 的意义与上述相同。上述 L，L_{max}，L_{min}，F，F_{max}，F_{min} 均根据有 Quin 2 负荷的 NP 悬液受到激发光连续照射后荧光读数的逐渐减小(光漂白作用，photobleaching)作相应校正，并扣除所加试剂产生的荧光读数。

RESULTS

Quin 2-AM 被导入细胞并被水解的鉴定 Quin 2-AM 加入到 NP 悬液后立即扫描发射光谱，峰值位于 430 nm。37℃孵育 1 h 后以 Hanks 液洗 NP 两次，此时的发射光谱峰值位于 492 nm，与文献报道[3]一致，说明 Quin 2-AM 已进入 NP 并被细胞内酯酶水解为 Quin 2，可在此基础上进行$[Ca^{2+}]_i$的测定(Fig 1)。

正常大鼠静息 NP 的$[Ca^{2+}]_i$及 Tet 和细胞外钙$[Ca^{2+}]_o$对$[Ca^{2+}]_i$的影响 正常大鼠静息 NP 在含 Ca^{2+} 的 Hanks 液中的$[Ca^{2+}]_i$为 186±

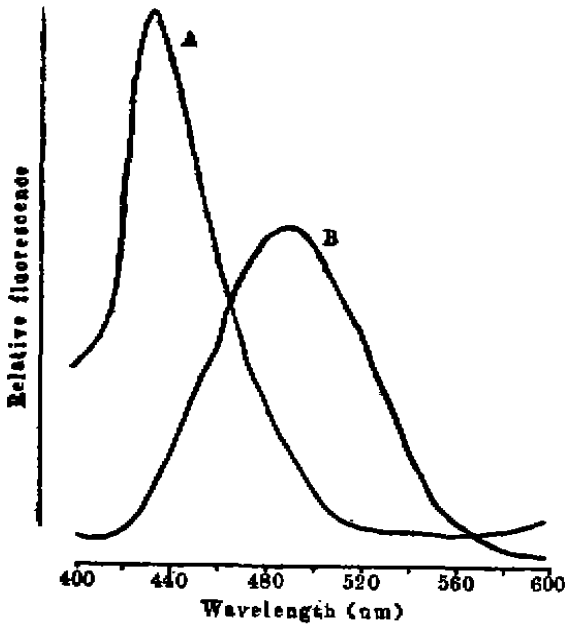

Fig 1. Fluorescent emission spectra of neutrophil suspension (A) just after the addition of Quin 2-AM and (B) after 1 h incubation at 37℃. Slits were 15 and 2 nm for E_x and E_m, respectively. E_x wavelength was 336 nm ($n=5$).

45 nmol/L.加 Tet 65 μmol/L, 5 min 对$[Ca^{2+}]_i$没有影响. 在含有 EGTA 0.3 mmol/L 的无 Ca^{2+} Hanks 液中, 正常大鼠静息的$[Ca^{2+}]_i$明显降低(Tab 1).

PGE_2, A-23187, LTB_4 和 PAF 对在含 Ca^{2+} 的 Hanks 液中 NP 的$[Ca^{2+}]_i$的影响及 Tet 的对抗作用 由 Tab 2 可见, 在含 Ca^{2+} 的 Hanks 液中, PGE_2 100 nmol/L 对 NP 的$[Ca^{2+}]_i$没有影响. A-23187 25 μmol/L, LTB_4 60 nmol/L 和 PAF 10 nmol/L 可使$[Ca^{2+}]_i$明显升高. 应用 Tet 32 μmol/L 或 65 μmol/L, 5 min 后再以相同浓度的 A-23187 攻击, $[Ca^{2+}]_i$的上升幅度降低. 同样, 应用 Tet 65 μmol/L 能对抗 LTB_4 60 nmol/L 和 PAF 10 nmol/L 升高 NP $[Ca^{2+}]_i$的作用(Fig 2).

Tab 1. Cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) of resting neutrophils from normal rats and the effects of tetrandrine (Tet) and extracellular calcium concentration ($[Ca^{2+}]_o$) on $[Ca^{2+}]_i$. $\bar{x}\pm SD$. $^*P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* control.

Drug	*n*	$[Ca^{2+}]_o$ (mmol/L)	Tet (μmol/L)	$[Ca^{2+}]_i$ (nmol/L)
Control	36	1.6	0	186±45
Tet	21	1.6	65	189±49*
EGTA	18	0	0	46±16***

Tab 2. Effects of prostaglandin E_2 (PGE_2), calcimycin (A-23187), leukotriene B_4 (LTB_4) and platelet activating factor (PAF) on $[Ca^{2+}]_i$ of neutrophils in Hanks' solution containing Ca^{2+} and antagonistic actions of Tet against A-23187, LTB_4 and PAF. $^*P>0.05$, $^{**}P<0.05$, $^{***}P<0.01$ *vs* before the addition of the agonist. $^{\dagger}P>0.05$, $^{\dagger\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.01$ *vs* control (without Tet).

Drug (nmol/L)	Tet (μmol/L)	*n*	$[Ca^{2+}]_i$(nmol/L) Before	After
PGE_2 (100)	0	5	206±18	207±14*
A-23187	0	7	185±54	814±67***
(25 μmol/L)	32	3	139±15	685±96††
	65	4	186±12	238±27†††
LTB_4 (60)	0	11	175±36	577±229***
	65	7	219±28	249±23†††
PAF (10)	0	13	188±54	540±174***
	65	10	169±60	187±51†††

化合物 48/80, LTB_4 和 PAF 对在无 Ca^{2+} 的 Hanks 液中的 NP$[Ca^{2+}]_i$的影响 化合物 48/80 3.2 μg/ml, LTB_4 300 nmol/L, PAF 10 nmol/L 及 PAF 5 nmol/L 加 LTB_4 150 nmol/L 对无 Ca^{2+} 的 Hanks 液中 NP 的 $[Ca^{2+}]_i$没有影响(Tab 3).

Tab 3. Effects of compound 48/80, leukotriene B_4(LTB_4) and platelet activating factor (PAF) on $[Ca^{2+}]_i$ of neutrophils in Ca^{2+}-free Hank's solution. $^*P>0.05$ *vs* before the addition of the agonist.

Drug (nmol/L)	*n*	$[Ca^{2+}]_i$ (nmol/L) Before	After
Compound 48/80(3.2 μg/ml)	8	45±13	43±12*
LTB_4 (300)	3	64±8	64±5*
PAF (10)	4	46±22	47±21*
PAF (5)+LTB_4 (150)	3	33±13	42±4*

DISCUSSION

本文测得在无 Ca^{2+} 溶液中 NP 的 $[Ca^{2+}]_i$

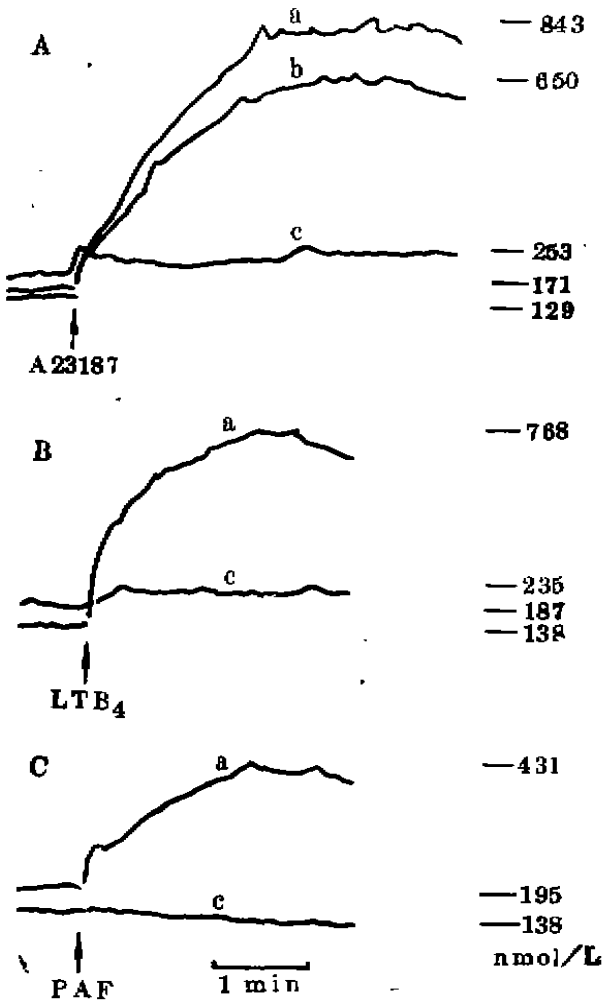

Fig 2. Antagonistic actions of Tet on increases of cytoplamic free calcium concentration ($[Ca^{2+}]_i$) induced by (A) calcimycin 25 μmol/L, (B) leukotriene B_4 (LTB_4) 60 nmol/L and (C) platelet activating factor (PAF) 10 nmol/L. The numbers on the right indicate $[Ca^{2+}]_i$ levels. (a) without Tet, (b)Tet = 32 μmol/L, (c) Tet = 65 μmol/L.

明显低于在含 Ca^{2+} 溶液中 NP 的$[Ca^{2+}]_i$，说明$[Ca^{2+}]_o$ 在维持 $[Ca^{2+}]_i$ 中有重要作用，这很可能是因为在无 Ca^{2+} 的溶液中 NP 膜上的 $Na^+ - Ca^{2+}$ 双向交换[8] 变成单向交换而使 $[Ca^{2+}]_i$ 降低。

NP 脱颗粒程度与$[Ca^{2+}]_i$ 呈明显的函数关系，能导致脱颗粒的 $[Ca^{2+}]_i$ 阈值为 200 – 300 nmol/L[7]。本文证实 LTB_4，PAF 和 A-23187 作用于 NP 后，$[Ca^{2+}]_i$ 远远超过上述阈值，因此可以作为 NP 激活的指标。设想体内的 NP 受到过敏和炎症时体内多种细胞产生的 PAF 和 LTB_4 的作用后即可被激活而表现出各种活性。PGE_2 也是一种重要的过敏和炎症介质，能增强白细胞的趋化性[9]，但本文首次报道 PGE_2 对 NP 的$[Ca^{2+}]_i$ 没有影响，推测 PGE_2 对白细胞的作用是通过其它途径实现的。NP 是否有足量的细胞内储存钙尚不明确[5]。本文发现在无$[Ca^{2+}]_i$ 溶液中，PAF，LTB_4 和化合物 48/80 不能使$[Ca^{2+}]_i$ 升高，可能是这些刺激物不能使大鼠 NP 释放储存钙，或是大鼠 NP 并无细胞内储存钙可释放。

本文首次发现 Tet 对 NP 的静息$[Ca^{2+}]_i$ 没有影响，但能对抗 PAF，LTB_4 和 A-23187 所致的$[Ca^{2+}]_i$ 的升高。因为 PAF，LTB_4 和 A-23187 所致的$[Ca^{2+}]_i$ 升高依赖于$[Ca^{2+}]_o$，说明 Tet 是通过抑制 Ca^{2+} 内流而抑制$[Ca^{2+}]_i$ 升高的钙拮抗剂，这很可能是它抗过敏作用[1]的机理。NP 的激活与炎症关系密切，Tet 能抑制 NP 的激活提示它可能有抗炎作用，但尚需整体和器官水平实验的证实。

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Effect of fluorocarbon blood substitute on neutrophil phagocytic function

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ABSTRACT Neutrophils were incubated at 37℃ for 2 h with fluorocarbon blood substitute or its main components *in vitro*. Neutrophil phagocytosis was determined by the method of chemiluminescence (CL) and the concentration of intracellular cAMP and cGMP were assessed. The results showed that the CL was inhibited while the level of cAMP was elevated. The alteration of cAMP seemed to be correlated with the inhibition of CL. Only did the emulsifier Poloxamer F-68 (F-68) of fluorocarbon blood substitute have the same effects. It is suggested that fluorocarbon blood substitute can inhibit neutrophil phagocytic function and the emulsifier F-68 may be responsible for it. The mechanism may be associated with the elevation of intracellular cAMP concentration.

KEY WORDS fluorocarbons; neutrophils; luminescence

Fluorocarbon blood substitute (FCBS) is capable of carrying and delivering substantial amounts of oxygen. It can perform the role of red blood cells in transporting oxygen throughout the body and has been applied increasingly in clinical usage[1]. However, FCBS had some adverse effects on neutrophil phagocytosis, chemotaxis and even metabolism[2-4]. The present study was undertaken to investigate whether this effect was due to FCBS itself or its components.

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MATERIALS AND METHODS

Fluorocarbons were obtained from Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, and emulsified with Poloxamer F-68 (F-68) in our laboratory. The emulsion contained (g/L): perfluorodecalin (FDC) 14, perfluorotripropylamine (FTPA) 6, F-68 45, glycerol 10, glucose 1.5, hydroxyethyl starch 30, NaCl 3.0, KCl 0.27, $CaCl_2$ 0.22, $MgCl_2$ 0.15, $NaHCO_3$ 0.5, with pH 7.4-7.6. The average diameter of the emulsion particles was less than 0.1 μm.

Neutrophils were separated from heparinized blood of healthy donors using a modified method of gradient centrifugation[5]. Neutrophil suspensions were washed and then suspended in Hank's solution at a concentration of 2×10^6 neutrophils/ml prior to incubation. Over 90% of neutrophils was yielded by microscope screening