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Tetrandrine differentially inhibits aggregation and ATP-release of rat platelets[1]

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AIM: To examine the effects of tetrandrine (Tet) on the aggregation and ATP-release of rat washed platelets induced by several platelet activators. **METHODS**: Gel-filtration (Sepharose 2B) was used to isolate washed platelets from adult rats and the platelet aggragation and ATP-release were measured simultaneously. **RESULTS**: In the presence of Ca^{2+} 1 mmol·L^{-1}, Tet 300 μmol·L^{-1} inhibited the aggregation induced by ADP (25 μmol·L^{-1}), collagen (2.5 g·L^{-1}), and thrombin (103 unit·L^{-1}) by 62 %, 60 %, and 34 %, respectively. It also inhibited arachidonic acid (1 mmol·L^{-1})-induced aggregation. Elevating intracellular Ca^{2+} concentration with the Ca^{2+} ionophore, calcimycin (30 μmol·L^{-1}), or by blocking the intracellular calcium pump with cyclopiazonic acid (5 μmol·L^{-1}) initiated platelet aggregation, which was also inhibited by Tet. In Ca^{2+}-free medium, Tet still elicited an inhibitory effect on aggregation induced by ristocetin (2.5 g·L^{-1}). Lower concentrations of Tet (30 nmol·L^{-1} to 3 μmol·L^{-1}) failed to inhibit the aggregation (requiring Tet 10 - 300 μmol·L^{-1}), but strongly suppressed ATP-release induced by ADP 10 μmol·L^{-1}, both of which were measured simultaneously in a single sample. **CONCLUSION**: Tet elicits a nonselective inhibitory effect on platelet aggregation not solely due to its Ca^{2+} antagonism and may act on a final common pathway leading to platelet aggregation. Furthermore, Tet is a much potent inhibitor of the release of ATP in platelets.

Tetrandrine (Tet), a bis-benzylisoquinoline alkaloid isolated from *Radix Stephania tetrandra* (S Moore), demonstrated Ca^{2+} antagonistic[1,2], antihypertensive[3], vasodilatory[4] and anti-arrythmic effects[5]. In view of its immunosuppressive and anti-inflammatory actions, the effect of Tet on aggregation induced by platelet activating factor (PAF) has been observed in platelet rich plasma[6]. Although Ca^{2+} is involved in many cellular and intracellular processes (such as in contraction and secretion) and Tet has been identified as a Ca^{2+} entry bloker[2,7], it is unclear whether its anti-aggregative effect is related to its Ca^{2+} antagonism. As PAF also plays a critical role in inflammatory and hypertensive diseases, attention has been directed to determining whether these therapeutic effects of Tet were related to changes in the function of platelets, such as aggregation[6,8]. Tetrandrine inhibited the aggregation induced by PAF, ADP, collagen (Col), thrombin (Thr), but not by arachidonic acid (AA) and calcimycin (Cal)[6]. Tet also elicited non-selective inhibition of Ca^{2+}-channels and other Ca^{2+}-dependent ion channels[9] and blockade of α-adrenoceptors[4]. The effect of Ca^{2+} antagonists on platelet aggregation also remained controversial[8,10,11]. For example, the aggregation of washed rat platelets was nonselectively inhibited by Ni^{2+}, but not by nifedipine (our unpublished data). Also, Ca^{2+} influx during platelet aggregation was practically insensitive to L-type Ca^{2+}-channel blockers and only partial aggregation was seen the absence of external Ca^{2+}[10].

The platelet activators are generally grouped based on their mechanisms of action[12]: (1) ADP, adrenaline, Thr and Col, act by binding to receptor (glycoprotein IIb and IIIb), then followed by secretion of ADP, ATP, and Ca^{2+}; (2) AA, induces the secretion reaction in platelet; (3) Cal, thapsigargin, CPA, elevate the intracellular Ca^{2+} level by

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changing membrane permeability or by blocking the intracellular Ca^{2+} pump[13]; (4) PAF elevates $[Ca^{2+}]_i$ through a possible effect on phospholipase followed by activating the phosphatidylinositol cycle[14]; (5) Ristocetin (Ris) and fibrinogen, act directly on the special receptor (glycoprotein Ib). The aggregations induced by most of these agents, except for Ris, are dependent upon the extracellular Ca^{2+}, *eg* ADP and Thr[12]. The aim of this work is to determine whether Tet elicits any nonselective effect on the aggregations induced by several typical platelet activators, which act via different mechanisms and to measure simultaneously the effects of Tet on ATP release platelet aggregation in washed platelets.

MATERIALS AND METHODS

Drugs and materials ADP, Cal, CPA and Sepharose 2B were obtained from Sigma Co. Thr, luciferin-luciferase, Col, ATP, Ris, AA were obtained from Chrono-Log Co (USA). Tet was a gift of Prof FANG Da-Chao, Tong-Ji Medical-University, China.

Tet was dissolved in HCl 0.05 mol·L^{-1} and diluted by distilled water to give a 30 mmol·L^{-1} stock solution. All of the platelet activators were prepared as stock solutions of ADP 1 mmol·L^{-1}, Col 1 g·L^{-1}, Thr 100 kunit·L^{-1}, CPA 0.1 mmol·L^{-1}, Cal 0.3 mmol·L^{-1}, AA 1 mmol·L^{-1}, and Ris 125 g·L^{-1}. The aggregometer was linked to an IBM 386 computer via the AGGRO/LINK computer interface (model 810A, Chrono-Log, Harvertown PA, USA).

Preparation of washed platelets Suspensions of washed rat platelets were prepared[9], with modification, from the blood of Sprague-Dawley rats (♂, 400 - 450 g) under pentobarbital sodium (60 mg·kg^{-1}, ip) anesthesia. Blood samples were collected into polypropylene centrifuge tubes containing 3.8 % sodium citrate in 1/10 of total volume. The citrated blood was centrifuged at 150 × *g* for 10 min to obtain the platelet-rich plasma (PRP), which was eluted by gel filteration chromatography on Sepharose-2B column (2 cm × 15 cm) with Ca^{2+}-free Tris-buffer (pH 7.2), containing NaCl 140 mmol·L^{-1}, $MgCl_2$ 1 mmol·L^{-1}, 0.35 % bovine serum albumin, 0.1 % glucose and Tris-HCl 50 mmol·L^{-1}. The platelet counts were adjusted to $(2-3)\times10^{11}$ cells·L^{-1} with Tris-buffer.

Platelet aggregation assay The platelet aggregation was carried out with lumi-aggregometer at 37 ℃. Cuvette containing 200 mL washed platelets was placed into the test well and the same volume of buffer as blank control into the reference well, with constant stirring at 150 × *g*. Tet (final concentration of 0.3 mmol·L^{-1}) was incubated with platelets for 3 min before experiments. The platelet aggregation was evoked by ADP 25 μmol·L^{-1}, Thr 1 kunit·L^{-1}, Col 2.5 mg·L^{-1}, CPA 5 μmol·L^{-1}, AA 1 mmol·L^{-1}, Cal 3 μmol·L^{-1} in the presence of Ris 2.5 g·L^{-1} in the absence of Ca^{2+}. The maximal transmittance (%) was measured automatically by computer and the data were stored in computer memory through the AGGRO/LINK interface for retrieval and analysis.

Measurement of ATP secretion The release of ATP induced by ADP 10 μmol·L^{-1} was measured in the presence of Tet (30 nmol·L^{-1} to 10 μmol·L^{-1}) by a luminesence assay. The measurement of ATP-release was also measured simultaneously with aggregation sample. Luciferin-luciferase mixture was added into the prewarmed sample at 37 ℃ to initiate the measurement of ATP. ATP standard (2 nmol) was always included for calibration before measurement of ATP-release.

ATP (nmol) = (Luminescence of test/Gain of test) × (Gain of ATP standard/Luminescence of ATP standard) × 2 nmol

RESULTS

Aggregations were induced by ADP (25 μmol·L^{-1}), Thr (1 kU·L^{-1}), Col (2.5 g·L^{-1}), CPA (5 μmol·L^{-1}), AA (1 mmol·L^{-1}) and Cal (3 μmol·L^{-1}) in the presence of Ca^{2+} 1 mmol·L^{-1} in washed platelet preparations. All of these aggregations were inhibited by pretreatment with Tet 300 μmol·L^{-1} for 3 min (Fig 1).

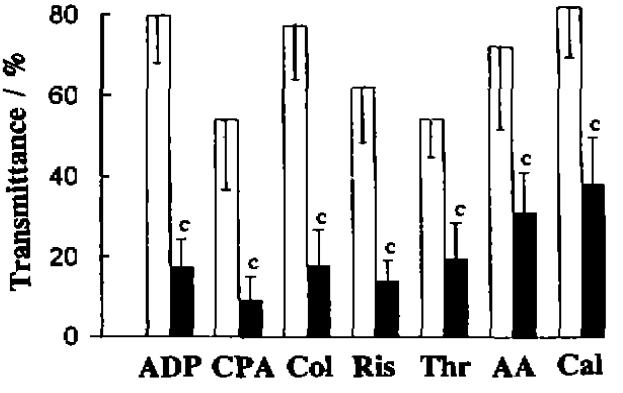

Fig 1. Inhibitory effects of Tet 300 μmol·L^{-1} on the platelet aggregation induced by ADP 25 μmol·L^{-1}, Col 2.5 g·L^{-1}, Thr 1 kunit·L^{-1}, CPA 5 μmol·L^{-1}, Cal 3 μmol·L^{-1}, AA 1 mmol·L^{-1} and Ris 2.5 g·L^{-1}. $n=6$, $\bar{x}\pm s$. □, no Tet; ■, with Tet. $^cP<0.01$ *vs* control.

At the same concentration, Tet also inhibited aggregation induced by Ris (2.5 g·L^{-1}) in the

absence of Ca^{2+} (−47 %, $P<0.01$). Tet inhibited the aggregation induced by ADP (from 80 ± 11 % to 18 ± 7 %, $P<0.01$), Col (from 77 ± 13 % to 18 ± 9 %, $P<0.01$), Thr (from 54 ± 10 % to 19 ± 9 %, $P<0.01$), respectively. Elevating intracellular Ca^{2+} concentration with Cal or by blocking the intracellular Ca^{2+}-pump with CPA initiated platelet aggregation, which was also inhibited by Tet 300 nmol · L^{-1} (− 44 % and −43 %, $P<0.01$, respectively). It also inhibited AA-induced aggregation (− 40 %, $P<0.01$).

Tet inhibited the release of ATP from washed platelets in a concentration-dependent manner (0.1 − 10 μmol·L^{-1}) (Fig 2, 3). The concentration of

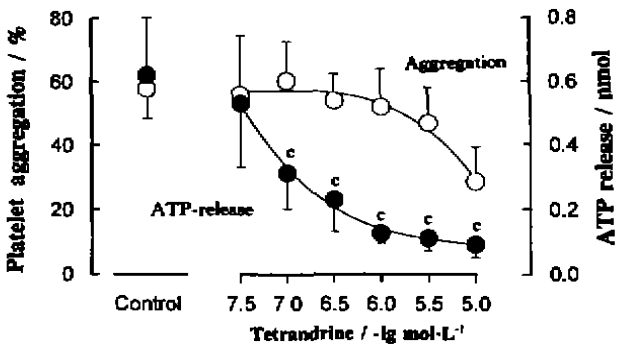

Fig 2. Effect of Tet on platelet aggregation and ATP-release measured simultaneously upon addition of ADP 10 μmol ·L^{-1}. $n=6$, $\bar{x}\pm s$. $^cP<0.01$ *vs* control.

Tet required to cause inhibition of ATP-release was 1 % that to elicit the inhibition of platelet aggregation induced by ADP 10 μmol·L^{-1}.

DISCUSSION

Tet 300 μmol · L^{-1} maximally inhibited the platelet aggregation not only by above mentioned activators in the presence of Ca^{2+} 1 mmol · L^{-1}, but also by Ris in Ca^{2+}-free medium. Therefore, this nonselective inhibitory effect of tetrandrine on platelet aggregations can not be solely explained by its Ca^{2+}-antagonistic action.

Secretion or release from cytoplasmic granules represent another physiological function of platelets during platelet activation. Intracellular Ca^{2+} is a key factor for platelet secretion[15], because ATP-release from the platelets under the same condition for aggregation did not depend on external Ca^{2+} or on Ca^{2+} influx[10]. Therefore, despite the interaction between the platelet aggregation and ATP-release, these 2 processes are separate entities and differentially regulated. This contention is also supported by our present study using simultaneous measurement of platelet aggregation and ATP-release. We observed a striking differential effect of Tet on these 2 cellular events in the platelets following ADP stimulation (Fig 2, 3).

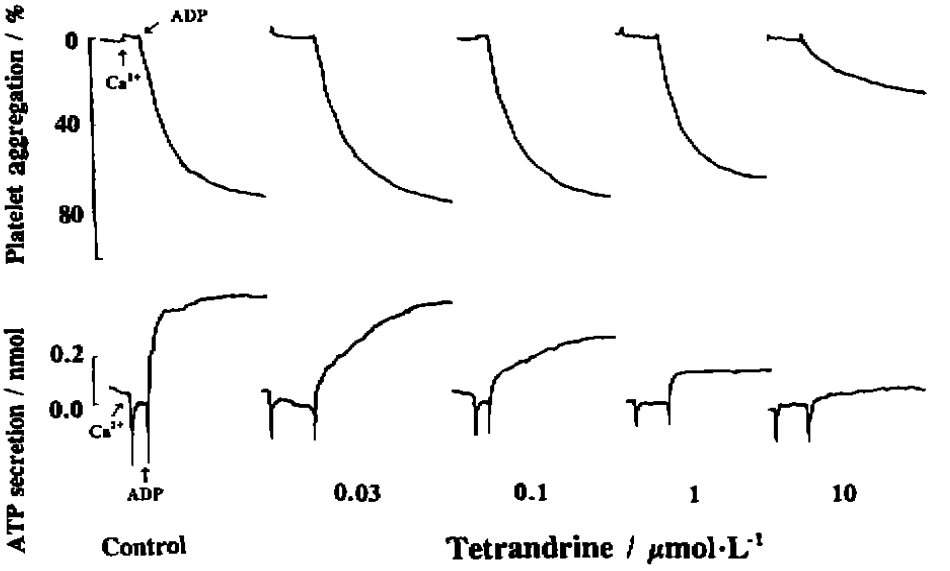

Fig 3. Simultaneous measurement of platelet aggregation and ATP-release induced by ADP 10 μmol·L^{-1} in the absence and presence of Tet.

These results prompt us to conclude that: (1) Tet inhibits aggregation induced not only by platelet activators, such as ADP, CPA, Thr, Col, AA or Cal, via Ca^{2+}-dependent pathways, but also by Ris, via a Ca^{2+}-independent process; (2) The inhibitory effect of Tet on ATP-release induced by ADP was much more sensitive to Tet than and dissociated from that on the aggregation in washed platelets. Furthermore, the IC_{50} for Tet in most of its Ca^{2+}-entry blocking events is 5 – 30 $\mu mol \cdot L^{-1}$. Since Tet inhibits ATP-release at concentrations (0.01 – 3.0 $\mu mol \cdot L^{-1}$) well below the reported IC_{50} values for its Ca^{2+}-channel antagonism, we suggest that the inhibition of ATP-release by Tet may involve other action of Tet on the mobilization of intracellular Ca^{2+}. Similarly, the inhibitory effect on platelet aggregation at higher concentration of Tet also led us to speculate that Tet may act via a common pathway leading to platelet aggregation, either on the membrane glycoprotein receptor or on the event distal to Ca^{2+}-channel antagonism.

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粉防己碱特异抑制大鼠血小板聚集和 ATP 释放

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关键词 粉防己碱；血小板聚集；卡西霉素；利托菌素

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