BIBLID: ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1995 May; 16 (3): 247-50

Inhibition of 5′-chloro-5′-deoxyadenosine on phosphoinositide and protein phosphorylation in swine platelets[1]

DING Ke-Hong[2], LIANG Nian-Ci
(Department of Biochemistry, Guangdong Medical College, Zhanjiang 524023, China)

AIM: To study the effects of adenosine on the phosphorylation of phosphoinositides and proteins in platelets. **METHODS:** In the presence of Mg^{2+} and/or Ca^{2+}, swine thrombocytic membranes were incubated with [γ-^{32}P]ATP at 30 ℃ for 3 min and the incorporations of ^{32}P into phospholipids or proteins were measured. **RESULTS:** 5′-Chloro-5′-deoxyadenosine decreased the formation of phosphatidylinositol 4-phosphate and phosphatidylinositol 4, 5-bisphosphate [IC_{50} 71 and 75 (95 % confidence limits 60-85 and 62-90) $\mu mol \cdot L^{-1}$, respectively], behaving as a competitive inhibitor to ATP, and inhibited the phosphorylation of pleckstrin (the major protein kinase C substrate) and myosin light chain [IC_{50} 75 and 82 (95 % confidence limits 62-90 and 66-102) $\mu mol \cdot L^{-1}$, respectively]. **CONCLUSION:** Adenosine affects the phosphoinositide signaling pathway in platelets, which helps to clarify the inhibition of adenosine on platelet activation.

KEY WORDS adenosine; blood platelets; phosphatidylinositols; protein kinase C; phosphorylation

Blood platelet activation by agonists such as thrombin and collagen is associated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) through the activation of phospholipase C and the subsequent production of the second messengers, 1,2-diacylglycerol (DG) and inositol-1, 4, 5-trisphosphate (IP_3)[1]. DG activates protein kinase C (PKC) in the presence of Ca^{2+} and phosphatidylserine, wherease IP_3 induces the release of Ca^{2+} from the dense tubular system. The two events are contributed in a synergistic manner to activate blood platelets, namely, shape change, secretion, and aggregation[2].

Adenosine, a natural purine metabolite that has potent antiplatelet activity, is continuously produced in the body by many tissues[3]. It is previously thought that adenosine actions in platelets are mediated via its binding to specific receptors (A_2) which stimulate membrane adenylate cyclase and increase intracellular cAMP levels. However, a change in cAMP is neither necessary nor sufficient for platelet activation[4]. Adenosine may result in activation of phospholipase C and/or mobilization of intracellular Ca^{2+} in many other cells[5]. In order to elucidate the mechanism of adenosine on antiplatelet actions, in this study we examined the effects of 5′-chloro-5′-deoxyadenosine (Cl-Ado) on phosphorylation of phosphoinositides and proteins in swine platelets. The analog was used because it is not metabolized by adenosine kinase or adenosine deaminase[6].

MATERIALS AND METHODS

Reagents Cl-Ado, ATP (Sigma). Phosphatidylinositol 4-phosphate (PI-4-P) and PI-4, 5-P_2 were prepared from bovine brain[7]. [γ-^{32}P]ATP (185 $GBq \cdot mol^{-1}$) was from Amersham. All other chemi-

[1] Project supported by the National Natural Science Foundation of China, № 3880443.

[2] Now in *Shanghai Institute of Physiology, Chinese Academy of Sciences, Shanghai 200031, China.*

Received 1994-06-06 Accepted 1994-09-30

cals were of AR.

Preparation of platelet membranes Thrombocytic membrane proteins were prepared from pig platelets by differential centrifugation[8].

Measurement of phosphoinositides Phosphoinositides were assayed at 30 ℃ in a 100-μL final reaction volume consisting of Tris-HCl 40 mmol·L⁻¹, pH 7.4, $MgCl_2$ 15 mmol·L⁻¹, KCl 100 mmol·L⁻¹, PI-4-P 50 μmol·L⁻¹ (sonicated with Tris-HCl 10 mmol·L⁻¹, pH 7.4, at 30 ℃ for 3 min previously), [γ-^{32}P]ATP 50 μmol·L⁻¹, and about 0.5 g·L⁻¹ proteins. The reaction was started by the addition of ATP and terminated 3 min later by addition of 500 μL of ice-cold chloroform/methanol/12 mol·L⁻¹ HCl (800 : 400 : 3, vol : vol). The lipids were extracted after addition of carrier polyphosphoinositides and separated by thin-layer chromatography. Lipids were detected with I_2 vapor. The spots were scraped out and were estimated by liquid scintillation counting.

Protein phosphorylation assay The phosphorylation assay was performed[8,9]. Briefly, the incubations were carried out at 30 ℃ for 3 min in a final volume of 100 μL. The reaction mixtures contained Tris-HCl 40 mmol·L⁻¹, pH 7.4, $MgCl_2$ 15 mmol·L⁻¹, $CaCl_2$ 5 mmol·L⁻¹, [γ-^{32}P]ATP 50 μmol·L⁻¹, about 100 − 200 μg proteins. Reactions were initiated by adding ATP and stopped by adding trichoroacetic acid to a final concentration of 8 %. Samples were centrifugalized at 10 000 × g for 5 min. The precipitates were washed twice with cold acetone, air dried, and mixed with 100 μL of the sample buffer (62.5 mmol·L⁻¹ Tris-HCl, pH 6.8, 2 % sodium dodecyl sulfate, 5 % 2-mercaptoethanol, 10 % glycerol, 0.001 % bromphenol blue). Proteins were electrophoresed on a 0.1 % SDS − 10 % polyacrylamide slab gels. Gels were stained with Coomassie brilliant blue, dried and analyzed by autoradiography. Radioactivity of ^{32}P-labeled proteins was quantified by liquid scintillation counting.

Protein estimation Protein concentrations were determined[10], using bovine serum albumin as standard.

Statistical analysis Unpaired *t*-test was used to analyze the differences between controls and Cl-Ado treated groups.

RESULTS AND DISCUSSION

Cl-Ado dose-dependently inhibited PI-4-P and PI-4,5-P_2 labeling (Fig 1), IC_{50} was 71 and 75 (95 % confidence limits 60−85 and 62−90) μmol·L⁻¹, respectively. The inhibition was particularly sensitive to PI-4-P formation at Cl-Ado 20−40 μmol·L⁻¹ (64 % of control incubations at 40 μmol·L⁻¹) and for PI-4,5-P_2 production at 20−60 μmol·L⁻¹ Cl-Ado (53 % of control incubations at Cl-Ado 60 μmol·L⁻¹).

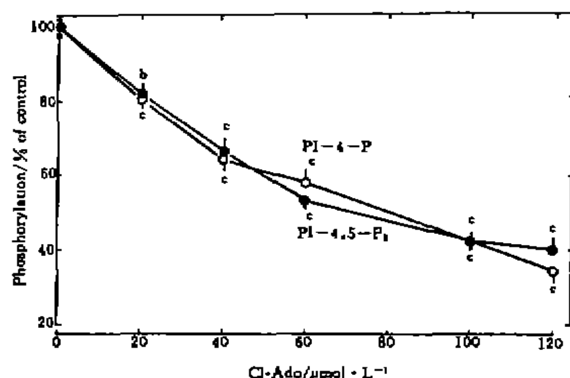

Fig 1. Inhibition of Cl-Ado on phosphoinositide phosphorylation. $n=6$, $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control. ^{32}P radioactivity in PI-4-P and PI-4,5-P_2 in control incubation was 28.7 ± 0.4 and 10.4 ± 0.2 nmol·min⁻¹/mg protein, respectively.

The inhibition of Cl-Ado on PI-4,5-P_2 formation showed in this experiment is implicated in the decreased production of the two second messengers. In addition, the level of PI-4-P, as a precursor of DG production, also influences DG of formation.

The inhibition of phosphoinositide phosphorylation by Cl-ado was competitive with respect to ATP (Fig 2), suggesting a direct interaction of the nucleosides with the kinases. The activation of phosphatidylinositol (PI) and PI-4-P kinases was shown in absence of Cl-Ado, the K_m for ATP was 90 and 80 μmol·L⁻¹, respectively, and the V_{max} was 0.83 and 0.57 nmol·min⁻¹, respectively.

The phosphorylation of 20- and 40-kDa proteins in platelets was inhibited with

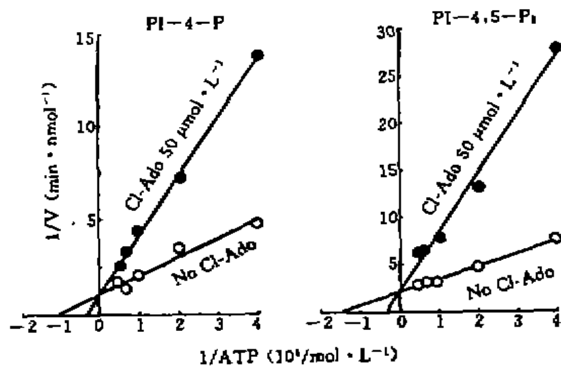

Fig 2. Inhibition of Cl-Ado on phosphoinositide phosphorylation: Lineweaver-Burk plot. $n=4$.

increasing Cl-Ado concentrations 20 — 160 $\mu mol \cdot L^{-1}$ (Fig 3). the inhibition of phosphorylation of 40-kDa protein by Cl-Ado was more sensitive than that of 20-kDa protein, IC_{50} for 20- and 40-kDa proteins were 82 and 75 (95 % confidence limits 66—102 and 62—90) $\mu mol \cdot L^{-1}$, respectively.

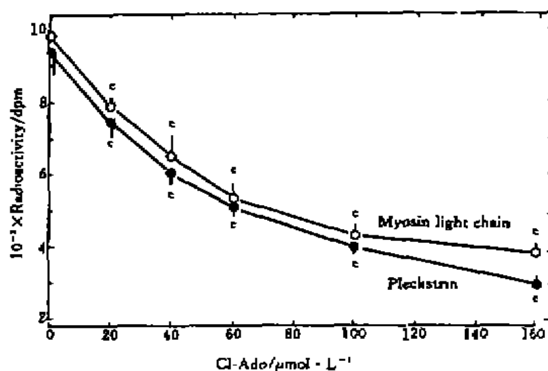

Fig 3. Inhibition of Cl-Ado on phosphorylation of myosin light chain (○) and pleckstrin (●) in platelets. $n=6$, $\bar{x} \pm s$. $^cP<0.01$ *vs* control.

It has been identified in platelets that 40 — 47 kDa protein termed pleckstrin, of unknown function, is the main substrate for PKC[11], and 20 kDa protein is myosin light chain (MLC), which is a substrate for both PKC and Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK)[12]. PKC and MLCK are associated with activation of blood platelets. The results indicated in our experiments suggest that Cl-Ado may act as an inhibitor of PKC and/or MLCK. As mentioned above, PI-4,5-P_2 and PI-4-P can activate PKC in a phosphatidylserine-dependent manner. A decrease of PI-4,5-P_2 and PI-4-P production by Cl-Ado may cause the reduction of phosphorylation of pleckstrin and MLC. The inhibition of PI-4,5-P_2 and PI-4-P formation may also cause the dissociation of PKC from membranes, which reduces the phosphporylation of PKC substrates, including pleckstrin and MLC in blood platelets.

In summary, this study demonstrated that Cl-Ado, behaving as a competitive inhibitor with respect to ATP, inhibited the turnover of phosphoinositides and the phosphorylation of pleckstrin and MLC in blood platelets, by which with effects on other possible steps in inositol phospholipid signaling pathway it inhibits blood platelet activation (Fig 4). This work primarily provides evidence that helps to clarify the mechanism of adenosine antiplatelet actions.

REFERENCES

1 Halbrugge M, Walter U. The regulation of platelet function by protein kinases. In: Huang CK, Sha'afi RI, editors. Protein kinases in blood cell function. Boca Raton: CRC, 1993: 245—98.

2 Walker TR, Watson SP. Synergy between Ca^{2+} and protein kinase C is the major factor in determining the level of secretion from human platelets. Biochem J 1993; **289**: 277—82.

3 Cusack NJ, Hourani SMO. Adenosine, adenine nucleotides, and platelet function. In: Phillis JW, editor. Adenosine and adenine nucleotides as regulators of cellular function. Boca Raton: CRC, 1991: 121—31.

4 Hourani SMO, Cusack NJ. Pharmacological receptors on blood platelets. Pharmacol Rev 1991; **43**: 243—98.

5 Harden TK. P_2-purinergic receptor signalling mechanisms. In: Imai S, Nakazawa M, editors. Role of adenosine and adenine nucleotides in the biological system.

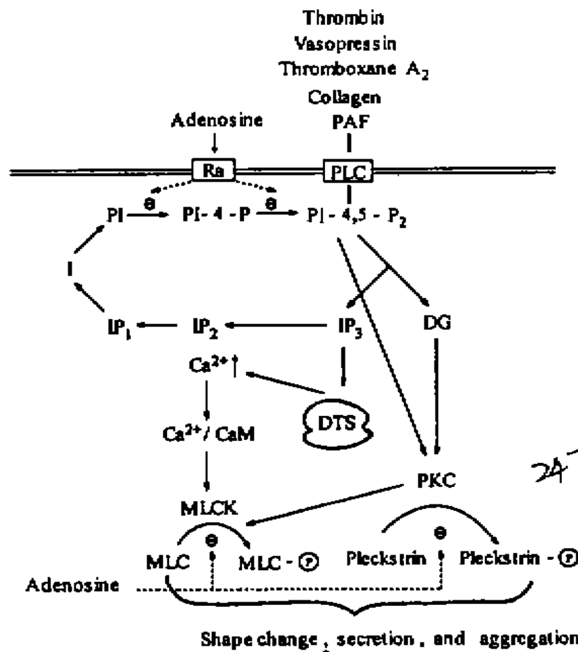

Fig 4. Mechanism of adenosine antiplatelet actions. CaM = calmodulin; DG = diacylglycerol; DTS = dense tubular system; I = inositol; IP_1, IP_2, and IP_3 = inositol-mono, bis, and trisphosphate; MLC = myosin light chain; MLCK = myosin light chain kinase; PI = phosphatidylinositol; PI-4-P = phosphatidylinositol-4-phosphate; PI-4, 5-P_2 = phosphatidylinositol-4, 5-bisphosphate; PKC = protein kinase C; PLC = phospholipase C; Ra = adenosine receptor.

Amsterdam: Elsevier, 1991: 33-41.

6 Doctrow SR, Lowenstein JW. Adenosine and 5'-chloro-5'-deoxyadenosine inhibit the phosphorylation of phosphatidylinositol and myosin light chain in calf aorte smooth muscle. J Biol Chem 1985; **260**: 3469-76.

7 Ding KH, Liang NC. Preparation of polyphosphoinositides from ox brain. Prog Biochem Biophys 1992; **19**: 482-3.

8 Ding KH, Liang NC. Effects of spermine on phosphorylation of phosphoinositide and 47 K protein in platelet membranes of pig. Acta Pharmacol Sin 1992; **13**: 452-4.

9 Ding KH, Liang NC. Inhibition of tetramethylpyrazine on the phosphorylation of phosphoinositide and 20 K protein in platelets. Chin Pharmacol Bull 1992; **8**: 377-9.

10 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; **193**: 265-75.

11 Tyers M, Rachubinski RA, Stewart MI, Varrichio AM, Shorr RG, Haslam RJ, *et al*. Molecular cloning and expression of the major protein kinase C substrate of platelets. Nature 1988; **333**: 470-3.

12 Naka M, Nishikawa M, Adelstein RS, Hidaka H. Phorbol ester-induced activation of human platelets is associated with protein kinase C phosphorylation of myosin light chains. Nature 1983; **306**: 490-2.

247-250

5'-氯-5'-脱氧腺苷对猪血小板中肌醇磷脂和蛋白质磷酸化的抑制作用

丁克宏, 梁念慈 R965.2
(广东医学院生化教研室, 湛江524023, 中国)

目的: 研究腺苷对血小板中肌醇磷脂和蛋白质磷酸化的影响. 方法: 在 Mg^{2+} 和/或 Ca^{2+} 存在下, 用猪血小板膜与[γ-^{32}P]ATP 在30 ℃下保温3 min, 测定^{32}P 掺入磷脂或蛋白质. 结果: 5'-氯-5'-脱氧腺苷减少磷脂酰肌醇-4-磷酸和磷脂酰肌醇-4,5-二磷酸的生成[IC_{50}分别为71和75 (95 %可信限分别为60-85和62-90) μmol·L^{-1}], 抑制与 ATP 呈竞争性, 并抑制 pleckstrin (C 激酶主要底物)和肌球蛋白轻链的磷酸化[IC_{50}分别为75和82 (95 %可信限分别为62-90和66-102) μmol·L^{-1}]. 结论: 腺苷影响血小板中肌醇磷脂信使通路, 这有助于阐明腺苷对血小板活化的抑制作用.

关键词 腺苷; 血小板; 磷肌醇类; 蛋白激酶C; 磷酸化作用