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THE THIENOPYRIDINE TICLOPIDINE SELECTIVELY PREVENTS THE INHIBITORY EFFECTS OF ADP BUT NOT OF ADRENALINE ON cAMP LEVELS RAISED BY STIMULATION OF THE ADENYLATE CYCLASE OF HUMAN PLATELETS BY PGE_1*

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Abstract—After oral administration, ticlopidine specifically inhibits ADP-induced platelet aggregation, prolongs the bleeding time and prevents thrombosis in man. Its mechanism of action is not well known. Ticlopidine inhibits ADP-induced binding of fibrinogen to platelet glycoprotein GP IIb–IIIa but not shape change and increases deaggregation. Ticlopidine has no direct effect on the GP IIb–IIIa complex. We studied the effects of ticlopidine (500 mg/day for 8 days) in four healthy male volunteers on washed platelet aggregation induced by 5 μM ADP or thrombin (0.1 units/mL) and potentiated by 1 μM adrenaline (Adr), on basal and 1 μM PGE_1-stimulated cAMP levels and on elevation of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). We found that: (i) ticlopidine inhibits aggregation by ADP but not the potentiation by Adr of ADP-induced aggregation; (ii) ADP, Adr or thrombin decreases cAMP levels raised by PGE_1, an effect inhibited by ticlopidine only for ADP and not for Adr or thrombin; and (iii) Ca^{2+} influx and Ca^{2+} mobilization from internal stores were not affected. These results suggested that ticlopidine or a metabolite impairs the coupling mechanism of the ADP aggregation pathway at an unknown level.

Ticlopidine is a potent inhibitor of platelet aggregation and platelet related thrombosis [1–3]. Its mechanism of action on platelet function remains unknown. Ticlopidine inhibits ADP-induced aggregation of washed platelets and this effect may explain the broad spectrum antiaggregating effect of the thienopyridine since ADP secretion plays a major role in platelet activation [4]. Ticlopidine is known to inhibit fibrinogen binding to its receptor *in vitro* [5] and *ex vivo* [6]. We have previously shown that PCR 4099, a structural analogue of ticlopidine, does not directly alter the fibrinogen binding site on the GP IIb–IIIa complex in man or in rat [7], which is not in favour of a thrombasthenic-like defect induced by the drug as suggested previously [8]. Several studies have been published on the effect of ticlopidine or analogous thienopyridines on second messengers such as cAMP [9–11] or intracellular Ca^{2+} movements [11–13]. The aim of our study was to examine the effects of oral administration of ticlopidine, 500 mg/day for 8 days in man, on washed platelet aggregation induced by ADP or thrombin and their potentiation by adrenaline (Adr, 1 μM) [14], on basal and PGE_1 (1 μM) stimulated total cAMP levels modulated by ADP or adrenaline or both and on cytosolic free Ca^{2+} elevation induced by ADP.

MATERIALS AND METHODS

Materials. The commercial form of ticlopidine, Ticlid® was provided by Laboratoires Millot-Solac (Paris, France), Adenosine 5′-diphosphate (ADP), prostaglandin E_1 (PGE_1), adrenaline, fatty acid free bovine serum albumin were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Quin 2-AM was from Lancaster Synthesis (Morecambe, U.K.). Trioctylamine was from Aldrich-Chemie (Steinheim, F.R.G.). Freon was kindly provided by C. Leray, Centre de Neurochimie (Strasbourg, France). [8-^{3}H]Adenosine 3′,5′-cyclic monophosphate (18.3 Ci/mmol) was purchased from Amersham, France. All other reagents were of analytical grade.

Volunteers. Four healthy male volunteers who had not taken any medication for the preceeding two weeks participated in the trial. Informed consent was given and the protocol was approved by the ethical committee of the Groupe de Recherche sur le Médicament, Université Louis Pasteur de Strasbourg. Ticlopidine, 250 mg twice, was taken orally for 8 days, all studies being performed 1 day before and at day 8, 2 hr after the last intake.

Bleeding time. The bleeding time was measured

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using a Simplate® device (Orthodiagnostics, France) under a constant pressure of 40 mm Hg on the arm.

Preparation of washed human platelets. Blood was collected from a forearm vein, six volumes of blood into one volume of acid–citrate–dextrose anticoagulant. Twice washed platelet suspensions were prepared as previously described [15]. The final resuspending medium (pH 7.35) was Tyrode's solution containing 2 mM Ca^{2+}, 1 mM Mg^{2+}, 0.35% human albumin (HSA, Centre Régional de Transfusion Sanguine, Strasbourg, France) and apyrase (2 μg/mL, a concentration that converted 0.25 μM ATP to AMP within 2 min at 37°).

The platelets were stored at 37° throughout the experiments. Platelet count was adjusted in the final suspension to 300,000/μL using a Baker 810 platelet counter (Baker Instruments, Allentown, PA, U.S.A.).

Platelet aggregation studies. Aggregation was measured at 37° by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Canada). A 0.45 mL aliquot of platelet suspension was stirred at 1100 rpm and activated by addition of 50 μL of ADP (5 μM final concentration) in the presence of DFP-treated human fibrinogen (0.8 mg/mL) or 50 μL of thrombin (0.1 units/mL) [15]. Aggregation was potentiated or not by previous addition of 10 μL of Adr (1 μM final concentration). The extent of aggregation was estimated quantitatively by measuring the maximum curve height, in millimetres, above baseline level.

cAMP assay. A 0.45 mL aliquot of washed platelets was stirred at 1100 rpm in the aggregometer cuvette. Reagents were added at 30 sec intervals (PGE_1 1 μM, adrenaline 1 μM, ADP 5 μM, thrombin 0.1 units/mL or solvent, a modified Tyrode's buffer containing no Ca^{2+} or Mg^{2+}). One minute later, the reaction was stopped by addition of 50 μL of ice-cold 6.6 N perchloric acid. The protein precipitate was eliminated by centrifugation, the supernatant was transferred to a 5 mL polystyrene tube and cAMP was extracted as described by Khym [16] using a mixture of trioctylamine and freon (~ v/v). Two millilitres of this mixture were added to the supernatant and mixed thoroughly for 2 min. The upper aqueous phase was transferred to an Eppendorf tube, lyophilized and the dry extract dissolved in the buffer provided in the commercial radioimmunossay kit (Amersham) used for cAMP measurement.

Measurement of cytoplasmic free calcium concentration. Platelets were loaded with Quin 2 by incubation with Quin 2-AM (10 μM) at 37° during the first step of the washing procedure. The platelets were finally suspended at 300,000/μL in Tyrode's Hepes buffer containing apyrase but no Ca^{2+}. Fluorescence measurements of platelet cytoplasmic free calcium were performed in the presence of 0.1% fatty acid free bovine serum albumin (BSA) because of the high background fluorescence of 0.35% HSA. The suspension of Quin 2 loaded platelets was placed in a square quartz cuvette at 37° in a Perkin-Elmer LS5 fluorescence spectrometer (Perkin-Elmer, Norwalk, CO, U.S.A.). Fluorescence was monitored with a standard monochromator at 340 nm excitation and 490 nm emission before and after the addition of $CaCl_2$ (1 mM) or EGTA (1 mM) and ADP (5 μM) plus Adr or solvent (saline). For Ca^{2+} calibration, platelets were solubilized by addition of 0.02% Triton X-100. Minimal fluorescence (F_{min}) was measured after adding 4 mM EGTA and increasing the pH to 8.3 with 0.1 M NaOH. Maximal fluorescence (F_{max}) was measured after addition of 6 mM $CaCl_2$. Intracellular calcium concentration $[Ca^{2+}]_i$, was calculated using the equation:

$$[Ca^{2+}]_i = K_D \frac{(F - F_{AF}) - (F_{min} - F_{min\,AF})}{(F_{max} - F_{max\,AF}) - (F - F_{AF})}$$

where K_D is the dissociation constant of the fluorescent probe for Ca^{2+} (K_D = 115 nM for Quin 2) and F_{AF}, $F_{max\,AF}$ and $F_{min\,AF}$ represent the respective autofluorescence of control platelets not loaded with the fluorescent probe [17].

RESULTS

Effects of ticlopidine on bleeding time

The bleeding time was significantly prolonged in all but one case. Compared to less than 8 min before the trial, it was above 20 min in two cases and above 9 min in one case after the trial. One subject had a poorly prolonged bleeding time, passing from 5 to 6 min, although ADP induced aggregation was reduced by 50%.

Effects of ticlopidine on washed platelet aggregation

After administration of 500 mg of ticlopidine per day for 8 days, 5 μM ADP induced aggregation was inhibited by about 70% when no Adr was added. Adr (1 μM) potentiated the ADP induced aggregation and this potentiation was not inhibited after ticlopidine administration (Fig. 1). In contrast, thrombin (0.1 units/mL) induced aggregation was not inhibited (data not shown).

Effects of ticlopidine on cAMP levels

As can be seen in Fig. 2, there was no detectable effect on cAMP levels of human platelets after ticlopidine administration when no PGE_1 was added. Basal levels were unchanged and neither ADP nor Adr modified them. However, when PGE_1 (1 μM) was added to platelets, prior to addition of ADP (5 μM) or Adr (1 μM) or both, PGE_1 raised the cAMP level from 1 to 5 pmol/10^8 platelets and this effect was reversed by ADP, by Adr or by both successively added to the system. Their action was not additional. After ticlopidine administration, the effect of ADP was strongly inhibited, whereas that of Adr was not. Likewise, the thrombin induced decrease in the cAMP level raised by PGE_1 was not inhibited by ticlopidine treatment (Fig. 3).

Effects of ticlopidine on Ca^{2+} movements

Ticlopidine had no detectable effect on basal levels of free cytoplasmic Ca^{2+} concentration (about 100 nM) in the four cases studied. In the presence of 1 mM extracellular Ca^{2+}, cytoplasmic free calcium rose rapidly from the basal level to about 300 nM in response to 5 μM ADP in both control and ticlopidine treated platelets. In the absence of external Ca^{2+}, where the fluorescence response corresponds to Ca^{2+} mobilization from internal stores, ADP

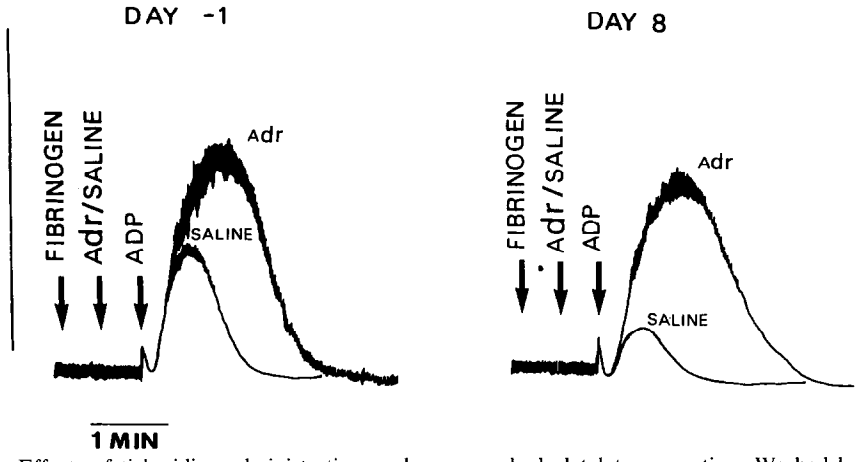

Fig. 1. Effects of ticlopidine administration on human washed platelet aggregation. Washed human platelets were incubated with fibrinogen (0.08%), 1 μM Adr or saline and 5 μM ADP. ADP induced aggregation was inhibited by about 70% at day 8 when no Adr was added. Adr potentiation of ADP induced aggregation was not inhibited after ticlopidine treatment. These data are from one experiment representative of four different experiments with identical results.

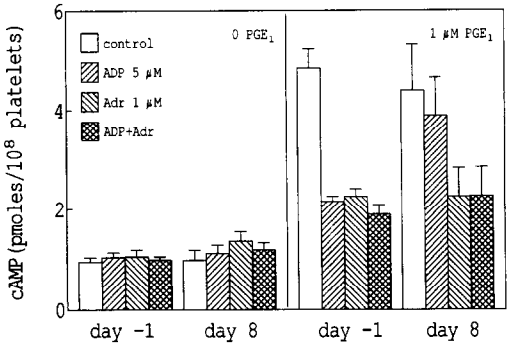

Fig. 2. Effects of ticlopidine (500 mg/day for 8 days) on cAMP levels in human platelets. Basal and 1 μM PGE_1 stimulated cAMP total levels were measured using a commercial radioimmunoassay kit. Basal levels (0 PGE_1) were unchanged by ADP, Adr or both. At day 8, no significant modification was observed. When 1 μM PGE_1 was added to platelets, cAMP levels rose from 1 to 5 pmol/10^8 platelets and this effect was reversed by ADP, Adr or both, without additional effect. At day 8, the action of ADP was strongly inhibited whereas the effect of Adr was not. Results are expressed as mean values ± SEM from four separate experiments where each measurement was in duplicate.

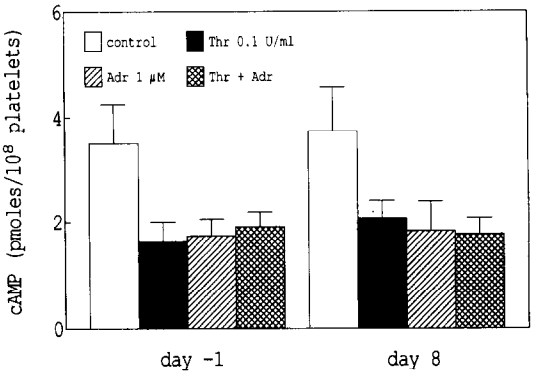

Fig. 3. Effect of thrombin on cAMP levels raised by PGE_1 in control and ticlopidine treated platelets. After 8 days of ticlopidine treatment, thrombin was still able to reverse PGE_1 stimulated cAMP levels of intact washed platelets equally as well as Adr. Results are expressed as mean values ± SEM from four separate experiments where each measurement was in duplicate.

stimulation induced an increase of $[Ca^{2+}]_i$ in both control and ticlopidine treated platelets from the basal level of about 50 nM to 100 nM (Fig. 4). Adr did not modify these effects. When added alone to the system, no increase of fluorescence was seen, while in combination with ADP, Adr did not increase Ca^{2+} influx or Ca^{2+} mobilization from internal stores (data not shown).

DISCUSSION

In a previous study we have shown that the effect of the thienopyridine PCR 4099 on fibrinogen binding to its receptor is probably not due to a direct modification of the GP IIb–IIIa complex either in man or in rat. We concluded that thienopyridines may act specifically on the ADP pathway at an unknown stage [7]. Studies performed in rat [12] and in rabbit [13] have shown that Ca^{2+} mobilization from internal stores is strongly inhibited by thienopyridine compounds, whereas they do not affect Ca^{2+} influx across the plasma membrane. The aim of our study was to evaluate the effects of ticlopidine administration in man, 500 mg/day for 8 days, on Ca^{2+} movements and on cAMP levels and to check for a possible link between inhibition of Ca^{2+} mobilization and cAMP levels. We also studied the effects of Adr and ADP on platelet aggregation and cAMP

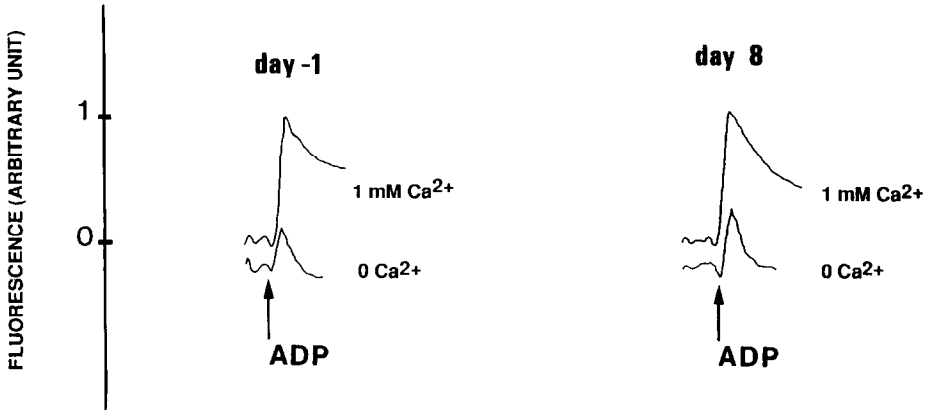

Fig. 4. Effects of ticlopidine (500 mg/day for 8 days) on calcium movements induced by 5 μM ADP. In the presence of 1 mM extracellular Ca^{2+}, cytoplasmic free Ca^{2+} concentration rose rapidly from the basal level of about 100 to 300 nM after addition of ADP. In the absence of external Ca^{2+}, ADP stimulation induced an increase of $[Ca^{2+}]_i$ from the basal level of about 50 to 100 nM. Ticlopidine treatment was without effect on these Ca^{2+} movements (day 8). The data are from one experiment representative of four different experiments with identical results.

levels in order to probe the selectivity of ticlopidine inhibition of the ADP pathway of human platelet activation.

In these studies, as expected, ticlopidine strongly inhibited ADP induced platelet aggregation. The potentiation of ADP induced aggregation by Adr was not affected, even at a low concentration of ADP (0.5 μM) which was not sufficient to induce aggregation alone either before or after ticlopidine treatment. This clearly suggests that Adr activates human platelets by mechanisms which are not directly related to the ADP pathway [14]. Basal cAMP levels were unchanged before and after ticlopidine treatment, which is in contradiction with several authors [11] but in agreement with others [9]. The addition of ADP, Adr or a combination of ADP and Adr did not change the basal cAMP levels of control or ticlopidine treated platelets. This may reflect the lack of sensitivity of total cAMP measurements in intact platelets using a radioimmunoassay. When PGE_1 was added to the platelet suspension, it caused a rise in cAMP levels from 1 to about 5 pmol/10^8 platelets in control and ticlopidine treated platelets. In contrast to previous studies [9, 10] we did not observe that ticlopidine increased the sensitivity of human platelets to PGE_1. ADP and Adr reversed the effect of PGE_1 on cAMP levels in control platelets. The effects of ADP and Adr were not additional, suggesting that inhibition of cAMP formation was maximal for each reagent. Ticlopidine prevented the inhibitory action of ADP on PGE_1 stimulated adenylate cyclase. In contrast, the effect of Adr on raised cAMP levels was not modified by ticlopidine and when thrombin was used, no inhibition of its effect on PGE_1 stimulated cAMP levels was observed. Adr acts on human platelet adenylate cyclase through the inhibitory GTP binding protein Gi [17]. Since thrombin has been found to exert its effect on the membrane adenylate cyclase through the same G-protein [18], we conclude from our results that ticlopidine does not directly alter this G-protein. It is not excluded that ADP may act on adenylate cyclase through the same G-protein. But if this is the case, one can speculate that ticlopidine impairs the interaction between Gi and the ADP receptor. If ADP does not act through Gi, it could act through another GTP binding protein which could be modified by ticlopidine, although such an inhibitory G-protein specifically linked to the human platelet ADP signal transduction pathway remains to be discovered. It is also possible that the ADP transduction system does not involve a G-protein.

In order to check for a possible link between the ticlopidine effect on cAMP levels and Ca^{2+} movements, we measured cytosolic free Ca^{2+} elevation induced by ADP. Ticlopidine had no detectable influence on basal Ca^{2+} levels, on Ca^{2+} fluxes across the plasma membrane or on intracellular Ca^{2+} mobilization. This is in contrast to previous studies [11–13] performed with rabbit and rat platelets, where intracellular Ca^{2+} mobilization was inhibited by ticlopidine. An explanation for this discrepancy might be that the animals were treated with very high doses of the drug and therefore the effect of ticlopidine or PCR 4099 on platelets was maximal. However, under the experimental conditions of our studies, where the rise of cytosolic free Ca^{2+} induced by exposure of human platelets to ADP was not prevented, platelet aggregation was inhibited. The discrepancy between calcium mobilization in platelets in man, rabbit or rat could thus be due to a species difference. We demonstrated that the potent effect of ticlopidine on ADP induced aggregation was accompanied by a specific and strong inhibition of the effect of ADP but not of Adr or thrombin on adenylate cyclase. Whether these results may help to explain the mechanism of inhibition of platelet aggregation by ticlopidine still requires further investigation but they provide a new lead to follow in order to understand

the action of ticlopidine and how the ADP aggregation pathway works.

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REFERENCES

1. Panak E, Maffrand JP, Picard-Fraire C, Vallée E, Blanchard J and Roncucci R, Ticlopidine: a promise for the prevention and treatment of thrombosis and its complications. *Haemostasis* **13**(suppl 1): 1–54, 1983.
2. Maffrand JP, Defreyn G, Bernat A, Delebassée D and Tissinier D, Reviewed pharmacology of ticlopidine. *Angiologie* **77**: 6–13, 1988.
3. Saltiel E and Ward A, Ticlopidine, a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in platelet-dependent disease states. *Drugs* **34**: 222–262, 1987.
4. Feliste R, Delebassée D, Simon MF, Chap H, Defreyn G, Vallée E, Douste-Blazy L and Maffrand JP, Broad spectrum antiplatelet activity of ticlopidine and PCR 4099 involves the suppression of the effects of released ADP. *Thromb Res* **48**: 403–415, 1987.
5. Lee H, Paton RC, Ruan C and Caen JP, The *in vitro* effect of ticlopidine on fibrinogen and factor VIII binding to human platelets. *Thromb Haemost* **46**: 590–592, 1981.
6. Dunn FW, Soria J, Soria C, Thomaidis A, Lee H and Caen JP, *In vivo* effects of ticlopidine on fibrinogen platelet cofactor activity and binding of fibrinogen to platelets. In: *Ticlopidine: Quo Vadis? Agents Actions* Suppl. Vol. 15 (Ed. Gordon JL), pp. 97–104. Birkhaüser Verlag, Basel, 1984.
7. Gachet C, Stierlé A, Cazenave J-P, Ohlmann P, Lanza F, Bouloux C and Maffrand J-P, The thienopyridine PCR 4099 selectively inhibits ADP-induced platelet aggregation and fibronogen binding without modifying the membrane glycoprotein IIb–IIIa complex in rat and in man. *Biochem Pharmacol* **40**: 229–238, 1990.
8. DiMinno G, Cerbone AM, Mattioli PL, Turco S, Lovine C and Mancini M, Functionally thrombasthenic state in normal platelets following the administration of ticlopidine. *J Clin Invest* **75**: 328–388, 1985.
9. Ashida SI and Abiko Y, Mode of action of ticlopidine in inhibition of platelet aggregation in the rat. *Thromb Haemost* **41**: 436–449, 1979.
10. Bonne C and Battais E, Ticlopidine and adenylate cyclase. In: *Ticlopidine: Quo Vadis? Agents Actions* Suppl. Vol. 15 (Ed. Gordon JL), pp. 88–96. Birkhaüser Verlag, Basel, 1984.
11. Morikawa M, Kojima T, Inoue M and Tsuboi M, Comparison of the inhibitory effects of aspirin and ticlopidine on platelet aggregation and Ca^{2+} mobilization in rat platelets. *Res Commun Chem Pathol Pharmacol* **54**: 275–278, 1986.
12. Feliste R, Simon MF, Chap H, Douste-Blazy L, Defreyn G and Maffrand JP, Effect of PCR 4099 on ADP-induced calcium movements and phosphatidic acid production in rat platelet. *Biochem Pharmacol* **37**: 2559–2564, 1988.
13. Derian CK and Friedman PA, Effect of ticlopidine *ex vivo* on platelet intracellular calcium mobilization. *Thromb Res* **50**: 65–76, 1988.
14. Lanza F, Beretz A, Stierlé A, Hanau D, Kubina M and Cazenave JP, Epinephrine potentiates human platelet activation but is not an aggregating agent. *Am J Physiol* **225**: 1276–1288, 1988.
15. Cazenave JP, Hemmendinger S, Beretz A, Sutter-Bay A and Launay J, L'agrégation plaquettaire: outil d'investigation clinique et d'étude phamacologique. *Ann Biol Clin* **41**: 167–179, 1983.
16. Khym JX, An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. *Clin Chem* **21**: 1245–1252, 1975.
17. Rink TJ, Smith SV and Tsien RY, Cytoplasmic free Ca^{2+} in human platelet: Ca^{2+} thresholds and Ca^{2+}-independent activation for shape-change and secretion. *FEBS Lett* **148**: 21, 1982.
18. Aktories K and Jakobs KH, Regulation of platelet cyclic AMP formation. In: *The Platelets: Physiology and Pharmacology* (Ed. Longenecker GL), pp. 271–278. Academic Press, Orlando, FL, 1985.
19. Aktories K and Jakobs KH, Ni-mediated inhibition of human platelet adenylate cyclase by thrombin. *Eur J Biochem* **145**: 333–338, 1984.