Finally, the use of the erythrocyte partitioning method provides an interesting alternative to the classical equilibrium dialysis or to ultrafiltration techniques. It is particularly useful for protein binding studies of drugs such as retelliptine, exhibiting non-specific adsorption on cellulosic membranes.

In summary. The binding of retelliptine hydrochloride to isolated serum proteins and erythrocytes was studied in order to predict free plasma concentration and to understand its blood distribution. Because of very important non-specific adsorption on cellulose, retelliptine binding was studied by using an erythrocyte partitioning technique. In serum, retelliptine was mainly bound to albumin and α_1-acid glycoprotein. In blood, it was bound to erythrocytes via a non-saturable process up to 200 μM and 45–50% of the drug was associated to the red cells. This last result could be explained by a high affinity for phospholipid layers.

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Lack of effect of heparin on TXA2 binding to platelets

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Heparin was reported to potentiate platelet aggregation [1] and to interfere with the antiaggregating effects of PGI_2, PGD_2 [2] and of other prostanoid (PGE_1) and non-prostanoid compounds [3]. Heparin has been demonstrated to bind to specific receptors on the surface of intact human platelets [4] and to interact with other platelet receptors, such as alpha adrenoceptors so reducing their binding affinity [5]. However, no inhibitory effect of heparin on the PGI_2 and PGD_2 binding to platelets was demonstrated [2], so that the mechanism of the proaggregatory effect has not yet been clarified. Lewy *et al.* [6] reported an increase in TxB_2 plasma levels after heparin intravenous administration in humans so suggesting that heparin might accelerate TxA_2 mediated proaggregating effects or coronary vasocontriction.

The aim of the present study was to establish if the proaggregating activity of heparin is related to change in the binding characteristics of TxA_2 released during platelet

aggregation. The interaction between heparin and TxA_2 binding to human platelet receptors was investigated *in vitro* by a direct radioligand assay method.

Materials and Methods

Blood sampling and platelet isolation. Blood was withdrawn by venipuncture from five non-smoker healthy volunteers (aged 44–62 years) and anti-coagulated with 15% (v/v) acid citrate dextrose (NIH formula A). No subject had taken any drug for at least the 15 days preceding the study. Platelets were washed and resuspended in assay buffer as previously described [7].

Binding studies. [^{125}I]PTA-OH binding. To evaluate the kinetics of association of [^{125}I]PTA-OH (9,11-dimethylmethano-11, 12-methano-16-(3[^{125}I]-4-hydroxyphenyl)-13, 14-dihydro-13-aza-15-tetra-nor-TxA_2) (Amersham, Bucks, U.K., 2000 Ci/mmol) to washed platelets, 0.5 nmol/L (final concentration, fc) [^{125}I]PTA-OH were incubated with 10^8 plt at 22° in a final volume of 0.2 mL. The reaction was stopped at selected times (Fig. 1) by adding four times 4-mL aliquots of ice-cold buffer to the tubes and the content was rapidly filtered under reduced pressure through Whatman GF/C glass microfiber filters. Filters were then counted in a Beckman gamma counter with an overall efficiency of 50%. Non-specific binding at each time was evaluated by adding 0.02 mL of ONO-11120 (9,11-dimethylmethane-11,12 - methane - 16 - phenyl - 13,14 - dihydro - 13 - aza - 15-tetranor-TxA_2) [8] (20,000 nmol/L, fc) (a kind gift from Prof. Narumiya, Kyoto, Japan).

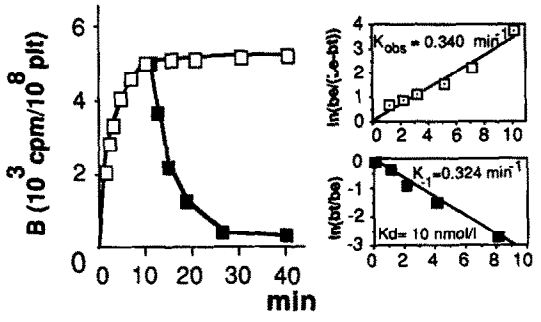

Fig. 1. Time course analysis of [^{125}I]PTA-OH. Platelets were incubated at 22° with 0.5 nmol/L [^{125}I]PTA-OH and specific binding was evaluated at indicated times. After 10 min incubation 20,000 nmol/L (fc) ONO-11120 was added to the incubation mixture and specific binding was evaluated at indicated times. The kinetic constants (K_{obs}, K_1 and K_{-1}) were calculated according to Weiland and Molinoff [9].

The kinetic of dissociation was evaluated in separate samples incubated under the same experimental conditions. After 10 min of incubation 0.02 mL of ONO-11120 (20,000 nmol/L, fc) were added to the reaction mixture. The residual binding was evaluated at selected times.

In competition studies platelet suspension (0.1 mL) was incubated in a final volume of 0.2 mL with [^{125}I]PTA-OH 0.05 nmol/L, (fc), plus ONO-11120 at increasing selected concentrations (0–4 × 10^{-6} mol/L) for 10 min at room temperature. The residual radioactivity after the addition of ONO-11120 2 × 10^{-5} mol/L (fc) was considered as non-specific binding. Non-specific binding of 0.05 nmol/L [^{125}I]PTA-OH amounted to 25–35% of total bound radioactivity.

After 10 min incubation the reaction was stopped and the content was filtered through Whatman GF/C filters. The entire washing procedure was completed within about 15 sec. Filters were counted in a Beckman gamma counter with an overall efficiency of 50%. Displacement curves of the [^{125}I]PTA-OH binding were obtained by U46619 (9,11-dideoxy-11α, 9α-epoxymethano- prostaglandin F2α) (Sigma Chemical Co., St. Louis, MO, U.S.A.) (0–2 × 10^{-6} mol/L), Prostacyclin (PGI_2) (Upjohn, Kalamazoo, MI, U.S.A.) (0–5 × 10^{-4} mol/L), and by unfractionated sodium heparin (Liquemin Roche, 5000 I.U./mL) (0–400 I.U./mL).

[^{3}H]U46619 binding. Platelet suspension was incubated in the same volume and under the above mentioned conditions with 20,000 nmol/L of [^{3}H]U46619 (22.4 Ci/mmol, NEN, Boston, MA, U.S.A.) 5 nmol/L (fc) plus unlabelled U46619 at increasing selected concentrations (0–4 × 10^{-6} mol/L). Residual radioactivity after the addition of 2 × 10^{-5} mol/L of unlabelled U46619 (fc) was considered as non-specific binding. Samples were processed as previously described [7] and filters were then suspended in a liquid scintillation solution (Aquasol, NEN, Dreieich, F.R.G.), and counted in a liquid scintillation spectrometer (Packard, LS 1800, Irvine CA, U.S.A.) with an overall efficiency of 40%. Displacement curves of the binding were obtained by ONO-11120 (0–2 × 10^{-6} mol/L), PGI_2 (0–5 × 10^{-4} mol/L) and heparin (0–400 I.U./mL).

Analysis of data. The kinetic constants (K_{obs}, K_1 and K_{-1}) were calculated according to Weiland and Molinoff [9].

The total binding for each concentration in displacement curve at equilibrium was determined by dividing the decay per min (dpm) of each platelet pellet by the calculated specific activity in dpm/mole [7]. The analysis of these binding isotherms was performed according to Scatchard [10]. The inhibitory constants upon the [^{125}I]PTA-OH and the [^{3}H]U46619 binding to platelets were calculated accord-

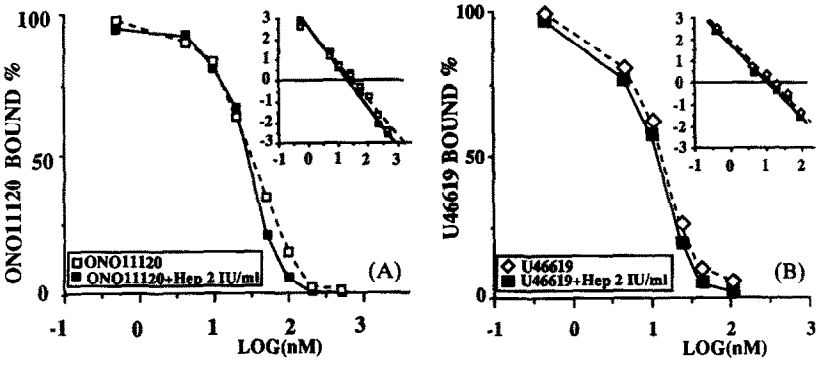

Fig. 2. Displacement of the specific [^{125}I]PTA-OH binding by increasing concentrations of ONO-11120 (A) and U46619 (B) in the presence and in the absence of heparin (2 I.U./mL).

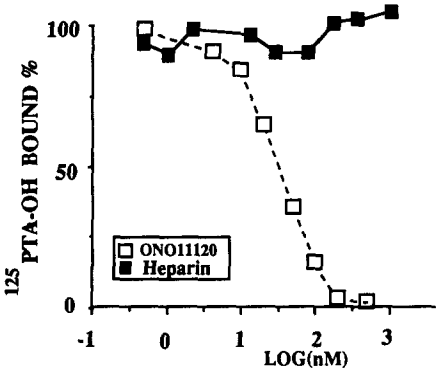

Fig. 3. Lack of displacement of the specific [^{125}I]PTA-OH binding by increasing concentration of heparin.

ing to Cheng and Prusoff [11]. Data are expressed as means ± SD.

Results and Discussion

In competitive binding studies ONO-11120 and U46619 showed a high affinity in the displacement of the [^{125}I]PTA-OH binding, whereas heparin did not displace the [^{125}I]PTA-OH binding to any extent (Fig. 2). The inhibitory constant (K_i) of ONO-11120 and U46619 were, respectively, 24.7 ± 4.2 and 18.4 ± 3.2 nmol/L (Fig. 1); when the competitive binding studies with ONO-11120 and U46619 were performed in the presence of heparin (2 I.U./mL), the inhibitory constants of the two analogues were, respectively, 21.9 ± 3.6 nmol/L and 16.8 ± 3.2 nmol/L and the equilibrium dissociation constant (K_d) and the maximum concentration of binding sites (B_{max}) determined by Scatchard analysis were 22.5 ± 6.2 nmol/L and 119 ± 23 fmol/10^8 plt (720 ± 138 rec/plt). When the incubation took place in the presence of heparin (2 I.U./mL) no changes in the [^{125}I]PTA-OH binding curve were observed and Scatchard analysis showed a K_d of 21.2 ± 4.8 with a B_{max} of 123 ± 25 fmol/10^8 plt.

The inhibitory power of ONO-11120, U46619, heparin and PGI_2 were evaluated also on the [^{3}H]U46619 binding. The K_i were 39.3 ± 11.1 nmol/L for ONO-11120 and 17.8 ± 5.6 nmol/L for U46619, whereas heparin and PGI_2 did not significantly inhibit the [^{3}H]U46619 binding to platelets (Fig. 1). Similarly competitive binding studies with ONO-11120 and U46619 in the presence of heparin (2 I.U./mL) failed to show changes in the inhibitory constants of the two analogues, which were respectively 36.9 ± 6.6 nmol/L and 16.8 ± 3.2 nmol/L. The K_d and the B_{max} determined by Scatchard analysis were 19.5 ± 4.1 nmol/L and 132 ± 42 fmol/10^8 plt (794 ± 252 rec/plt). The presence of heparin did not affect the [^{3}H]U46619 binding and K_d and B_{max} were, respectively, 17.1 ± 3.8 nmol/L and 142 ± 38 fmol/10^8 plt.

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The present study shows that the two labelled stable TxA_2 analogues, [^{3}H]U46619 and [^{125}I]PTA-OH, were displaced by low concentrations of the two unlabelled compounds (U46619 and ONO-11120), while the presence of heparin did not affect the characteristics of the binding of the two analogues either in the saturation curve (i.e. the total number of receptors was not changed) or in competitive binding studies.

In conclusion, heparin does not potentiate platelet activation by directly interacting with TxA_2 receptors and the mechanism of its proaggregatory effect, possibly related to the interaction of its highly dense negatively charged groups with aminoacidic structures and possibly mediated by a plasma cofactor, remains to be defined.

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