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# DETERMINATION OF SPECIFIC INHIBITORS OF THROMBOXANE $A_2$ FORMATION

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Activation of platelets by contact with collagen leads to the simultaneous formation of a prostaglandin and thromboxane ($TXA_2$), which can be assayed biologically with the superfused rabbit aorta and rat stomach strip. By this method it has been shown that a cyclo-oxygenase inhibitor, aspirin, inhibited formation of both prostaglandin and $TXA_2$ while a specific $TXA_2$ synthetase inhibitor, clotrimazole, inhibited only $TXA_2$ formation. The method is suitable as a primary screen in the search for $TXA_2$ specific synthetase inhibitors.

**Introduction** The determination of thromboxane $A_2$ ($TXA_2$) is difficult because it is labile, having a half-life of only about 30 s (Hamberg, Svensson & Samuelsson, 1975). The most commonly used method of measuring $TXA_2$ formation is by radioimmunoassay of its stable metabolite $TXB_2$. However, it is possible that other metabolic pathways of $TXA_2$ exist so that radioimmunoassay of $TXB_2$ is not necessarily always an accurate measurement of the formation of $TXA_2$.

It has been established that platelets constitute an important source of the enzyme which converts prostaglandin-endoperoxides to $TXA_2$ (Hamberg *et al.*, 1975). Furthermore, collagen is known to cause activation of human platelets leading to the release reaction (Caen, Legrand & Sultan, 1970) which therefore involves $TXA_2$ formation. Gryglewski, Korbut, Ocetkiewicz & Stachura (1978) have used these facts to devise a method of measuring platelet aggregation onto a strip of collagen placed in an extracorporeal circulation. It seems likely that the platelet aggregation in their experiments was caused at least partly by $TXA_2$ formation.

Piper & Vane (1969) used the rabbit isolated aorta to measure the release of an unknown labile substance (RCS) which has since been shown to consist mainly of $TXA_2$. The rabbit aorta is insensitive to prostaglandins and is at least 50 times more sensitive to $TXA_2$ than to the prostaglandin endoperoxides (Needleman, Moncada, Bunting, Vane, Hamberg & Samuelsson, 1976).

In this investigation we have attempted to activate platelets *in vitro* with a strip of collagen placed in a superfusion cascade system, measuring $TXA_2$ on the rabbit isolated aorta. In the same cascade a rat stomach strip was included to measure prostaglandin formation.

**Methods** *Platelets.* Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared from fresh rabbit blood according to the method described by Cooper, Lewis, Lieberman, Webb & Westwick (1979). The platelet count was adjusted to $5 \times 10^8$ platelets/ml and they were used as soon as the PRP was prepared. The platelets were found to remain stable for about 2.5 h, during which time they were stored at 37°C.

*Experimental tissues* A strip of the central part of longitudinally-cut rabbit Achilles tendon (approx. 30 × 3 mm) as described by Gryglewski *et al.* (1978) was mounted first in a cascade system. This tissue was perfused with Krebs solution alone (4 ml/min). The perfusate from the collagen was subsequently superfused over a spirally-cut rabbit aorta (RA) and a rat stomach strip (RSS). These latter were also superfused with Krebs (4 ml/min) containing a mixture of inhibitors and antagonists including indomethacin 1 μg/ml, propranolol 2 μg/ml, hyoscine 0.1 μg/ml, mepyramine 0.1 μg/ml, dibenzamine 0.1 μg/ml and methysergide 0.2 μg/ml.

**Results** When PRP or PPP were added directly to the fluid superfusing the RA and RSS there was no response but when PRP (but not PPP) was added to the fluid perfusing the collagen there was a subsequent contraction of both RA and RSS, indicating the release of products from the platelets after activation by contact with the collagen.

Figure 1 (a) and (b) show the response to 0.3 ml PRP added to the superfusion fluid first directly over the assay tissues and secondly over the collagen strip. The contractions of RA and RSS in the presence of a mixture of inhibitors and antagonists indicate the formation of $TXA_2$ and a prostaglandin respectively.

The responses of the assay tissues could be repeated several times by fresh additions of the PRP to the fluid superfusing the collagen strip.

Further evidence that the active principles were derived from arachidonic acid via the cyclo-oxygenase pathway can be seen in Figure 1a. This figure shows

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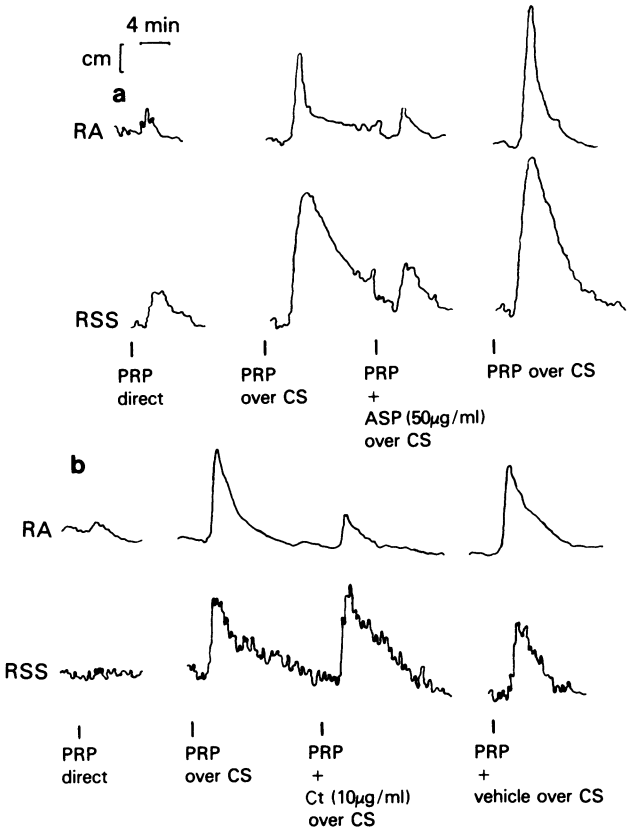


**Figure 1** Responses of the spirally-cut rabbit aorta (RA) and the rat stomach strip (RSS) perfused with Krebs solution containing indomethacin, propranolol, hyoscine, mepyramine, dibenzamine and methysergide to platelet-rich plasma (PRP). In the upper tracings (a) (i) PRP was added to the fluid directly perfusing the assay tissues, (ii) and (iv) PRP was first superfused over a strip of collagen (CS) before superfusing the assay tissues, (iii) PRP was first incubated with aspirin (Asp) 50 μg/ml for 5 min before coming into contact with the CS. In the lower tracings (b) PRP was added in the same way as for (a) except that in (iii) the PRP was incubated with clotrimazole (Ct) 10 μg/ml for 10 min before coming into contact with the CS.

that the formation of the active agents was inhibited when the platelets were incubated with aspirin (50 μg/ml) before passing them over the collagen.

Two additional pieces of evidence indicate that $TXA_2$ was responsible for the responses of the RA to the activated platelets. Firstly, if the PRP was kept for a few min after activation by the collagen strip before being superfused over the assay tissues, the RA contracting activity disappeared whereas the RSS still contracted. This agrees with the finding that $TXA_2$ is extremely labile. Secondly, if the platelets were incubated with imidazole (100–400 μg/ml) before being brought into contact with the collagen strip, the contraction of the RA was reduced much more than that of the RSS. This is consistent with the finding that imidazole inhibits the conversion of the endoperoxides to $TXA_2$ (Moncada, Bunting, Mullane, Thorogood, Vane, Raz & Needleman, 1977).

Clotrimazole (1-(1-0-chlorophenyl-1, 1-diphenylmethyl) imidazole, Bayer), a derivative of imidazole, which is known to inhibit $TXA_2$ formation (personal communication E.C. Ku), was found to inhibit $TXA_2$ formation in lower concentrations and more specifically than imidazole itself. The experiment illustrated in Figure 1(b) shows that when platelets were incubated for 10 min with 10 μg/ml of clotrimazole, the response of the RA was considerably reduced whereas the response of the RSS was not reduced but slightly enhanced. In 6 experiments in which the effect of aspirin (50 μg/ml) on platelets was compared with that of clotrimazole (10 μg/ml), the response of the RA was reduced after treatment with aspirin or clotrimazole by 62% ± 10 (s.e. mean) and 56% ± 6 respectively. In contrast, the response of the RSS was reduced by 57% ± 9 after treatment with aspirin but enhanced by 34% ± 12 after clotrimazole.

These findings suggest that clotrimazole inhibits $TXA_2$ synthetase activity specifically resulting in a diversion of endoperoxide metabolism towards prostaglandin formation.

**Discussion** The simultaneous measurement of $TXA_2$ and prostaglandin formation during platelet activation provides a simple estimation of specific inhibitors of $TXA_2$ formation. We have not yet used the technique to estimate $IC_{50}$ values in respect of inhibition of $TXA_2$ synthetase and cyclo-oxygenase, but such a study is now being made.

In view of the importance given to $TXA_2$ in current ideas on platelet aggregation and thrombosis *in vivo*, the development of specific inhibitors of $TXA_2$ synthetase assumes a considerable therapeutic potential. The method outlined here might well prove to be a useful primary screen for such compounds.

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