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Requirement of ADP for arachidonic acid-induced platelet aggregation: studies with selective thromboxane-synthase inhibitors

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Arachidonic acid induces human platelet aggregation [1]. It was originally suggested that this occurred through the formation of cyclic prostaglandin endoperoxides [2] but later on it was shown that these unstable compounds generated thromboxane A2 (TxA2), an extremely potent aggregating agent in platelets [3]. Evidence was presented that arachidonic acid-induced platelet aggregation is dependent on ADP [4–6], the major endogenous amplifier of platelet aggregation response [7]. Other studies, however, indicated direct platelet activation by cyclic endoperoxides and/or TxA2 [8–15]. From these conflicting results the role of ADP released during platelet aggregation induced by AA remains unclear. We show here that selective pharmacological blockade of platelet TxA2-synthase did not result in any apparent modification of the platelet aggregatory response to arachidonic acid, provided ADP was available. We suggest that cyclic endoperoxides, accumulating when TxA2 synthesis is selectively prevented, have an absolute requirement for ADP, while TxA2 can induce platelet aggregation independent of this nucleotide. Thus, the various amplifying pathways during platelet stimulation have more or less relevance not by themselves but rather in relation to the availability of the others.

Materials and methods

Citrated human platelet-rich plasma (PRP) was stimulated for 3 min in a Born aggregometer (Elvi 840, Elvi Logos, Milan, Italy) under constant magnetic stirring (1000 rpm) with sodium arachidonate (AA, Sigma, >99% pure) [16], or the endoperoxide stable analogue U-46619 [17] or adenosine-5′-diphosphate (ADP, Sigma) [16]. For each individual PRP sample the Threshold Aggregating Concentration (TAC) of the stimulus that induced a 70% increase in light transmission was selected [16]. PRP was preincubated for 3 min at 37° with dazoxiben (UK 37,248-01, Pfizer Central Res., Sandwich, U.K., 40 μM) or OKY-046 (Ono Pharmaceutical Co., Japan, 40 μM) or solvent (Tris–HCl, 150 mM, pH 7.4) before AA addition. Creatine phosphate (Sigma 5 mM, CP) and creatine phosphokinase (Sigma 10 U/ml, CPK) were freshly dissolved in Tris–HCl 15 mM pH 7.4. Potato apyrase prepared according to Molnar and Lorand [18] hydrolysed 2 nmoles ATP/min/μg protein. The addition of CP/CPK or apyrase was able to completely prevent 10 μM ADP-induced platelet aggregation. Ketanserin (Janssen Pharmaceutica, Beerse, Belgium) was dissolved in redistilled water.

An aliquot of PRP was deproteinized by addition of equivolume of absolute ethanol and TxB2 and PGE2 measured by specific radioimmunoassay [19]. 5-Hydroxytryptamine (5-HT) release was assayed in the platelet pellet obtained by rapid centrifugation of PRP, treated with 0.4 N HCl, sonicated and centrifuged. 5-HT was assayed in this clear supernatant by high performance liquid chromatography with electrochemical detection as described [20].

Results and discussion

Human platelet aggregation induced by AA was accompanied by generation of TxB2, the stable derivative of TxA2 and the release reaction, as measured by the release of endogenous 5-HT (Table 1). Two selective TxA2-synthase inhibitors, dazoxiben and OKY-046, completely suppressed TxB2 generation, but only partially prevented the release reaction. Cyclic endoperoxides accumulated concomitantly as suggested by the 10–20-fold increase of platelet PGE2 generation [21, 22] (Table 1).

The ADP-removing enzymatic system CP/CPK had no effect on AA-induced platelet aggregation, but almost completely prevented it in platelets preincubated with either

Table 1. TxB2 and PGE2 generation and 5-HT release in human PRP stimulated by TAC of AA (alone or in the presence of a TxA2-synthase inhibitors) or of U-46619

Release (%)	TxB2 (pmol/ml)	PGE 2 (pmol/ml)	5-HT
AA (0.4–0.6 mM)	900–2500	80–150	40–50
AA + Dazoxiben (40 μM)	6–15	700–1200	29–35
AA + OKY-046 (40 μM)	3–12	1300–1700	24–34
U-46619 (180–270 nM)	<5	<5	14–25

Figures are ranges from 6 samples.
5-HT release is % of total platelet 5-HT content [20].

TxA2-synthase inhibitor (Fig. 1). Similar results were obtained when apyrase, instead of CP/CPK, was used. The selective contribution of ADP secreted from platelet dense bodies together with 5-HT was supported by the observation that selective blockade of platelet 5-HT receptor by ketanserin (40 μM) [23] did not result in any modification of platelet aggregation or release reaction, independent of whether TxA2 was generated or not (data not shown).

The contribution to AA-induced platelet aggregation of ADP (secreted or present in plasma) has been excluded by several previous studies [9–15]. In these studies, however, TxA2 formation by platelets was not excluded. In our approach to this issue we questioned whether the importance of ADP could be affected by the availability of a normal platelet TxA2-synthase capacity. This was indeed the case, as shown above.

Our interpretation is reinforced by the following experiment: U-46619, a stable cyclic endoperoxide analogue [17, 24], at concentrations inducing only a small, reversible platelet aggregation was strongly potentiated by subthreshold concentrations of ADP. The irreversible full response induced by the combination of U-46619 and ADP was completely reversed by either CP/CPK or apyrase (Fig. 2).

In summary, AA induces platelet aggregation independent of ADP, provided TxA2 is normally generated. This nucleotide becomes crucial, however, in the absence of TxA2. The possible therapeutical implication of our observations is that the pharmacological blockade of TxA2 synthesis by selective inhibitors may have no consequences on platelet function if other measures to block or remove ADP effect on platelets are not associated.

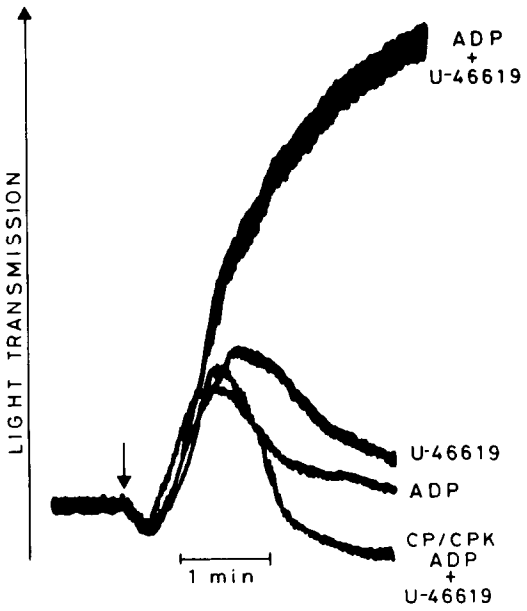

Fig. 2. Representative tracings of platelet aggregation induced by combination of ineffective concentrations of U-46619 (120 nM) and ADP (0.5 μM) and effect of preincubation with CP (5 mM)-CPK (10 U/ml).

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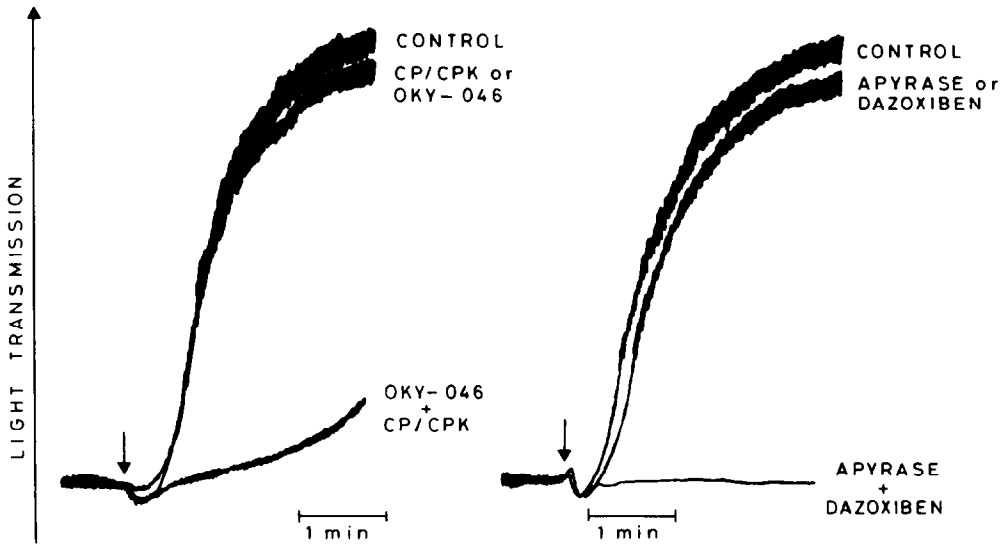

Fig. 1. Representative tracings of human platelet aggregation induced by sodium arachidonate and effect of preincubation with a TxA2-synthase inhibitor and/or an ADP-removing enzymatic system. TAC of AA was 0.4 mM in this particular experiment, representative of 5 experiments. OKY-046 (40 μM) (left) or dazoxiben (40 μM) (right) did not affect platelet aggregation. Preincubation of PRP with CP/CPK (5 mM/10 U/ml) or apyrase (50 μl) did not affect AA-induced platelet aggregation. The combination of a TxA2-synthase inhibitor with ADP-removing enzymatic system completely blocked platelet aggregation induced by TAC of AA.

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Laboratory of Cardiovascular Clinical Pharmacology
Istituto di Ricerche Farmacologiche Mario Negri
Via Eritrea 62
20157 Milano, Italy

CHIARA CERLETTI
SALVADOR MINOLDO
FRANCESCA BUCCHI
ALDO DEL MASCHIO
GIOVANNI DE GAETANO

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Effect of doxorubicin on calcium binding sites in guinea-pig heart

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There is strong evidence that the acute cardiotoxic effect of Doxorubicin (DXR) is mediated by an inhibitory effect on cell calcium flux. In fact, kinetic studies demonstrated that the cardiotoxic activity of DXR and of its 4′-epi- and 4′-deoxy-analogues is linearly correlated with their capacity to inhibit the fast-exchanging calcium compartment [1].

It was also demonstrated that DXR acutely inhibits calcium influx and the associated magnitude and duration of the slow action potential [2, 3], and moreover inhibits the Na^+/Ca^{2+}-exchange in the sarcolemma [4].

The present investigations were undertaken in order to get a further insight in the DXR–Ca interaction in the myocardiac cell.

Materials and methods

Since previous experiments demonstrated that up to 360/ μM DXR does not modify the heart rate in guinea pig, the present investigations were performed in spontaneously beating isolated guinea-pig atria. Incubation was made in Tyrode solution of the following composition: 11.1 mM glucose, 136.8 mM NaCl, 5.37 mM KCl, 051 mM $MgCl_2$, 11.9 mM $NaHCO_3$, 0.46 mM NaH_2PO_4, 1.87 mM $CaCl_2$; the medium was gassed with a 95% O_2 + 5% CO_2 mixture to maintain a pH value of 7.4. The organs were loaded with 1.0 g tension and allowed equilibrating for 60 min.

The contractile responses developed by the organs were recorded by means of an isometric tension recording system; dF/dt was used as a contractility index.

A preliminary set of trials indicated a calcium concentration of 5×10^{-5} M as the maximum calcium concentration resulting in a non-working heart. Starting from this concentration, curves of increasing contractile force were obtained by a stepwise increase of calcium levels up to a final cumulative concentration of 1.2×10^{-2} M. For each calcium concentration, the preparations were allowed to stabilize until a new dF/dt value was obtained.

Calcium titration curves were also obtained in the presence of 25–200 μM DXR.

Results and discussion

The stepwise addition of calcium to non-working organs equilibrated with 5×10^{-5} M Ca yields a sigmoid-shaped curve of increasing contractile force reaching its maximum at about 9×10^{-3} M Ca. Different concentrations of DXR bring about a moderate shift of this curve to the right and a dose-dependent depression of the maximal contractile response (Fig. 1). This behaviour is different from that of the typical Ca-antagonist, verapamil, which brings about a competitive calcium antagonism: in fact, the depression of contractile force induced by this drug can be reversed by