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SHORT COMMUNICATIONS

Why do some β adrenergic agonists inhibit generation of thromboxane A_2 in incubates of platelets with arachidonic acid?

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Platelets are a rich source of potential inflammatory substances [1] including the cyclic endoperoxides [2, 3], the prostaglandins (PG) [4] and thromboxane A_2 (TxA_2) [5]. These substances are unique because they are synthesized *de novo* from added or endogenous arachidonic acid (AA) and thus are not stored, in contrast to other potential inflammatory substances [1]. Thromboxane A_2 has been implicated in platelet aggregation particularly when triggered by added AA [6]. Generation of PG [4] and of TxA_2 [5] in platelets, and elsewhere [7], is inhibited by non steroidal anti-inflammatory drugs (NSAID) such as aspirin or indomethacin and, accordingly, aggregation is suppressed [8]. Since NSAID inhibit release of other potential inflammatory mediators during the so-called release reaction accompanying platelet aggregation [9, 10] and since an anti-inflammatory effect can be obtained by accelerating the generation of PGE_2 from the endoperoxides, at the expenses of TxA_2 [11], a case can be made for a pivotal role for TxA_2 in the initial cellular responses of inflammation. Release of potential inflammatory mediators, such as amines or lysosomal enzymes, from various cell types, is also inhibited by β adrenergic agonists and this is considered to be due to their ability to increase the intracellular content of cyclic AMP [12]. It has been demonstrated previously that dibutyryl cyclic AMP reduces the generation by platelets of TxA_2 activity from AA [13, 14]. We now studied the influence of β adrenergic agonists on TxA_2 formation and bring evidence for an as yet undescribed type of interaction of platelets and adrenergic mediators.

Experiments were performed with dog platelets which constitute a suitable test system since, contrary to those of other animal species, they fail to aggregate when challenged with AA under standard conditions [15, 16] but still generate abundant amount of TxA_2 [17]. Use of these non-aggregating platelets thus allows to work without disturbing the TxA_2 bioassay and its generation with other substances released from platelets during aggregation. Dog citrated platelet-rich plasma (PRP) was prepared as described previously [15] and incubated for 2 min with AA in presence or absence of adrenergic stimulants and of their pharmacological antagonists. The incubates were bioassayed for TxA_2 activity on isolated strips of rabbit aorta or on rabbit PRP pre-incubated with indomethacin (0.5 mM) in order to suppress any effect of transferred AA [15–17]. (Details in Fig. and Table legends).

As seen in Fig. 1, the yield of TxA_2 in incubates of dog platelets with AA was reduced, concentration dependently, by D,L-isoproterenol or by salbutamol. Inhibition increased with duration of incubation (Table 1). Similar results were obtained when rabbit platelets were used as a detecting system for TxA_2. In the latter case 1 mM of dihydroergotamine was added to rabbit PRP in order to prevent α adrenergic-mediated potentiation of aggregation due to transferred D,L-isoproterenol [13]. The percent inhibition of TxA_2 generation detected by rabbit platelet aggregation, in presence of D,L-isoproterenol (2 mM) or of salbutamol (1 mM) were respectively of 52 ± 17 and 42 ± 26 for a 5 min incubation (mean ± S.D. of six experiments). Thromboxane A_2 generation by human platelets was also inhibited in presence of D,L-isoproterenol. Thus, a 2 mM concentration of the latter, incubated with PRP for 5 min at 37° reduced the yields of TxA_2 from AA (0.5 mM) by 70 ± 24 per cent (mean ± S.D. of four experiments).

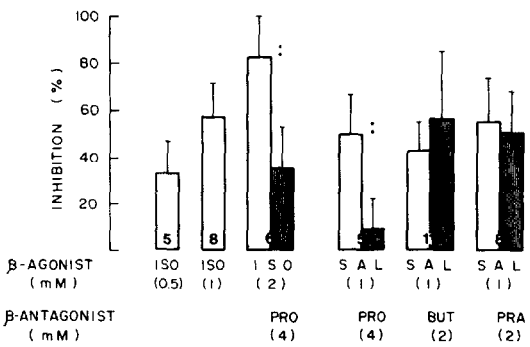

Fig. 1. The β agonists, D,L-isoproterenol (ISO) (Sigma) and salbutamol (SAL) (Glaxo) were incubated at the indicated concentrations with 0.4 ml of dog platelet rich plasma for 5 min at room temperature. Arachidonic acid (0.2 mM) (Sigma) was then added and the incubate was stirred for 2 min and bioassayed for thromboxane A_2 activity on superfused rabbit aorta strips [17]. Percent inhibition was calculated from control experiments in which β agonists were added to the incubates 5 sec before the bioassay, when TxA_2 was clearly formed. The β antagonists, D,L-propranolol (PRO) (Avlocardyl® ICI), butoxamine (BUT) (Borroughs Wellcome, courtesy of Dr. S. Moncada) and practolol (PRA) (ICI, courtesy of Dr. B. Lacoume), were added to dog PRP one minute before the β agonists, and inhibition was compared to control experiments in which the β antagonists and the β agonists were added to the incubates 5 sec before testing. Columns, (□) for inhibition by β agonists and (■) for reversion of this inhibition by the β antagonists), indicate the mean ± S.D. The number of separate experiments is indicated in each column, and statistical significance at $P > 0.01$ by **Student's 't' test). Drugs were dissolved as follows: D,L-isoproterenol in an ascorbic acid solution (17 $\mu g.ml^{-1}$), salbutamol in H_2SO_4 1N (final pH being around 9), practolol in polyethylene glycol 300 (Merck–Schuchardt), D,L-propranolol and butoxamine in 0.9% (wt. vol.$^{-1}$) NaCl and arachidonic acid in 95% ethanol–3 mM sodium carbonate (1:6, v/v), to which $NaCo_3$ crystals were added until complete clarification of the solution.

Inhibition of TxA_2 formation by D,L-isoproterenol and by salbutamol was prevented by the $\beta1$–$\beta2$ antagonist D,L-propranolol [18] but not by the $\beta1$ antagonist practolol [19] nor by the $\beta2$ antagonist butoxamine [20] both up to 2 mM (Fig. 1). Since the $\beta2$ agonist salbutamol [21], prevented TxA_2 formation, failure of the $\beta2$ antagonist, butoxamine, to reverse inhibition was a surprising finding. A similar profile, i.e. effectiveness of

Table 1. Time-dependent inhibition of thromboxane A_2 generation by D,L-isoproterenol and by salbutamol

Adrenergic agonist	Duration of incubation, min 1	3	5
D,L-isoproterenol 2 mM	28 ± 22*	50 ± 21†	73 ± 15†
Salbutamol 1 mM	50 ± 21†	61 ± 4 †	66 ± 26†

D,L-isoproterenol and salbutamol were added to 0.4 ml of dog platelet rich plasma, at the indicated concentrations, 1, 3 or 5 min before arachidonic acid (0.2 mM). The incubates were stirred at room temperature for 2 min before being tested for thromboxane A_2 generation on rabbit aorta strips. Percent inhibition was calculated from control experiment in which the β agonists were added just before thromboxane A_2 determination. Each figure represents the mean ± S.D. of 4–7 experiments. The differences between the yield of thromboxane A_2 activity in the presence and in the absence of the β agonists was statistically significant at $P < 0.05$*, and at $P < 0.01$† when indicated.

D,L-isoproterenol and its reversal by D,L-propranolol (but not by practolol nor by butoxamine) has been recently demonstrated in the cat urinary bladder [22].

The yield of TxA_2 in PRP-AA incubates was maximal within 2 min [15] and was reduced by D,L-isoproterenol (2 mM incubated for 5 min) to a similar extent if measured within 1, 2 or 5 min after addition of AA. Thus, inhibitions in per cent, as compared to controls, were respectively of 70 ± 23, 60 ± 26 and 63 ± 26 (mean ± S.D. of 5–7 experiments). This ruled out pseudo-inhibition by slowing down of TxA_2 generation. The integrity of platelets was required for inhibition, since sonicated platelets generated as much TxA_2 in presence as in absence of the β adrenergic agonists. This suggested that the inhibitory activity involves a multistep mechanism triggered by stimulation of the β adrenoceptors. Substances known to inhibit aggregation by activation of the adenyl cyclase [18] such as prostaglandin E_1 [23], dibutyryl cyclic AMP or papaverine [25] failed to inhibit TxA_2 generation at doses which suppressed aggregation by adenosine diphosphate (ADP) (Table 2). Moreover, salbutamol failed to antagonize aggregation of dog platelets by ADP (1–50 μM) and thus presumably failed to increase the level of cyclic AMP, but inhibited the generation of TxA_2 (Table 2).

In order to pin-point the mechanism of inhibition by the β adrenergic agonists, the two propranolol isomers were used. Both the L-isomer, which is the active form of the β antagonist, as well as the D-isomer, which is practically inactive in comparison [26], counteracted inhibition by D,L-isoproterenol of generation of TxA_2 (Fig. 2), suggesting that a common property, other than β antagonism, was involved. One such property is the membrane stabilizing effect [27] and to verify its relevance, the local anaesthetic lidocaine was used and also shown to reverse inhibition due to D,L-isoproterenol (Fig. 2). Since relatively high concentrations of the drugs were needed (2–4 mM) cell damage might have occurred [27, 28] and explain failure of inhibition by D,L-isoproterenol, as observed in cell lysates. This was ruled out, since microscopic and photometric observations of PRP treated with propranolol and with lidocaine failed to reveal differences as compared to control PRP.

Table 2. Comparison between the inhibition of generation of thromboxane A_2 activity and of ADP-induced aggregation by substances that increase the platelet cyclic AMP level

Drug	Concentration (mM)	Activity in % of controls Thromboxane A_2 generation	Platelet aggregation
Salbutamol	1	34 ± 28†	95 ± 7
Dibutyryl cyclic AMP	5	104 ± 20	3 ± 6†
Prostaglandin E_1	0.001	96 ± 14	0 ± 0†
Papaverine	0.05	96 ± 10	4 ± 9†

Salbutamol, dibutyryl cyclic AMP (Boehringer, Mannheim) prostaglandin E_1 (Ono) and papaverine (Bruneau) were added to 0.4 ml of platelet rich plasma, at the indicated concentration, 5 min before arachidonic acid (0.2 mM) or ADP (Sigma) (50 μM), and the resulting thromboxane A_2 generation or platelet aggregation respectively, were measured. Thromboxane A_2 generation was measured within 2 min on a superfused rabbit aorta strip. In control experiments, drugs were added to the incubates just before the bioassay. The percentage of inhibition was calculated by comparison with those controls. ADP induced aggregation was measured by turbidimetry on dog platelet rich plasma [15]. Drugs were incubated at room temperature for 5 min before the addition of ADP (50 μM). Percent inhibition was calculated by comparison with control experiments in which 0.9% NaCl replaced the potential inhibitors. Each figure is the mean ± S.D. of 3–6 experiments; statistical significance as in Table 1.

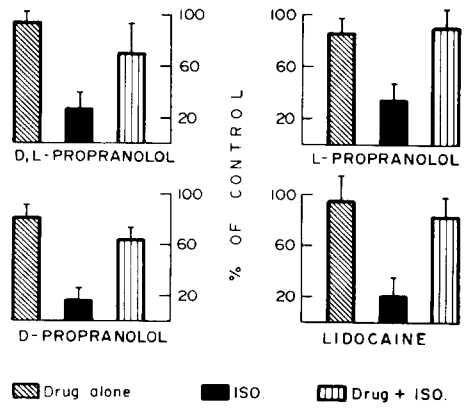

Fig. 2. Drugs as indicated (4 mM) were added to dog platelet rich plasma 1 min before D,L-isoproterenol (2 mM) and incubates were left for 5 min at room temperature, before the addition of 0.2 mM of arachidonic acid. Two min later the incubates were tested for thromboxane A_2 activity on superfused rabbit aorta strips. In controls incubates D,L-isoproterenol (ISO) and the reversing agent were added 5 sec before the bioassay. When the reversing agent was added alone 6 min before AA and D,L-isoprenaline 5 sec before the bioassay, (▨) a marginal reduction of thromboxane A_2 generation was observed, but when D,L-isoproterenol was added alone 5 min before AA and the reversing agent 5 sec before the bioassay, the generation of Thromboxane A_2 was inhibited markedly (■). Inhibition was reversed when the reversing agent was added to platelet rich plasma 1 min before D,L-isoproterenol (▥). Each column represents the mean ± S.D. of 4–5 experiments. D,L-propranolol, D-propranolol, L-propranolol (ICI, courtesy of Dr. M. Wharmby) and lidocaine (Xylocaine® Lab. R. Bellon, Paris) were dissolved in 0.9% NaCl. Other legends as in Fig. 1.

Generation of TxA_2 from AA by dog platelets is thus reversibly inhibited by two β adrenergic agonists. Inhibition is unlikely to be mediated by the classic β adrenoceptors, involving adenyl cyclase activation [18] since, (1) it was not reversed by specific $\beta 1$ and β 2 antagonists, (2) it was unaffected by drugs known to increase the intracellular cyclic AMP level (3) propranolol isomers and lidocaine were equieffective in reversing inhibition. The local anaesthetic activity is a common property of propranolol and lidocaine and is likely to be involved in reversion of blockade by the β agonists. Since cell lysis due to high concentrations of the reversing agents was ruled out, interference with Ca^{2+} mobilisation [29, 30] and/or the membrane protective effect, remain as alternative explanations. In this case, propranolol and lidocaine may interfere with the access of the adrenergic agonists to the sites where they exert their inhibitory activity, through membrane interaction, as reported for protection against erythrocyte damage [27]. The relatively high concentrations of D,L-isoproterenol which were needed, as compared to low requirements in other systems [18] might have indicated an unspecific effect, such as anti-oxydation exerted by the catechol moiety. Effectiveness of salbutamol, which has a methoxy substituted hydroxyl, reversion by various agents and uneffectiveness of β stimulants in absence of plasma or in platelet lysates ruled out this possibility but failed to uncover the mechanism of inhibition of TxA_2 generation.

Our experiments did not demonstrate whether inhibition was at the cyclo-oxygenase or at the thromboxane synthetase level, since formation of the final product, TxA_2, was monitored. D,L-propranolol fails to reverse inhibition of cyclo-oxygenase by aspirin and by indomethacin (unpublished results), and inhibition by β agonists is thus different from that due to anti-inflammatory drugs. The high concentrations of β agonists needed to influence TxA_2 formation renders less likely a physiological relevance, but under conditions of pathological stress, the potential protective role of β stimulation on platelets, counteracting the deleterious effects of α stimulation, may bear some importance.

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Deamination of dopamine and its 3-*O*-methylated derivative by human brain monoamine oxidase

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In recent years two forms of the mitochondrial monoamine oxidase, which selectively deaminates 5-hydroxytryptficities have been identified [1–4]. The A form of the oxidase, which selectively deaminates 5-hydroxy-tryptamine (5-HT) and norepinephrine, is selectively inhibited by clorgyline, and the B form of MAO, which selectively degrades phenylethylamine (PEA), is inhibited by deprenyl [5, 6]. Substrates which are deaminated exclusively by either the A or B forms of MAO display simple sigmoidal plots when inhibited by these selective inhibitors. In contrast, amines such as tyramine, which are metabolized by both forms of the oxidase, display a biphasic inhibition pattern when incubated in the presence of clorgyline or deprenyl.

The putative neurotransmitter, dopamine, has been reported to be deaminated in rat liver by both forms of the mitochondrial oxidase when inhibited by clorgyline, as evidenced by biphasic plots [7]. Yang and Neff [8] also reported that dopamine is deaminated by both the A and B forms of rat brain MAO. In contrast, Braestrup *et al.* [9] and Waldmeier *et al.* [10] reported that dopamine is primarily metabolized by the A form of rat brain MAO. However type B MAO of human brain was suggested to be the principle form which degrades this catecholamine [11, 12]. The 3-*O*-methylated derivative of dopamine, 3-methoxy-4-hydroxyphenylethylamine (3-MHPEA) has been suggested to be deaminated by both forms of MAO in rat liver [7], although a similar *O*-methylated derivative of benzylamine is primarily degraded by the B type of MAO [7].

Since an imbalance of brain dopamine has been implicated in the symptomatology of behavioral [13] and neurological disorders [14], the pathway by which this neurotransmitter is degraded may be important in regulating brain dopamine levels. Because conflicting reports [9–12] exist in the literature as to which form of MAO degrades dopamine, it was decided to re-examine the form of human brain MAO which deaminates this catecholamine and its catechol *O*-methyl transferase (COMT) degradative product, 3-MHPEA.

Human brain mitochondria obtained from frontal lobes were isolated as described previously [15]. These preparations were stored frozen at −20° in 0.1 M potassium phosphate buffer, pH 7.4. MAO was assayed by a modification of the procedure of Roth *et al.* [16]. In brief, reaction mixtures consisting of 0.125 mM ascorbic acid and varying amounts of clorgyline or deprenyl were preincubated in a total volume of 0.4 ml of 0.062 M potassium phosphate buffer, pH 7.4, for 12 min with human brain mitochondria prior to addition of radioactively labeled amine substrates. After addition of substrate, reaction mixtures were incubated for an additional 10 min when PEA was substrate, and 60 min when either dopamine, 5-HT or 3-MHPEA was employed as substrate. The concentrations of PEA and 5-HT used are near their reported K_m values [17, 18]. Deamination of amines was terminated by the addition of 50 μl of 0.4 M HCl, and 0.2-ml aliquots of the mixture were chromatographed through Bio-Rex 70 cation-exchange resin columns. The resin was washed with 2.8 ml water which was combined with the original 0.2 ml eluate, and to this solution 10 ml Aquasol was added. The radioactive deaminated product was measured by liquid scintillation spectrometry.

The effects of clorgyline on 5-HT, PEA, dopamine and 3-MHPEA deamination are illustrated in Fig. 1, panels A and B. As reported previously [11], clorgyline inhibits 5-HT and PEA metabolism in a sigmoidal, predictable manner based on the known relative specificities of this drug for the A and B forms of MAO. In contrast, both dopamine and 3-MHPEA are degraded in a biphasic pattern by clor-

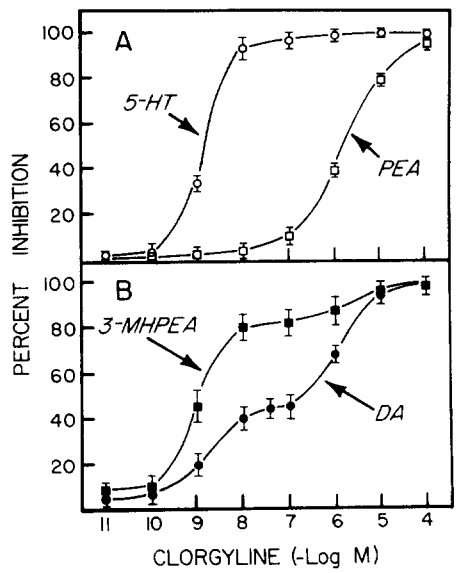

Fig. 1. Per cent inhibition of human brain mitochondrial deamination of (A) 5-hydroxytryptamine (5-HT) and phenylethylamine (PEA) and (B) dopamine (DA) and 3-methoxy-4-hydroxyphenylethylamine (3-MHPEA) at varying concentrations of clorgyline. Reaction mixtures consisting of human brain mitochondria and clorgyline were preincubated for 12 min prior to the addition of 5 μM [^{14}C]PEA or 10^{-4} M [^{14}C]5-HT, [^{14}C]DA or [^{3}H]3-MHPEA. Incubation time was 10 min with PEA and 60 min with the other substrates. In the absence of clorgyline, 82.7, 3.56, 29.3 and 30.4 nmoles of deaminated product were formed from deamination of 5-HT, PEA, DA and 3-MHPEA respectively.