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The effects of imidazoline agents on the aggregation of human platelets

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Abstract

Clonidine (2-[(2,6-dichlorophenyl)amino]-2-imidazoline), an imidazoline α_2-adrenoceptor agonist, is known to exert complex effects on human platelet aggregation distinct from those of the catecholamines, which are non-imidazoline α-adrenoceptor agonists. This study has investigated the aggregatory/anti-aggregatory effects of various imidazolines on human platelets. Blood samples were taken from normal volunteers and platelet aggregation was assessed by a turbidimetric method using a Chronolog aggregometer. Noradrenaline (2 μM) and adenosine diphosphate (1 μM) were used as aggregating agents. The results showed that, with the exception of moxonidine, all of the imidazoline agents used (with or without α_2-adrenoceptor activity) were able to inhibit noradrenaline-induced platelet aggregation. Compared with the non-imidazoline α_2-adrenergic antagonist, yohimbine, the rank order of potency was: efaroxan ($IC50 = 3.07 \times 10^{-8}$ M) > idazoxan ($IC50 = 1.74 \times 10^{-7}$ M) > tolazoline ($IC50 = 3.90 \times 10^{-7}$ M) > clonidine ($IC50 = 1.49 \times 10^{-6}$ M) $\cong$ antazoline ($IC50 = 1.77 \times 10^{-6}$ M) > yohimbine ($IC50 = 3.19 \times 10^{-6}$ M) > rilmenidine ($IC50 = 1.27 \times 10^{-5}$ M) > moxonidine ($IC50 > 10^{-4}$ M). Clonidine-displacing substance (CDS), a putative endogenous ligand at imidazoline receptors, was found to inhibit noradrenaline-induced platelet aggregation. Harmane, norharmane and agmatine, putative candidates for the active principle of CDS, had no effect on noradrenaline-induced platelet aggregation. In contrast to noradrenaline-induced aggregation, ADP-induced platelet aggregation was neither potentiated nor inhibited by the imidazoline agents, with the exceptions of clonidine and moxonidine. In conclusion, most imidazoline agents effectively inhibit noradrenaline-induced human platelet aggregation. The lack of effect of moxonidine and the proposed endogenous ligands suggested this effect was mediated by an 'atypical' non-adrenoceptor imidazoline-binding site. The results indicated an anti-aggregatory role of imidazoline compounds on noradrenaline-induced human platelet aggregation. In addition, CDS might be an endogenous modulator that prevented platelet hyper-reactivity to catecholamine stimulation.

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Introduction

In man, it is known that adrenaline/noradrenaline-induced platelet aggregation is mediated by α_2-adrenoceptors (Ardlie et al 1966; Grant & Scrutton 1979). The response varies widely between catecholamines and other α_2-adrenoceptor agonists, in particular, clonidine, an imidazoline compound known to possess agonist activity at α_2-adrenoceptors. Clonidine binds with high affinity to platelet α_2-adrenoceptors but is ineffective in inducing an aggregation response, in contrast to the effect of natural agonists such as adrenaline (Grant & Scrutton 1979; Hsu et al 1979). However, clonidine inhibits the aggregatory actions of noradrenaline and adrenaline on platelets (Stump & Macfarlane 1983), whereas it is able to potentiate ADP-stimulated platelet aggregation at the concentrations that inhibit adrenaline-induced aggregation (Shattil et al 1981). The mechanism underlying this discrepancy between the effects of clonidine and adrenaline/noradrenaline on platelet aggregation is unclear. Several studies reported the involvement of phospholipase A_2 activation (Banga et al 1986) or inhibition of adenylyl cyclase in the actions of adrenaline/noradrenaline on platelets (Clare et al 1984). Another platelet stimulator, ADP, initiates aggregation by simultaneous activation of three different receptors. P2Y1, coupled to the Gq protein, mediates ADP-induced shape change,

phospholipase C activation and calcium flux (Jin et al 1998). P2Y12 signals through Gi and inhibits adenylyl cyclase activity (Jin & Kunapuli 1998), and the $P2X_1$ receptor mediates rapid Ca^{2+} influx (Vial et al 1997). Inhibition of adenylyl cyclase is not solely responsible for the aggregatory action of α_2-adrenoceptor agonists in human platelets (Clare et al 1984).

Imidazoline binding sites have been found in several tissues, however their functions are still unclear. The I_1 site is thought to play a role in controlling systemic blood pressure. The I_2 sites are found to be allosteric sites on monoamine oxidase. Their exact functions remain to be determined with selective ligands. A more recent finding is the subtype I_3, which modulates insulin secretion and is reported to be the first functional site to be pharmacologically defined by selective agonists and antagonists (Eglen et al 1998). The existence of non-adrenergic, imidazoline binding sites (I_1 and I_2 receptors) that are pharmacologically distinct from α_2-adrenoceptors in human platelets has been reported (Piletz et al 1991; Piletz & Sletten 1993). Previous studies have suggested that imidazoline compounds may interact with non-α_2-adrenoceptor binding sites on human platelets, since two imidazoline compounds, KUM32 (2-(2,6-dichlorobenzylidenehydrazino)-2-imidazoline) and CBS1276 (2-(4-hydroxy-2-methylbenzylidenehydrazino)-2-imidazoline), were found to inhibit platelet adenylyl cyclase through non-α_2-adrenoceptor mechanisms and the effects could not be blocked by the α_2-adrenoceptor antagonist, yohimbine (Ferry et al 1986). Imidazoline compounds may, therefore, interact with non-adrenoceptor binding sites and produce responses distinct from those of the catecholamines.

Therefore, this study investigated whether imidazoline compounds (with or without α_2-adrenergic activity) possessed aggregatory/anti-aggregatory effects on human blood platelets induced by two aggregating agents, noradrenaline and ADP. In addition, clonidine-displacing substance (CDS), a putative endogenous ligand for imidazoline receptors, was examined for aggregatory/anti-aggregatory effects. CDS has been partially purified from calf brain since 1984 and was found to recognize α_2-adrenoceptors and imidazoline receptors (Atlas & Burstein 1984). It has been reported to possess several biological activities; in particular, it is involved in blood pressure regulation (Atlas 1990). The presence of CDS in human plasma (Synetose et al 1991) makes CDS a possible endogenous modulator in the cardiovascular system. Harmane, norharmane (Hudson 1999; Musgrave & Badoer 2000) and agmatine (Li et al 1994) have been suggested as putative active components of CDS and the effects of these agents on platelet aggregation were examined also.

Materials and Methods

Materials

The following drugs were purchased from Sigma: (±)-noradrenaline, adenosine-5′-diphosphate sodium salt, sodium citrate, idazoxan hydrochloride, clonidine hydrochloride, yohimbine hydrochloride, antazoline hydrochloride, tolazoline hydrochloride, efaroxan hydrochloride, moxonidine hydrochloride, rilmenidine hemifumarate, harmane hydrochloride, norharmane hydrochloride and agmatine sulfate.

The following is a summary of the binding properties of imidazoline and non-imidazoline α-adrenergic agents examined in this study. Antazoline is an imidazoline binding site ligand, which interacts with atypical 'I_3' sites in the pancreas. It possesses H_1-antagonistic activity and is devoid of α_2-adrenoceptor activity (Schultz & Hasselblatt 1989; Berdeu et al 1995). Clonidine is an α_2-adrenoceptor partial agonist and prototypical I_1 imidazoline receptor ligand (Ernsberger et al 1987); pK_i values were 7.21, 7.16, 6.87, 7.25 and 6.02 for α_{2A}, α_{2B}, α_{2C}, I_1 and I_2 receptors, respectively (Eglen et al 1998). Efaroxan is a potent and selective α_2-adrenoceptor antagonist. It is also an imidazoline I_1 receptor ligand and promotes insulin secretion at the putative I_3 site (pK_i values were 7.87, 7.42, 5.74, 7.28 and < 5 for α_{2A}, α_{2B}, α_{2C}, I_1 and I_2 receptors, respectively) (Chapleo et al 1984; Eglen et al 1998). Idazoxan is an α_2-adrenoceptor antagonist (pK_i 8.01, 7.43 and 7.7 for α_{2A}, α_{2B}, α_{2C}-adrenoceptor, respectively) and an I_2 imidazoline binding site ligand (pK_i 7.22) selective over the I_1 site (pK_i 5.9) (Michel & Ernsberger 1992). Moxonidine is a selective imidazoline I_1 receptor ligand (pKi 8.37) with only low affinity for I_2 and α_2-adrenoceptors ($pK_i < 5$ for I_2, α_{2B}, α_{2C} and 5.4 for α_{2A}-adrenoceptor, respectively) (Ernsberger et al 1992; Eglen et al 1998). Rilmenidine is an I_1 binding site selective ligand with greater I_1 selectivity (pK_i 7.22) over I_2 (pK_i 5.96) than clonidine. It possesses low α_2-adrenoceptor agonist activity (Bricca et al 1989; Michel & Ernsberger 1992); pK_i values were 5.80, 5.76 and 5.33 for α_{2A}, α_{2B}, α_{2C}-adrenoceptors, respectively (Eglen et al 1998). Tolazoline (2-benzyl-2-imidazoline) is a mixed α_1, α_2-adrenergic antagonist (Tranquilli & Thurmon 1984) and interacts with histamine H_2-receptors (Sanders et al 1975).

Blood sample collection and platelet preparation

Blood samples were taken from healthy volunteers who had not taken any medication for at least two weeks before blood collection. Whole blood samples were transferred into siliconized glass tubes containing 3.2% sodium citrate in a ratio of 9:1 (v/v). Platelet-rich plasma (PRP) was prepared by centrifugation at 150 *g* for 15 min. The upper supernatant was collected and used as PRP. The remaining lower portion was further centrifuged at 2000 *g* for 10 min and the clear supernatant was used as platelet-poor plasma (PPP).

Aggregation studies

Platelet aggregation studies were performed as previously described (Hsu et al 1979). Briefly, this study used a turbidimetric method by means of a Chronolog aggregometer (Model 550, Chronolog corporation, Haverton, PA). PRP was pre-warmed at 37 °C before addition of aggregating agents. Minimum light transmission was set with PRP (0%), and maximum with PPP (100%). All the drugs used were finally dissolved in normal saline. Samples (5 μL) of normal saline, imidazoline, α-adrenergic agents (10^{-8}–10^{-4} M) or CDS were added to 250 μL PRP and

pre-incubated for 1 min at 37 °C. A 5 μL sample of aggregating agent (ADP or noradrenaline) was then added to initiate aggregation. For the studies of the combined effects of α_2-adrenoceptor agonists and antagonists, antagonist was added 1 min before addition of the agonist. Equal volumes of normal saline were added instead of agonist and antagonist as a control for noradrenaline-induced aggregation. The extent of aggregation was the maximum light transmission found after the addition of ADP or noradrenaline and expressed as percentage of ADP or noradrenaline response. Aggregation was recorded for 4 or 5 min for ADP and noradrenaline, respectively, after the addition of aggregating agents.

Preparation of porcine brain clonidine-displacing substance (CDS)

The method for preparation of clonidine-displacing substance (CDS) was as described (Singh et al 1995; Pinthong et al 1995; Pinthong 2000). Briefly, porcine brains were obtained from a local abattoir immediately after slaughter of the animal. The brain was kept in ice during transportation. Half a porcine brain (70–80 g wet weight) was taken and the cerebellum and all pia mater removed before chopping into small pieces. Cerebral cortex was cut into pieces and homogenized in 6 vol distilled water using a Polytron homogenizer. The homogenate was centrifuged at 65 000 *g* for 30 min at 4 °C. The supernatant was boiled for approximately 5 min to precipitate soluble protein and then cooled to room temperature. The resulting solution was centrifuged at 65 000 *g* for 30 min at 4 °C. The resulting supernatant was dried using an evacuated centrifuge (Speed Vac plus SC110A). The dried material was extracted with methanol (HPLC grade) 2 × 20 vol (w/v) by sonication for 30 min at room temperature. The methanolic extracts were combined and centrifuged at 4000 rev min^{-1} for 5 min to remove any particulate matter and then evaporated to dryness using a rotary evaporator. The dry methanolic extract containing CDS was reconstituted in double-distilled water and stored at −20 °C until required for use. CDS activity of the extract was determined by competition for [^{3}H] clonidine binding to α_2-adrenoceptors in a porcine cerebral cortex membrane preparation (Atlas & Burstein 1984; Pinthong et al 1995; Pinthong 2000). One unit of CDS was defined as the amount of the extract that produced 50% inhibition of [^{3}H]clonidine (1 nM) binding to porcine cerebral cortex membranes.

Data analysis

All data were expressed as means ± s.e.m. of five to eight experiments. The concentration of the agent causing 50% inhibition of aggregation (IC50) for each curve was obtained from the data using the non-linear curve fitting routines in the Kaleidagraph (Synergy Software) package on a Macintosh computer. The values were processed further for statistical purposes.

The equation used was

$$Y = (m1)(m0^{m2})/(m0^{m2}) + (m3^{m2})$$

where Y was the % inhibition of aggregation, m0 was an independent variable, m1 was the % maximum inhibition, m2 was the slope of the curve, and m3 was the IC50. A curve of best fit was calculated for each agent on % inhibition of noradrenaline-induced aggregation curve using this equation from Kaleidagraph. Significant differences among group means were analysed by analysis of variance. Individual differences between the concentrations were evaluated using Tukey's post hoc test. Differences were considered significant at $P < 0.05$.

Results

Effects of imidazoline agents and non-imidazoline α-adrenergic agents on noradrenaline-induced human platelet aggregation

Noradrenaline (10^{-8}–10^{-4} M) was examined for its ability to induce human platelet aggregation. A concentration close to the EC50 (2 μM) of noradrenaline was chosen to induce platelet aggregation since it produced optimal aggregatory responses in most platelet preparations (data not shown).

All imidazoline and related compounds (including clonidine), in the absence of noradrenaline, failed to affect platelet aggregation. In contrast, almost all imidazoline compounds, irrespective of their activity at α_2-adrenoceptors, were able to inhibit the aggregatory response to noradrenaline (Figure 1). Table 1 shows the concentrations of drugs that inhibited 50% of noradrenaline-induced aggregation (IC50). All values were significantly different from 100% except those of clonidine and antazoline. The results for some agents were omitted from Figure 1 to aid clarity.

The rank order of potencies (based on IC50) of drugs in inhibiting noradrenaline-induced aggregation in human platelets was efaroxan > idazoxan > tolazoline > clonidine ≅ antazoline > yohimbine > rilmenidine > moxonidine. Efaroxan and idazoxan, which are imidazoline α_2-adrenergic antagonists, were approximately 100-and 18-fold, respectively, more potent than the non-imidazoline α_2-adrenergic antagonist, yohimbine. The imidazoline compound antazoline, which is devoid of α_2-adrenergic activity, showed the ability to inhibit noradrenaline-induced platelet aggregation. Rilmenidine and moxonidine were less potent with regard to the inhibitory effect. Agmatine, norharmane and harmane, putative endogenous ligands at imidazoline receptors, did not show any inhibitory effect, except at a high concentration of harmane (10^{-5}–10^{-4} M), which produced only 20% inhibition of noradrenaline-induced platelet aggregation.

Effects of clonidine-displacing substance (CDS) on noradrenaline-induced human platelet aggregation

The CDS-containing extract alone did not show any effect on platelet aggregation (data not shown) whereas, in the

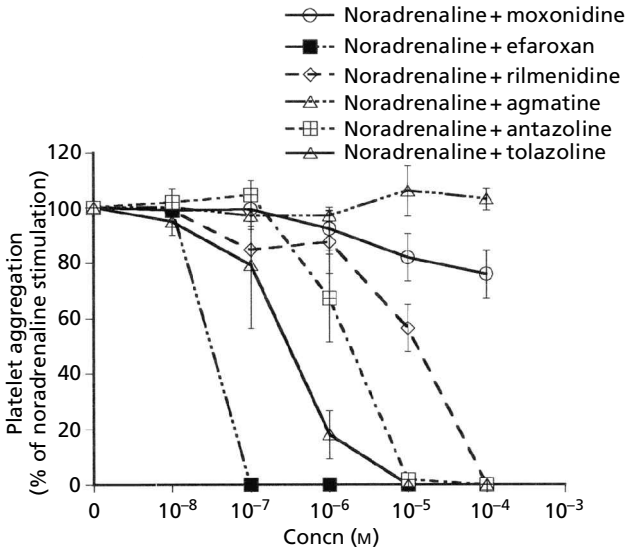

Figure 1 Concentration-related effects of imidazolines and non-imidazoline α-adrenergic agents and putative endogenous ligands at imidazoline receptors on noradrenaline (2 μM)-induced human platelet aggregation. Each agent was added 1 min before addition of noradrenaline. Noradrenaline-induced aggregation (100%) was used as control. The effects of compounds on noradrenaline-induced platelet aggregation are shown as percentages of control. Values shown are means ± s.e.m. of five to eight experiments (two-way analysis of variance, Tukey's post hoc test, $P < 0.05$). To aid the clarity, not all lines are included.

Table 1 Potencies of imidazoline agents and non-imidazoline α-adrenergic agents in inhibiting human platelet aggregation induced by noradrenaline (2 μM). Each value indicates the IC50 (means ± s.e.m.) from five to eight experiments (one-way analysis of variance, Tukey's test, $P < 0.05$).

	Drug	IC50 (• 10^{-8} M)
Imidazoline agents	Efaroxan	3.07 ± 0.20
	Idazoxan	17.4 ± 1.22
	Tolazoline	39.0 ± 4.87
	Clonidine	149 ± 11.14
	Antazoline	177 ± 7.93
	Rilmenidine	1270 ± 64.43
	Moxonidine	> 10 000
Non-imidazoline agent	Yohimbine	319 ± 15.12
Putative clonidine-displacing substances	Agmatine	> 10 000
	Harmane	> 10 000
	Norharmane	> 10 000

presence of noradrenaline, the CDS extract was found to inhibit noradrenaline-induced human platelet aggregation in a concentration-dependent manner (Figure 2). CDS activity, 0.75 U, inhibited the noradrenaline response by 82 ± 4% (n = 8). The addition of yohimbine (10^{-6} M) was unable to reverse but, in fact, significantly potentiated the inhibitory effect of CDS on noradrenaline-induced platelet aggregation.

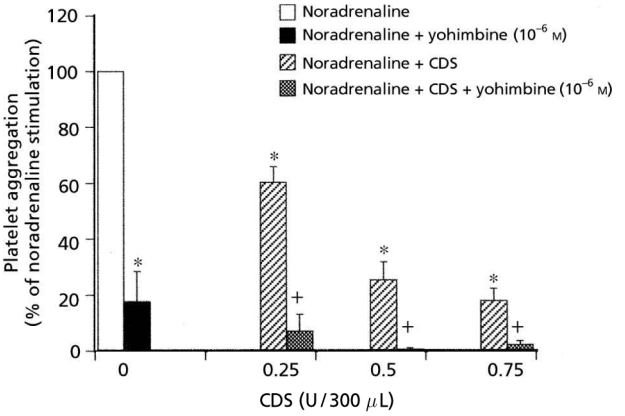

Figure 2 The effects of clonidine-displacing substance (CDS)-containing extract on noradrenaline (2 μM)-induced human platelet aggregation. Noradrenaline alone-induced aggregation = 100%. The effect of yohimbine on noradrenaline alone-induced aggregation and the effects of CDS on noradrenaline-induced aggregation are shown as percentages of noradrenaline response in the absence or the presence of yohimbine. Values shown are means ± s.e.m. of five to eight experiments (one-way analysis of variance, Tukey's post hoc test, $P < 0.05$). *Significant difference from noradrenaline-induced platelet aggregation. $^{+}$Significant difference from CDS effect on noradrenaline-induced platelet aggregation in the absence of yohimbine.

Effects of imidazoline agents and non-imidazoline α-adrenergic agents on ADP-induced human platelet aggregation

ADP (1 μM) was used to induce human platelet aggregation. Clonidine and moxonidine showed similar potentiating effects on ADP-induced platelet aggregation (Figure 3). Rilmenidine showed a slightly smaller potentiating effect. Efaroxan, idazoxan, antazoline, tolazoline, agmatine, harmane and norharmane had no effect.

Discussion

It is known that adrenaline/noradrenaline-induced platelet aggregation in man is mediated by α_2-adrenoceptor stimulation since it is antagonized by α_2-adrenoceptor antagonists, such as yohimbine (Figure 2), phentolamine and idazoxan, but not by α_1-adrenoceptor antagonists such as phenoxybenzamine and prazosin (Grant & Scrutton 1979; Hsu et al 1979; Lasch & Jakobs 1979). In addition, binding studies with radiolabelled adrenoceptor agonists and antagonists have demonstrated the existence of α_2-adrenoceptors on human platelet membranes (Shattil et al 1981; Motulsky et al 1980; Lanza & Cazenave 1985). Despite having no effect alone on platelet aggregation, all of the imidazoline compounds examined in this study inhibited the full platelet aggregation induced by noradrenaline, but some had opposite effects on ADP-induced aggregation. This is in agreement with the previously reported inhibition of catecholamine-induced aggregation by the imidazoline α_2-adrenoceptor agonist

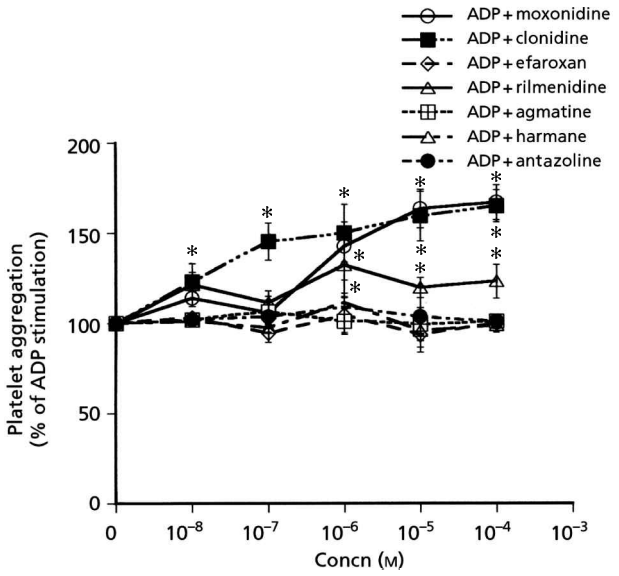

Figure 3 Concentration–effect curves of imidazoline and non-imidazoline α-adrenergic agents on ADP (1 μM)-induced human platelet aggregation. Each agent was added 1 min before addition of ADP. ADP-induced aggregation (100%) was used as control. The effects of imidazoline compounds on ADP-induced platelet aggregation are shown as percentages of control. Values shown are means ± s.e.m. of five to eight experiments. To aid clarity, not all lines are included. *Significant difference from ADP-induced human platelet aggregation (two-way analysis of variance, Tukey's post hoc test, $P < 0.05$).

clonidine (Stump & Macfarlane 1983; Piletz & Sletten 1993).

Based on pK_i values from previous studies, the rank order of affinity of imidazoline and non-imidazoline agents at α_2-adrenoceptors is; RX821002 > idazoxan > efaroxan > clonidine > yohimbine > rauwolscine > KU14R > rilmenidine > moxonidine > 2-BFI > agmatine. The rank order of affinity of imidazoline and non-imidazoline agents at I_1 sites is; moxonidine > agmatine ≅ efaroxan ≅ oxymetazoline > clonidine = rilmenidine > idazoxan, whereas at I_2 sites, 2-BFI ≅ BU-224 > idazoxan > clonidine ≅ rilmenidine > efaroxan ≅ moxonidine ≅ agmatine (as reviewed in Eglen et al (1998)). The rank order of potency of imidazoline and non-imidazoline agents in inhibition of noradrenaline-induced aggregation is; efaroxan > idazoxan > tolazoline > clonidine ≅ antazoline > yohimbine > rilmenidine > moxonidine, which is similar to the rank order of affinity of these ligands at α_2-adrenoceptors. The potency of imidazoline and non-imidazoline agents in potentiation of ADP-induced aggregation was not fitted with any rank order. In this case only moxonidine, clonidine and rilmenidine potentiated ADP-induced aggregation.

Although the rank order of potency is close to the related affinities of these ligands at the α_2-adrenoceptor, these effects are not likely to be mediated through α_2-adrenoceptors. This study found that antazoline, an imidazoline compound devoid of α_2-adrenoceptor activity, inhibited noradrenaline-induced platelet aggregation. This suggested that antazoline interacted with non-α_2-adrenoceptor sites on platelets. Although agents which possessed α_2-adrenoceptor antagonistic activity (efaroxan, idazoxan) inhibited platelet aggregation (but with 100- and 18-fold potency compared with yohimbine), agents that possessed α_2-adrenoceptor agonist activity showed similar effects (clonidine). Given the similarity of the responses to agonists and antagonists it is difficult to see how the α_2-adrenoceptor could be the mediator of the responses. By implication, the other imidazoline compounds could have a similar action. It is attractive to speculate that the binding sites mediating these non-adrenoceptor effects might be the I_1 or I_2 imidazoline receptors which have been identified on human platelets (Piletz et al 1991; Piletz & Sletten 1993). However, the subtype involved is likely to be an 'atypical' non-adrenoceptor imidazoline binding site since the effect on noradrenaline-induced aggregation showed that the more selective I_1 ligands (moxonidine, rilmenidine) produced less inhibitory effects but the converse was the case for ADP-induced aggregation; clonidine, moxonidine and rilmenidine potentiated ADP-induced aggregation.

The mechanism by which putative imidazoline receptors might interact with α_2-adrenoceptors to inhibit human platelet aggregation remains to be clarified. The general sequence of events following exposure to an aggregating agent is shape change, appearance of fibrinogen receptors, aggregation and release of dense granules (resulting in enhanced activation due to released ADP) and α-granules (resulting in enhanced stabilization of the fibrinogen bridges between platelets and irreversible aggregation). The platelet α-adrenoceptors have been characterized pharmacologically as α_2-adrenoceptors, which is in keeping with their affinity for blood-borne catecholamines and their inhibitory coupling to adenylyl cyclase, although a decrease in cyclic AMP alone may not be the only cause of aggregation (Cusack & Hourani 1982). Stimulatory agonists, in general, act via the phospholipase C pathway to generate the second messengers Ca^{2+} and diacylglycerol, whereas inhibitory agonists act via stimulation of adenylyl cyclase to generate the second messenger cyclic AMP. Increases in cyclic AMP levels inhibit and reverse the increases in cytoplasmic Ca^{2+} caused by aggregating agents (Feinstein et al 1983). However, there is no direct evidence for imidazoline receptor occupation leading to activation of the cyclic AMP signalling pathway. On the contrary, a previous report on human platelets showed that two clonidine derivatives (KUM32 and CBS1276) inhibited platelet adenylyl cyclase through non-α_2-adrenoceptor mechanisms, since their effects were not blocked by the α_2-adrenoceptor antagonist, yohimbine (Ferry et al 1986). The results of studies on imidazoline receptor signal transduction have been rather controversial. For example, in studies involving chromaffin cells activated by I_1 imidazoline agonists, there were no effects on the accumulation of cAMP, cGMP or inositol phosphates (Regunathan et al 1991). In contrast, other studies have shown that binding to I_1 imidazoline sites is sensitive to guanine but not to adenine nucleotides, implying coupling to a G-protein. This has been described in

various tissues and cell lines, including human platelets (Jin & Kunapuli 1998; Ernsberger et al 1995). More recently, Greney et al (2000) suggested that I_1-agonists lowered cAMP levels. However, decrease of the cAMP level is not the only mechanism involved in platelet aggregation since previous studies found that an inhibitor of adenylyl cyclase did not potentiate platelet agonist-induced aggregation and secretion (Haslam et al 1978). The interactions between the signalling pathway activated by imidazoline agents and catecholamines clearly remain to be clarified.

The concentration of adrenaline/noradrenaline needed to induce platelet aggregation in-vitro is in the micromolar range, considerably higher than the highest concentrations ever likely to be found in the circulation (nanomolar range). This might suggest that aggregation induced by adrenaline/noradrenaline is unlikely to have any significance in-vivo, especially because it only occurs at reduced extracellular Ca^{2+} concentrations (Glusa & Markwardt 1980). However, the synergistic effects of other pro-aggregatory agonists, such as 5-HT and vasopressin with noradrenaline (Culliver & Ardlie 1981), bring the required adrenaline/noradrenaline concentration within a plausible range and probably reflect the physiological situation in blood more accurately. This synergistic effect implies that circulating adrenaline/noradrenaline has a role in sensitizing platelets, which can be influenced by imidazoline drugs.

The imidazoline compounds had a quite different effect on ADP-induced aggregation. Moxonidine behaved like clonidine and, to a lesser extent, rilmenidine, in potentiating ADP-induced aggregation. ADP initiates aggregation by simultaneous activation of three purinoceptors: P2Y1, coupled to Gq, mediates ADP-induced shape change, phospholipase C activation and calcium flux (Jin et al 1998); P2Y12, which signals through Gi and inhibits adenylyl cyclase activity (Jin & Kunapuli 1998); and the $P2X_1$ receptor, which mediates rapid Ca^{2+} influx (Vial et al 1997). It is known that ADP causes increases in intracellular Ca^{2+}, although these seem to be via influx, rather than a mobilization from internal stores, due to activation of receptor-operated Ca^{2+} channels and not any second-messenger system (Sage & Rink 1987). It is not known whether the imidazoline compounds have a positively modulating effect on purinoceptor-mediated Ca^{2+} influx.

The inability of some of the other imidazolines to potentiate ADP-induced aggregation suggested heterogeneity within the binding sites mediating the inhibitory and potentiating responses to noradrenaline and ADP, respectively. This is supported by the lack of effect of agmatine, harmane and norharmane on either noradrenaline or ADP-induced platelet aggregation, although they have been shown to have significant affinities for imidazoline receptors (Hudson 1999; Molderings et al 1999). Further studies using selective imidazoline receptor ligands, such as 2-BFI or BU224 (Hudson et al 1999), would be interesting.

A porcine brain extract containing clonidine-displacing substance (CDS), a putative endogenous ligand of imidazoline receptors, was found to inhibit noradrenaline induced platelet aggregation, like the imidazoline compound. CDS displayed a marked, concentration-dependent effect on noradrenaline-induced platelet aggregation, 0.75 U of the extract reducing aggregation by more than 80% (Figure 2). The results from this study were partly consistent with those of Diamant et al (1987), who used HPLC-purified bovine CDS. However, this study showed that the effect of CDS was not likely to be due to α_2-adrenoceptor activation, as suggested by Diamant et al (1987), since yohimbine did not reverse its inhibitory effect. On the contrary, it potentiated the anti-aggregatory effect of CDS (Figure 2). In addition, the CDS preparation from our laboratory was found to inhibit 1 μM ADP-induced human platelet aggregation in a concentration-dependent manner and the effect was not reversed by yohimbine (data not shown). This is somewhat different from the findings of Diamant's group; their CDS inhibited adrenaline-induced human platelet aggregation but potentiated ADP-induced aggregation, as did clonidine. However, their effects were found to be via α_2-adrenoceptors, since they were reversed by yohimbine (Diamant et al 1987).

Agmatine, harmane and norharmane have been identified as putative clonidine-displacing substances (Li et al 1994; Hudson 1999), but their inability to show any effect on noradrenaline-induced platelet aggregation, in contrast to the CDS extract, casts some doubt upon this identity. There are several lines of evidence that showed that the proposed endogenous ligands lacked efficacy in some models or only produced effects at very high concentrations, although they did have high binding affinity for the receptors (Eglen et al 1998). CDS, however, has been found in human serum (Synetose et al 1991) and it may have a regulatory role in inhibiting noradrenaline-induced platelet aggregation and preventing vascular occlusions.

Conclusion

Imidazoline compounds displayed complex effects on human platelet aggregation, inhibiting responses to noradrenaline and potentiating ADP stimulation. These effects might have been mediated by an 'atypical' non-adrenoceptor imidazoline binding site. The results suggested that imidazoline compounds could have a regulatory role in preventing platelet hyper-reactivity induced by catecholamines. In addition, CDS might be an endogenous modulator, preventing this hyper-reactive condition.

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