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THE EFFECTS OF MONOVALENT AND DIVALENT CATIONS ON THE α-ADRENOCEPTOR OF INTACT HUMAN PLATELETS

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1 We have examined the effects of monovalent and divalent cations and purine nucleotides on the binding of agonists and antagonists to the α-adrenoceptor of intact human platelets.

2 Replacement of Na^+ (150 mM) by NH_4^+ (150 mM) in the incubation medium significantly reduced the binding affinity of [^{3}H]-dihydroergocryptine ($P<0.05$) but did not alter the binding capacity. The competitive binding affinity of adrenaline and noradrenaline was unaltered.

3 The addition of Ca^{2+} (1 mM) or Mg^{2+} (1 mM) to the platelet suspension significantly reduced the platelet α-adrenoceptor capacity as indicated by either [^{3}H]-dihydroergocryptine ($P<0.05$) or [^{3}H]-yohimbine ($P<0.01$; Ca^{2+} only).

4 The addition of Ca^{2+} (1 mM) or Mg^{2+} (1 mM) had no effect on the binding affinity of [^{3}H]-dihydroergocryptine but significantly reduced that of [^{3}H]-yohimbine ($P<0.05$). The competitive affinity of adrenaline and noradrenaline determined by inhibition of [^{3}H]-dihydroergocryptine binding, was unchanged in the presence of either cation.

5 Addition of the purine nucleotides ADP, ATP, GDP or GTP (final concentration 10 μM), either alone or in the presence of 1 mM Ca^{2+} or 1 mM Mg^{2+}, had no effect on the binding of [^{3}H]-dihydroergocryptine or on the competitive affinity of adrenaline or noradrenaline.

6 We conclude that the α-adrenoceptor of intact human platelet displays the binding characteristics of the α_{2_L} form of the receptor previously identified in the platelet lysate preparation.

Introduction

The modulation of receptor binding affinity by monovalent and divalent cations and by purine nucleotides has been described for a number of receptors (Pert & Snyder, 1974; Maguire, Van Arsdale & Gilman, 1976; U'Prichard & Snyder, 1978; Peroutka, Lebovitz & Snyder, 1979). In the human platelet lysate preparation the affinity of adrenaline for the α-adrenoceptor is increased by the presence of Mg^{2+} and subsequently decreased by the addition of guanosine triphosphate (GTP) or its imido-analogue guanyl-5′-yl-imidodiphosphate (Gpp(NH)p) (Tsai & Lefkowitz, 1979). Similar changes have been reported in the rabbit platelet lysate preparation, though in addition the affinity of adrenaline is reduced by the presence of Na^+ ions (Tsai & Lefkowitz, 1978; Michel, Hoffman & Lefkowitz, 1980).

Using the labelled α-adrenoceptor antagonists [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine, we have previously described the binding characteristics of the α-adrenoceptor on intact human platelets, (Boon, Elliott, Grahame-Smith, Outlaw & Stump, 1981; Elliott & Grahame-Smith, 1982). In most respects the properties of the platelet α-adrenoceptor observed in intact cells match those reported for the lysate preparation (Newman, Williams, Bishopric & Lefkowitz, 1978; Alexander, Cooper & Handin, 1978; Motulsky, Shattil & Insel, 1980; Daiguchi, Meltzer & U'Prichard, 1981). The outstanding exception in this comparison is the difference in affinity of agonists in the two systems. The studies in intact cells indicate an affinity for adrenaline which is more than ten fold lower than that reported in the lysate preparation. Previous studies showed that this difference was not due to the metabolism or uptake of adrenaline by the intact platelets (Elliott & Grahame-Smith, 1982).

We have therefore investigated the effects of various monovalent and divalent cations and purine nucleotides on the binding characteristics of the α-adrenoceptor in intact human platelets in an attempt to resolve this discrepancy. A preliminary report of this study was presented to the British Pharmacological Society (Elliott & Grahame-Smith, 1981).

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Methods

Tissue preparation

Blood was taken using a 19-gauge needle from the antecubital vein of healthy, young, male volunteers and anticoagulated by addition of nine volumes of blood to one volume of disodium edetate (EDTA) (1% w/v) in isotonic saline. Platelet-rich plasma was prepared by centrifugation of the blood at 180 *g* for 12 min at 20°C. The platelets were then separated from the plasma by further centrifugation at 1700 *g* for 7 min at 10°C and resuspended in the incubation medium by repeated vortex mixing. This process of centrifugation and resuspension was then repeated. The incubation medium normally comprised 2.5 mM disodium EDTA, 150 mM NaCl, pH 7.5 except for the study using sodium-free medium when this was replaced by 2.5 mM EDTA free acid, 150 mM NH_4Cl fixed at pH 7.5 using NH_4OH. Platelet cell density was routinely estimated by means of a Coulter Counter Industrial-D model and was always in the range $0.6-1.0 \times 10^8$ cell/ml.

Radioligand binding assay

Intact human platelets were incubated with [^{3}H]-dihydroergocryptine or [^{3}H]-yohimbine for 20 min at 37°C as described previously (Boon *et al.*, 1981; Elliott & Grahame-Smith, 1982). Specific binding was identified as that inhibited by 5 μM phentolamine and constituted 20–30% total binding in the case of [^{3}H]-dihydroergocryptine and 60–80% total binding for [^{3}H]-yohimbine. Binding characteristics of each radioligand were determined by Scatchard analysis of specific binding observed over the range 1–15 nM free concentration. Each incubate was replicated four fold for both total and non-specific binding within each assay.

The binding affinities of adrenaline and noradrenaline were determined from the inhibition of total and non-specific binding of [^{3}H]-dihydroergocryptine at 5 nM concentration. Each compound was assayed at between four and eight concentrations in order accurately to determine the IC_{50} value.

The effects of divalent cations on radioligand binding characteristics were determined by parallel studies of a control sample, Ca^{2+} addition and Mg^{2+} addition all assayed simultaneously on platelets obtained at one venepuncture. This study was repeated six times in the case of [^{3}H]-dihydroergocryptine and four times for [^{3}H]-yohimbine. The divalent cations were added at the beginning of the incubation to a final concentration of 1 mM in the presence of 2.5 mM EDTA. This contrasts with the investigation of monovalent cations where the platelets were resuspended in a medium containing either Na^+ or NH_4^+. The binding characteristics of [^{3}H]-dihydroergocryptine were then determined for four subjects examined in the Na^+ and NH_4^+ media on different occasions.

Analysis of data

The equilibrium binding characteristics of [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine were calculated from Scatchard analysis of the specific binding data according to the method of least squares linear regression. Comparisons between the binding characteristics of platelets resuspended in Na^+ and NH_4^+ salts were made on the basis of Student's unpaired *t* test. Comparisons between the binding characteristics of platelets resuspended in the normal incubation medium, for both [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine, and those to which Ca^{2+} or Mg^{2+} had been added were determined on the basis of Student's paired *t* test.

Probit analysis was used to linearise the competitive inhibition curves for adrenaline and noradrenaline and obtain an estimate of the IC_{50}. The binding affinity for each compound was then calculated from

$$K_i = \frac{IC_{50}}{1 + \frac{[D]}{K_d}}$$

(Cheng & Prusoff, 1973), where IC_{50} = concentration producing 50% inhibition of specific binding of [^{3}H]-dihydroergocryptine; [D] = free concentration of [^{3}H]-dihydroergocryptine; and K_d = dissociation equilibrium binding constant for [^{3}H]-dihydroergocryptine on intact human platelets in the same incubation medium.

The Hill coefficient (n_H) for each inhibition curve was calculated from the slope of the Hill curve where the ordinate represented log (% inhibition/100% − % inhibition) and the abscissa log (Agonist concentration).

Drugs

[^{3}H]-dihydroergocryptine (specific activity 25.7–38.8 Ci/mmol) and [^{3}H]-yohimbine (specific activity 82.6–84.4 Ci/mmol) were obtained from New England Nuclear Ltd.

Phentolamine hydrochloride was donated by Ciba-Geigy Ltd., (−)-adrenaline bitartrate, (−)-noradrenaline bitartrate, adenosine-5′-diphosphate (ADP), adenosine-5′-triphosphate (ATP), guanosine-5′-diphosphate (GDP) and guanosine-5′-

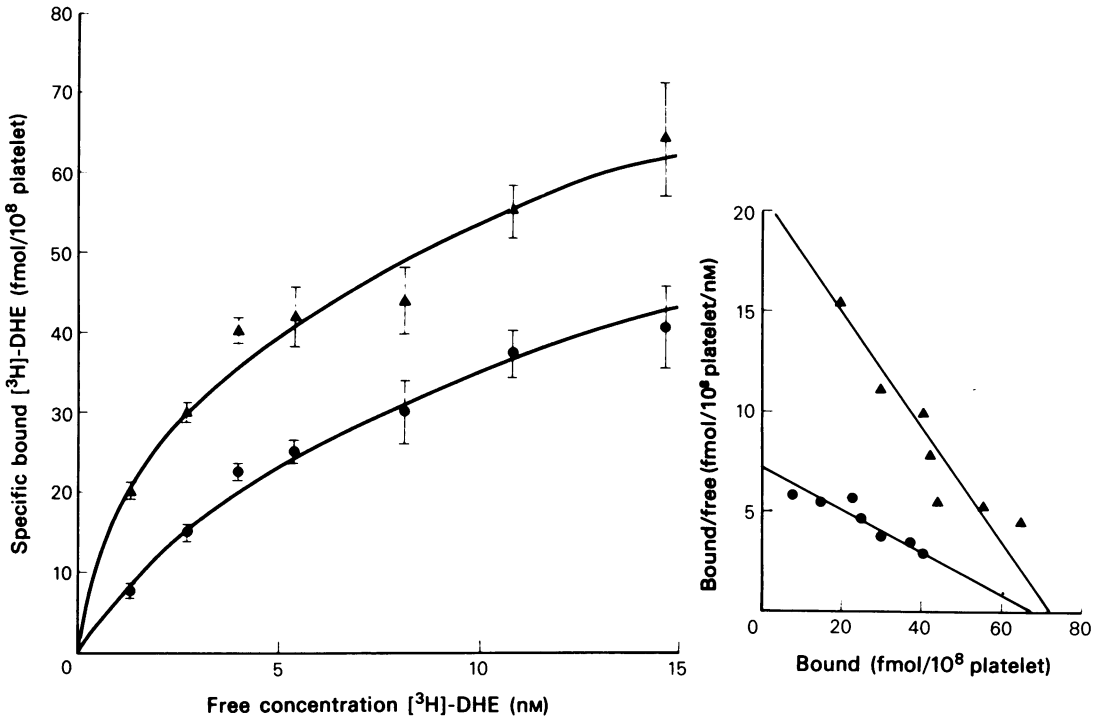

Figure 1 Effects of Na^+ and NH_4^+ on the binding characteristics of [^{3}H]-dihydroergocryptine ([^{3}H]-DHE). Intact human platelets were resuspended in 2.5 mM EDTA and 150 mM NaCl (▲) or 150 mM NH_4Cl (●) as described in the text and incubated with [^{3}H]-DHE (range 1–15 nM) for 20 min at 37°C. Each point represents the mean from 4 separate experiments; vertical lines indicate s.e.mean. Inset is a Scatchard plot of the same data.

triphosphate (GTP) were obtained from Sigma London Chemical Company. All inorganic compounds were obtained from BDH Chemicals Ltd. and were of analytical reagent grade.

Results

Effects of Na^+ and NH_4^+ on [^{3}H]-dihydroergocryptine binding

The substitution of NH_4^+ for Na^+ in the incubation medium did not alter the binding capacity of [^{3}H]-dihydroergocryptine on intact human platelets but significantly ($P<0.05$) reduced the radioligand affinity (Figure 1), indicated by an increase in the value of the equilibrium dissociation constant (K_d) from 3.45 nM to 9.16 nM (Table 1).

Inhibition of [^{3}H]-dihydroergocryptine binding to intact human platelets by adrenaline and noradrenaline was tested in both the Na^+ and NH_4^+ incubation media. The affinities of both agonists were lower in the NH_4^+ than Na^+ media, but these differences were not statistically significant (Table 1). Analysis of these inhibition curves according to the Hill plot indicated a gradient close to unity in all cases.

Effects of Ca^{2+} and Mg^{2+} on the α-adrenoceptor binding characteristics of intact human platelets

Addition of Ca^{2+} or Mg^{2+} to the normal incubation medium caused a concentration-dependent reduction in both specific and non-specific binding of [^{3}H]-dihydroergocryptine to intact human platelets. At a final estimated concentration of 0.5 mM neither Ca^{2+} nor Mg^{2+} caused any significant change in the affinity or binding capacity of [^{3}H]-dihydroergocryptine.

In the presence of 1 mM Ca^{2+} or Mg^{2+} the affinity of [^{3}H]-dihydroergocryptine was unchanged but the binding capacity was reduced, Ca^{2+} being more potent than Mg^{2+} (Figure 2). This effect was observed in each of the six subjects studied and proved significantly different ($P<0.05$) from the control studies for both Ca^{2+} and Mg^{2+} (Table 2).

When [^{3}H]-yohimbine was used to label the α-adrenoceptors a similar decrease in binding capacity was observed for both divalent cations (Figure 3), although the decrease caused by Mg^{2+} was not statis-

Table 1 Binding characteristics of [^{3}H]-dihydroergocryptine ([^{3}H]-DHE) and binding affinity of adrenaline and noradrenaline on intact human platelets resuspended in Na^+ or NH_4^+ based incubation media

EDTA (2.5 mM) incubation medium containing	*[^{3}H]-DHE binding characteristics* *Affinity (K_d) (nM)*	*Capacity (B_{max}) (fmol/10^8 platelet)*
Na^+ (150 mM) ($n=4$)	3.45 ± 0.60	72 ± 6
NH_4^+ (150 mM) ($n=4$)	9.16 ± 1.56*	68 ± 7

	Competitive binding affinity *Adrenaline*		*Noradrenaline*	
	K_i (μM)	n_H	K_i (μM)	n_H
Na^+ (150 mM) ($n=9$)	4.1 ± 0.4	1.03 ± 0.08	14.9 ± 2.0	0.97 ± 0.10
NH_4^+ (150 mM) ($n=5$)	6.9 ± 1.7	0.97 ± 0.09	20.3 ± 3.7	0.99 ± 0.12

All values shown indicate mean ± s.e.mean from n determinations. All comparisons between results in Na^+ and NH_4^+ media are not statistically significant except *$P<0.05$.

tically significant. However, unlike [^{3}H]-dihydroergocryptine, the affinity of [^{3}H]-yohimbine for the platelet α-adrenoceptor was significantly reduced by the presence of both Ca^{2+} and Mg^{2+} (Table 2).

The affinity of adrenaline in the presence of 1 mM Ca^{2+} ($K_i = 4.3$ μM) and Mg^{2+} ($K_i = 3.7$ μM) and noradrenaline in the presence of 1 mM Ca^{2+} ($K_i = 14.0$ μM) and Mg^{2+} ($K_i = 12.0$ μM), as determined by inhibition of [^{3}H]-dihydroergocryptine binding, were similar to the values observed in the standard EDTA/saline medium. Similarly the Hill coefficient for each of the agonists in the presence of either Ca^{2+} or Mg^{2+} was close to unity. These values represent the means of two determinations, each of which differed by less than 10% from their mean.

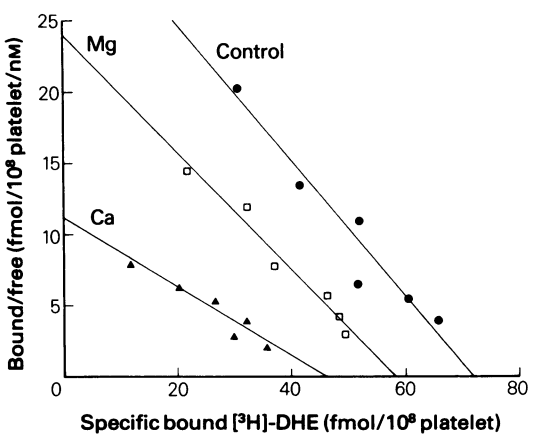

Figure 2 Effect of Ca^{2+} and Mg^{2+} on the binding characteristics of [^{3}H]-dihydroergocryptine ([^{3}H]-DHE) on intact human platelets. Final concentration of Ca^{2+} or Mg^{2+} was 1 mM in the presence of 2.5 mM EDTA. Results shown represent Scatchard analysis of the specific binding in a single subject and are typical of 6 such experiments.

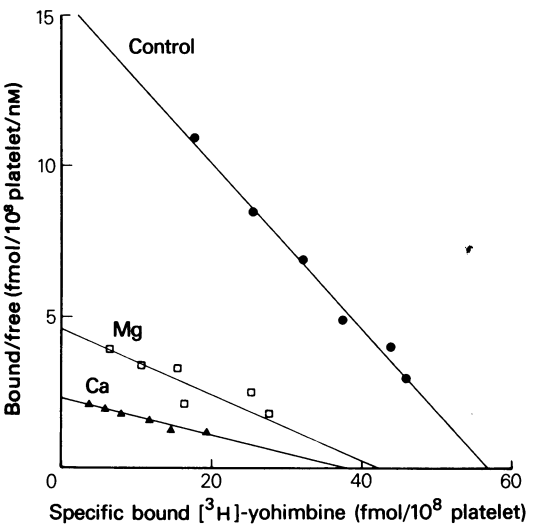

Figure 3 Effect of Ca^{2+} and Mg^{2+} on the binding characteristics of [^{3}H]-yohimbine on intact human platelets. Final concentration of Ca^{2+} or Mg^{2+} was 1 mM in the presence of 2.5 mM EDTA. Results shown represent Scatchard analysis of the specific binding in a single subject and are typical of 4 such experiments.

Table 2 Effect of Ca^{2+} and Mg^{2+} on the binding characteristics of [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine on intact human platelets

	[^{3}H]-dihydroergocryptine	
	K_d (nM)	B_{max} (fmol/10^8 platelet)
Contol	2.66 ± 0.27	76 ± 7
Ca^{2+} (1 mM)	3.61 ± 0.24	52 ± 5*
Mg^{2+} (1 mM)	3.25 ± 0.27	59 ± 6*

*$P < 0.05$ t-paired v control; $n = 6$

	[^{3}H]-yohimbine	
	K_d (nM)	B_{max} (fmol/10^8 platelet)
Control	3.37 ± 0.15	54 ± 7
Ca^{2+} (1 nM)	10.73 ± 1.05**	28 ± 8**
Mg^{2+} (1 mM)	11.30 ± 1.65*	45 ± 7

*$P < 0.05$
**$P < 0.01$ t-paired v control; $n = 4$

All values indicate mean ± s.e.mean from n determinations.

Effect of purine nucleotides alone and in conjunction with divalent cations

Addition of the adenine nucleotides ADP and ATP (final concentration 10 μM) did not affect the binding of [^{3}H]-dihydroergocryptine to intact human platelets and did not alter the binding affinity of adrenaline or noradrenaline.

Similarly the guanine nucleotides GDP and GTP (final concentration 10 μM) were without effect on the binding of [^{3}H]-dihydroergocryptine or on the affinities of adrenaline and noradrenaline, whether tested alone or in the presence of 1 mM Ca^{2+} or Mg^{2+}.

Discussion

Unlike the platelet lysate preparation, these results demonstrate that the binding affinity of agonists for the α-adrenoceptor of intact human platelets is not altered by the presence of Na^+ or Mg^{2+} ions or guanine nucleotides. In both human and rabbit platelet lysates it has been shown that Mg^{2+} increases the binding affinity of agonists for the α-adrenoceptor and that subsequent addition of GTP reduces the apparent agonist affinity (Tsai & Lefkowitz, 1979; Michel *et al.*, 1980). This decrease in affinity is accompanied by an increase in the Hill coefficient of the inhibition curve from a value significantly less than unity to approximately 1.0. Consequently it has been proposed that the α_2-adrenoceptor may exist in two forms, one with a high affinity for agonists (α_{2H}) and the other with a lower agonist affinity (α_{2L}), the conversion of α_{2H} to α_{2L} being mediated by GTP (Hoffman, Mullikin-Kilpatrick & Lefkowitz, 1980; Lynch & Steer, 1981). The affinity of α-adrenoceptor agonists on intact human platelets as described here differs considerably from the values first identified in the platelet lysate preparation (Newman *et al.*, 1978; Alexander *et al.*, 1978) but agree closely with the values more recently determined for the α_{2L} sites (Hoffman *et al.*, 1980; Michel *et al.*, 1980). Furthermore the Hill coefficient for inhibition by these agonists in intact cells is close to unity, suggesting a single class of agonist binding sites unlike the mixture of α_{2H} and α_{2L} sites in the simple lysate preparation. It appears, therefore, that the binding characteristics of the α-adrenoceptor of intact human platelets resemble those of a homogeneous population of α_{2L} receptors and that this may account for the discrepancy in the agonist affinities between the intact and broken cell preparations. The existence of the platelet α-adrenoceptor in the α_{2L} form in the intact cell suggests that this is the dominant form of the receptor *in vivo* and questions the significance of investigations of the α_{2H} form of the receptor as labelled by α-adrenoceptor agonists.

The presence or absence of Na^+ has little effect on the affinity of α-adrenoceptor agonists on intact human platelets, whereas in the rabbit platelet lysate Na^+ reduces agonist affinity by a mechanism quite distinct from that of GTP (Tsai & Lefkowitz, 1978; Michel *et al.*, 1980). The affinity of [^{3}H]-dihydroergocryptine, however, was significantly reduced by replacement of Na^+ by NH_4^+ ions. This may be due to a direct action of Na^+ on the α-adrenoceptor or to some indirect effect of Na^+ removal, such as the destruction of the membrane potential as also reported to affect the α-

adrenoceptor binding characteristics of intact rat parotid cells (Strittmatter, Davis & Lefkowitz, 1977).

The reduction in α-adrenoceptor capacity of intact platelets by Ca^{2+} or Mg^{2+} has not been observed in platelet membrane preparations, suggesting that this effect may also depend on the functional integrity of the cell. Divalent cations have been reported to affect platelet morphology (Born, Dearnley, Foulks & Sharp, 1978) and our own preliminary studies indicate a correlation between the concentration of Ca^{2+} or Mg^{2+} inducing shape-change and that affecting the α-adrenoceptor capacity. Further investigation of this relationship is currently in progress.

The differential effects of divalent cations on the affinities of [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine on intact human platelets suggest possible differences in the binding sites associated with each ligand. In the platelet lysate preparation Mg^{2+} does not affect the binding of [^{3}H]-dihydroergocryptine (Tsai & Lefkowitz, 1979) whereas it reduces the binding affinity of [^{3}H]-yohimbine (Daiguchi *et al.*, 1981). In intact platelets we have described a consistent difference in the α-adrenoceptor binding capacities identified by [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine in some individuals (Boon *et al.*, 1981) and a similar discrepancy has been reported in platelet lysates (Daiguchi *et al.*, 1981). One interpretation of these data would be that [^{3}H]-yohimbine and [^{3}H]-dihydroergocryptine label different sites. However, both ligands display inhibition profiles typical of the α_2-adrenoceptor, indeed the affinities of many compounds are identical when determined by each ligand (Boon & Elliott, unpublished observations). An alternative explanation is that the binding sites for [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine on the α-adrenoceptor are differentially sensitive to divalent cations, although this does not explain the difference in binding capacity. Further analysis of these discrepancies is currently under investigation and caution is indicated when extrapolating results of [^{3}H]-dihydroergocryptine binding to that of [^{3}H]-yohimbine and *vice versa*.

In conclusion, the binding affinity of agonists for the α-adrenoceptor of intact human platelets, as indicated by [^{3}H]-dihydroergocryptine, resemble that of the α_{2L} form of the receptor identified from subcellular studies and is not altered by the presence of Na^+ or Mg^{2+} ions or GTP. The presence of divalent cations reduces the capacity of both tritiated ligands on intact cells whereas the affinity of [^{3}H]-yohimbine is reduced without effect on the affinity of [^{3}H]-dihydroergocryptine. This discrepancy in the binding characteristics of [^{3}H]-dihydroergocryptine and [^{3}H]-yohimbine on intact human platelets remains unresolved.

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