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Further insights into the anti-aggregating activity of NMDA in human platelets

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1 In the present study the effect of N-methyl-D-aspartate (NMDA) on thromboxane B_2 synthesis and on $[Ca^{2+}]_i$ was studied in human platelets.

2 NMDA (10^{-7} M) completely inhibited the synthesis of thromboxane B_2 from exogenous arachidonic acid (AA), while it did not interfere with the aggregating effect of the thromboxane A_2 receptor agonist U-46619.

3 NMDA (0.1 μM – 10 μM) dose-dependently increased intracellular calcium in washed platelets pre-loaded with fura 2 AM, and this effect was not additive with that of AA.

4 NMDA shifted the dose-response curve of AA to the right. At the highest AA concentrations platelet aggregation was not inhibited.

5 The antiaggregating effect of NMDA was not antagonized by N^G-monomethyl-L-arginine (L-NMMA), a nitric oxide synthase (NOS) inhibitor.

6 Finally, NMDA (0.01 nM – 100 nM) associated with either aspirin or indomethacin significantly potentiated the antiaggregating activity of both cyclo-oxygenase inhibitors.

7 It was concluded that NMDA is a potent inhibitor of platelet aggregation and thromboxane B_2 synthesis in human platelet rich plasma (PRP).

Keywords: Platelets; aggregation; glutamate; NMDA receptors; thromboxane; arachidonic acid; $[Ca^{2+}]_i$

Introduction

It was recently found that glutamate exerts an antiaggregating effect on human platelets exposed to arachidonic acid (AA), adenosine 5′-diphosphate (ADP) or platelet activating factor (PAF), through the activation of N-methyl-D-aspartate (NMDA) receptors (Franconi *et al*., 1996). NMDA receptors were initially described and characterized in the central nervous system (CNS), but recently their presence and role have also been studied in peripheral organs of animals (Erdo, 1991) and man (Franconi *et al*., 1996). In fact, they have been localized in the mesenteric plexus (Moroni *et al*., 1986) and the ileal longitudinal muscle of guinea-pigs (Luzzi *et al*., 1988), as well as in the adrenal glands (Yoneda & Ogita, 1986) and the peripheral cholinergic system of rat bronchial smooth muscles (Aas *et al*., 1989). Moreover, it has been shown that NMDA receptor activation mediates both the release of insulin and of glucagon from rat pancreas (Bertrand *et al*., 1992; 1993) and the glutamate induced increase of $[Ca^{2+}]_i$ in rat cardiac myocytes (Winter & Baker, 1995).

Peripheral NMDA receptors seem to have some functional characteristics different from those considered as paradigmatic of CNS receptors. For instance, in human platelets the presence of glutamate or glycine in the medium, either alone or in association, does not modify the binding of [^{3}H]-MK-801 (Franconi *et al*., 1996), while in neuronal membranes such binding is potentiated by either glutamate or glycine (Foster & Wong, 1987; Kemp & Leeson, 1993), being maximal in the presence of both. Moreover, the antiaggregating effect of NMDA is antagonized by unusually high concentrations of MK-801, a non-competitive NMDA receptor inhibitor (Wong *et al*., 1986), while the competitive antagonist (AP5) is ineffective (Franconi *et al*., 1996). Thus, it was concluded that, relative to neurones, platelet NMDA receptors have an agonist-preferring state conformation (Franconi *et al*., 1996).

The antiaggregating effect of glutamate and NMDA observed in human platelet rich plasma (PRP) seems related to a calcium dependent adenylyl cyclase stimulation. In fact, NMDA increases adenosine 3′:5′-cyclic monophosphate (cyclic AMP) levels in intact platelets only in the presence of calcium in the medium, and such an effect is not antagonized by calmodulin inhibition with trifluoperazine (Franconi *et al*., 1996). Thus, considering the role played by Ca^{2+} in the process of platelet aggregation, it was essential to verify the effect of NMDA receptor stimulation on $[Ca^{2+}]_i$ in platelets. Moreover, since many effects of NMDA in the CNS are mediated by nitric oxide (NO) (Bredt & Snyder, 1989; Schuman & Madison, 1994), the effect of NO synthase (NOS) inhibition on NMDA antiaggregating activity was investigated. Finally, since the pattern of inhibition of NMDA on platelet aggregation appears compatible with that of a compound which interferes with the thromboxane A_2 (TxA_2) pathway, its effect on the aggregation induced by U-46619, a stable TxA_2 mimetic, and on thromboxane synthesis was evaluated. We also considered the possible theoretical and clinical importance of the study of NMDA antiaggregating activity in association with classical cyclo-oxygenase inhibitors.

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Methods

Preparation of platelets

PRP was prepared according to Franconi *et al*. (1995). In brief: blood was drawn from the antecubital vein of healthy volunteers who had taken no medication for at least two weeks before blood collection. Blood was gently mixed in 3.8% trisodium citrate (9 : 1 v/v ratio), and PRP was obtained by 800 r.p.m. centrifugation for 10 min at room temperature. The residual plasma was further centrifuged at $3000 \times g$ for 10 min to yield platelet poor plasma (PPP). The platelet count was adjusted to about 280,000 cell μl^{-1} by the addition of PPP.

Aggregation procedure

Aggregation was measured turbidimetrically according to Born and Cross (1963) by use of a Daichii PA-3220 apparatus (Kyoto, Japan). To express the aggregation of platelets and the inhibitory action exerted by the drugs, the absorption of PRP itself was set at 0%, while that of PPP was set at 100% (Franconi *et al*., 1996). The aggregating agent used was the sodium salt of arachidonic acid (AA) at the concentrations presented in the figures and tables. Aggregation was quantified by measuring the peak increase, the maximum rate of increase in light transmission (slope) and the lag-time after the addition of AA. A dose-response curve for AA was performed before each experiment in order to determine the EC_{50}. Since in our experience the antiaggregating effect of NMDA does not vary within a 5–40 min interval, non-cumulative concentration-response-curves for AA were started 10 min after the addition of the amino acid to the medium, and aggregation was monitored during the following 6 min. At the end of each experiment AA induced aggregation was carried out again and if results differed by more than 10% with respect to initial values, the experiment was discarded.

The same experimental conditions were used to study U-46619-induced aggregation.

In a second set of experiments PRP was preincubated for 15 min with 1 mM N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS (Radomski *et al*., 1990) and further incubated for 10 min with NMDA then AA, at the final concentration corresponding to the respective EC_{50}, was added.

Finally, in order to study a possible interaction of NMDA with other antiaggregating agents, two classic inhibitors of cyclo-oxygenases, i.e. acetyl salicyclic acid (ASA) and indomethacin (Insel, 1995), were used. A preliminary dose-response curve for each compound, incubated for 10 min, was carried out with 1 mM AA as agonist, and the respective antiaggregating EC_{50} concentrations were evaluated. The EC_{50} of both compounds have been used in association with NMDA on aggregation induced by 1 mM AA. PRP was preincubated for 10 min with different NMDA concentrations (0.01 nM – 100 nM); then it was incubated with cyclo-oxygenase inhibitors for 10 min before the addition of 1 mM AA.

TxB_2 determination

TxB_2 synthesis was measured in PRP preincubated for 10 min with NMDA (10^{-7} M), both in the presence and absence of 10 min incubation with AA (0.5 mM). Indomethacin (10^{-5} M) was used as the positive control. Fifty microlitres of PRP were diluted in 450 μl of ice-cold phosphate buffered saline, pH 7.4, and quickly frozen. The samples were subsequently processed for TxB_2 by radioimmunoassay, by use of a commercial kit (Thromboxane B_2 Cayman Chemical Company, Ann Arbor, MI, U.S.A.). Determinations were carried out in duplicate.

Intracellular calcium determination

Intracellular calcium was measured in washed platelets. The procedure used for isolation and dye loading of platelets with fura 2 AM (Molecular Probe) was described by Ivanova *et al*. (1993). Briefly, platelets were obtained from PRP by centrifugation at $2000 \times g$ for 10 min, and resuspended in Tyrode-HEPES assay buffer (pH 7.4) containing 1 mM EGTA, 145 mM NaCl, 5 mM KCl, 5.6 μM glucose, 0.5 mM Na_2HPO_4, 10 mM HEPES. Dye loading was standardized by incubating platelets with 3 μM fura 2 AM for 30 min at room temperature. Platelets were then washed with Tyrode-HEPES buffer and the assay was performed on a stirred aliquot (1.5 ml) containing 100,000 platelets μl^{-1} with the use of a Hitachi F-2000 spectrofluorometer (Tokyo, Japan). The excitation and emission wavelengths used were 340 – 380 nm and 510 nm, respectively, detected every 500 ms and stored in separate memories of the F2000 spectrofluorometer. A data Manager was used to monitor the fluorescent signal of fura 2 AM-loaded platelets. Platelets were preincubated at 37°C for 1 min in a medium containing 1 mM $CaCl_2$. Basal and stimulated cytosolic calcium were quantified according to Grynkiewicz *et al*. (1985) by use of the ratio technique and $K_d = 224$ nM as dissociation constant of fura 2 AM; maximal and minimal values of fluorescence were evaluated after the addition of 0.3% TritonX100 and 50 mM EGTA, respectively. Hitachi 2000 software was used for calculation.

Platelets were incubated for 100 s in the absence or in the presence of increasing NMDA concentrations (0.1 μM – 10 μM). At the end of each experiment the response to the agonist was redetermined and, if the response differed by more than 20% from the initial value, the results were discarded. Resting calcium concentration was evaluated before the addition of the compound to be studied.

Chemicals

The sodium salt of arachidonic acid, NMDA, L-NMMA, ASA and indomethacin were purchased from Sigma (St Louis, Mi, U.S.A.) and MK-801 (+)-5-methyl-10, 11-dihydro-5H-dibenzyl [a,d] cyclohepten-5,10-imine hydrogen maleate) from RBI (Natick, Ma, U.S.A.). U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; Upjohn, Kalamazoo, MI, U.S.A.) was kindly donated by Prof. Gretele (Perugia, Italy).

Statistical analysis

Data are expressed as mean $\pm$ s.e.mean. Statistical comparisons were made by ANOVA, followed by a *post hoc* analysis by use of Dunnet's ($P < 0.05$), or by Kruskal-Wallis non parametric test followed by Dunn's multiple comparisons test ($P < 0.05$) (Instat 2.01 for Macintosh, GraphPad Software, Inc.).

Results

The effect of different concentrations of NMDA on intracellular calcium was measured in washed platelets loaded with fura 2 AM and incubated in a medium containing 1 mM $CaCl_2$. Basal values were comparable to those previously obtained (Tan *et al*., 1995). NMDA increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Figure 1).

In the presence of 1 mM $CaCl_2$, AA (5 mM – 15 mM) dose-dependently increased $[Ca^{2+}]_i$ from a basal value of about 100 nM up to more than 800 mM (Table 1). Such an increase was biphasic; in fact, 5 mM AA produced the initial peak that was followed by a gradual decrease, but the basal value of $[Ca^{2+}]_i$ was not reached.

A concentration curve of NMDA on AA-induced calcium increase was carried out by using the lowest active dose of the fatty acid. In fact, at high concentrations AA produced a $[Ca^{2+}]_i$ increase which sometimes resulted in platelet aggregation. Table 2 shows that preincubation of washed platelets with different NMDA concentrations (0.1 – 10 μM) did not significantly modify AA-induced calcium increase.

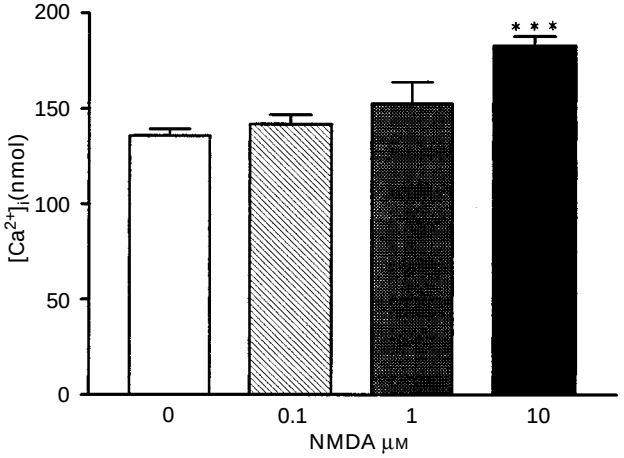

Figure 1 Enhancement of $[Ca^{2+}]_i$ induced by NMDA in human washed platelets in the presence of 1 mM $CaCl_2$. Values are the means ± s.e.mean of 4 experiments. ANOVA followed by Dunnett's test: $***P<0.001$.

Table 1 Dose-response curve for effect of AA on $[Ca^{2+}]_i$ in washed human platelets

AA (μM)	$[Ca^{2+}]_i$ (nM)
0	123 ± 3
5	202 ± 19**
10	332 ± 37***
15	820 ± 64***

Experiments were carried out in the presence of 1 mM $CaCl_2$. Values are the means ± s.e.mean of at least 4 experiments. ANOVA followed by Dunnett's test: $**P<0.01$, $***P<0.001$, with respect to basal values.

Table 2 Effect of NMDA preincubation on AA (5 μM)-induced $[Ca^{2+}]_i$ increase in washed human platelets

NMDA (μM)	$[Ca^{2+}]_i$ (nM) *(peak value)*	$[Ca^{2+}]_i$ (nM) *(value at 200 s)*
0	70 ± 8	28 ± 2
0.1	68 ± 8	21 ± 3
1	53 ± 5	39 ± 8
10	51 ± 16	9 ± 10

Calcium was measured at peak value and after 200 s following AA addition to the medium. Values represent the difference between the values obtained in the presence and in the absence of AA and are the means ± s.e.mean of at least 4 experiments.

NMDA, added to human PRP at the concentrations of 0.01 μM and 1 μM, shifted the AA concentration-response curve to the right when the slope or the maximum amplitude was evaluated (Figure 2a and b) and prolonged the lag phase (Table 3). At the highest AA concentrations platelet aggregation was not inhibited.

The effect of NMDA on TxB_2 synthesis and on TxA_2 receptor agonists induced aggregation was studied. Table 4 shows that the amino acid failed to affect platelet aggregation induced by the EC_{50} of U-46619, a TxA_2 receptor agonist. The basal production of TxB_2 was significantly stimulated by 0.5 mM AA. Indomethacin at a concentration which completely inhibited aggregation, prevented the AA-induced TxB_2 synthesis. Finally, 10^{-7} M NMDA completely inhibited AA enhanced TxB_2 production in human PRP (Table 5).

The effect of the NOS inhibitor (Forstermann *et al.*, 1991) L-NMMA on the antiaggregating effect of NMDA was studied. The EC_{50} concentration of AA induced an average value ($n=3$) of 55 ± 8% aggregation which was inhibited by NMDA (8 ± 8%) and this effect was not antagonized by L-NMMA (8 ± 8%).

The antiaggregating activity of NMDA was also studied in association with ASA and indomethacin, two classical antiaggregating agents which are known to act by inhibiting the cyclo-oxygenase cascade. Table 6 shows the potentiating effect of increasing NMDA concentrations (0.01 nM – 100 nM) on the antiaggregating activity of ASA and indomethacin. The two cyclo-oxygenase inhibitors were added to the medium at

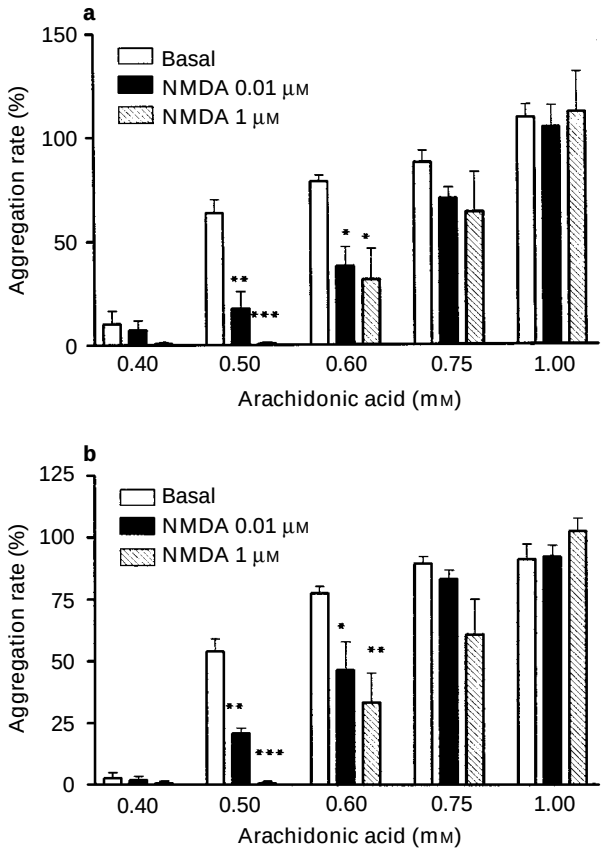

Figure 2 (a) Dose-response curve for effect of AA on the rate of aggregation of human platelets in the absence and presence of NMDA (0.01 μM, 1 μM). (b) Dose-response curve for AA-induced platelet aggregation in the absence and presence of NMDA (0.01 μM, 1 μM). The results are the means ± s.e.mean of at least 6 experiments. Kruskal-Wallis followed by Dunn's test: $*P<0.05$; $**P<0.01$; $***P<0.001$.

Table 3 Effect of 2 different NMDA concentrations on the lag-time of AA-induced PRP aggregation

	Lag-time (s) *AA* (mM)				
	0.4	*0.5*	*0.6*	*0.75*	*1*
NMDA 0	320±21	127±11	89±11	45±3	33±3
NMDA 0.01 μM	>360	281±40**	184±15*	97±5*	35±5
NMDA 1 μM	>360	>360	263±24**	88±26	40±5

Values are the means±s.e.mean of at least 6 experiments. ANOVA followed by Dunnett's test: *$P<0.05$, **$P<0.01$, with respect to AA alone.

Table 4 Failure of NMDA to antagonize U-46619-induced platelet aggregation

	Aggregation (%)	*Rate (%)*
U-46619	48±5	63±3
U-46619+NMDA 0.1 μM	53±7	75±7
U-46619+NMDA 1 μM	47±8	65±6

Values are mean±s.e.mean of at least 3 experiments. Platelet aggregation was stimulated by U-46619 added to the

Table 5 Effect of NMDA on AA-induced TxB_2 synthesis in human PRP

Treatments	*TxB_2* (ng ml^{-1})
None	1.7±0.4
AA 0.5 mM	561±40***
NMDA 100 nM+AA	4±0.8***
Indomethacin 10 μM+AA	75±13***

Values are the mean±s.e.mean experiments, each one performed in duplicate. ANOVA followed by Dunnett's test; ***$P<0.001$ versus none.

Table 6 Effects of ASA and indomethacin, alone or associated with different concentrations of NMDA, on platelet aggregation induced by 1 mM AA

Treatments	*Maximum Aggregation* (%)	*Rate of aggregation* (%)
AA 1 mM	84±2	71±1.5
AA+ASA 20 μM	54±2§	40±2§
AA+ASA+NMDA 0.01 nM	38±5*	32±3*
AA+ASA+NMDA 0.1 nM	31±4**	24±3**
AA+ASA+NMDA 1 nM	7±1**	7±0.9**
AA+ASA+NMDA 100 nM	1±0.7**	2±0.8**
AA+indo 1 μM	40±1§	33±2§
AA+indo+NMDA 0.01 nM	37±1	30±0.7
AA+indo+NMDA 0.1 nM	21±0.9$	16±0.8$
AA+indo+NMDA 1 nM	13±1$$	8±0.8$$
AA+indo+NMDA 100 nM	2±0.6$$	1±0.4$$

Values are the mean±s.e.mean of at least 6 experiments. §$P<0.01$ compared to AA alone. *$P<0.05$ **$P<0.01$, compared to AA+ASA. $$P<0.05$, $$$P<0.01$, compared to AA+indomethacin.

their EC_{50} concentrations, detected by using an aggregating concentration of AA (1 mM) insensitive to NMDA alone. The association of 100 nM NMDA with either ASA or indomethacin completely antagonized AA-induced platelet aggregation, and the amino acid significantly increased the efficacy of both compounds up to a concentration of 0.1 nM.

Discussion

The present results confirm that NMDA receptor stimulation antagonizes the aggregating effect of AA in human PRP. The lack of activity of NMDA on U-46619-induced aggregation indicates that the effect of the amino acid is not mediated by an interaction with the TxA_2/PGH_2 receptors present in platelets (Hanasaki & Arita, 1991). Moreover, our findings show that NMDA inhibited the increase in TxB_2 induced in PRP by exongenous AA. Thus evidence is provided that the mechanism of the antiaggregating effect of NMDA may be related to an influence on platelet TxA_2 synthesis. The rate limiting factor in the biosynthesis of prostaglandins and related compounds is the availability of the fatty acid precursor. Thus, the reduction of thromboxane production may derive from the inhibition of either diacylglycerol lipase, i.e. the enzyme which controls AA availability (Prescott & Majeras, 1983), or an enzyme of the cyclo-oxygenase/thromboxane-synthase cascade. The present results show that NMDA reduced thromboxane formation induced by exogenously added AA in platelets, indicating that the amino acid may act at the cyclo-oxygenase/thromboxane-synthase level. Hence the effects of NMDA on TxA_2 biosynthesis could be due to interference with the access of the exogenous AA to the cyclo-oxygenase, or to inhibition of cyclo-oxygenase or thromboxane-synthase. Whether the effect of NMDA is receptor-mediated is not clear from out data, but the experiments with U-46619 exclude the possibility that NMDA was acting as an antagonist of thromboxane receptors. Interestingly, in ASA- and in indomethacin-treated platelets NMDA dose-dependently increased the efficiency of cyclo-oxygenase inhibitors.

Since NMDA receptor stimulation increases cyclic AMP formation in platelets (Franconi *et al*., 1996), it may be proposed that the effect of NMDA on AA-induced aggregation is mediated by both inhibition of thromboxane synthesis and cyclic AMP-dependent mechanisms. In fact, cyclic AMP is known to inhibit in platelets: (a) the induction and the membrane exposure of fibrinogen receptors (Hawiger *et al*. 1880); (b) agonist-induced diacylglycerol formation (Siess, 1989); (c) the rise of $[Ca^{2+}]_i$ mediated by membrane receptor stimulation and not by A23187 (Pannocchia & Hardisty, 1985); thus suggesting that it may also stimulate calcium transport from cytosol into intracellular organelles (Siess, 1989); (d) the mobilization of intracellular calcium stores (Abbracchio & Burnstock, 1994).

Human platelets contain constitutive NO synthase (Metha *et al*., 1995), and NO inhibits aggregation by stimulating cyclic

GMP accumulation in platelets (Mellion *et al*., 1981). Moreover, NO also inhibits phospholipase C activity in platelets, which would result in diminished TxA_2 formation (Durante *et al*., 1992). The failure of L-NMMA to affect the antiaggregating activity of NMDA indicates that these mechanisms are not involved in the effects of the amino acid on platelet aggregation. Many central effects of NMDA receptor stimulation are mediated by Ca-calmodulin activation of NO synthase and the consequent increase of NO (Bredt & Snyder, 1989). Thus, the present data further outline the differences between neuronal and non-neuronal NMDA receptors. Such a difference is also confirmed by the fact that the effect of NMDA on resting calcium was obtained in a medium without glycine, while the binding of glycine appears to be an absolute requirement for NMDA channel activation (Kemp & Leeson, 1993) in the CNS. The functional differences observed between CNS and peripheral NMDA receptors leaves open the question of whether one or more peripheral forms of NMDA receptors exist, which may be coded by already known or yet uncloned genes.

In washed platelets 10^{-5} M NMDA induced a modest but significant increase in intracellular calcium. Similar high concentrations of NMDA were necessary to increase calcium concentration in rat cardiac myocytes (Winter & Baker, 1995) and anterior pituitary cells (Villabos *et al*., 1996). The increase in intracellular calcium was very modest and it did not induce aggregation, although it appeared to be sufficient to increase cyclic AMP levels (Franconi *et al*., 1996). Therefore, the calcium-dependent increase in cyclic AMP observed in intact platelets (Franconi *et al*., 1996) could be explained by the existence of discrete intracellular compartments (Tsunoda *et al*., 1988). However, NMDA failed to affect the $[Ca^{2+}]_i$ increase produced by 5 μM AA in washed platelets. Discrepancies between results obtained in washed platelets and PRP have been observed previously. Paradoxically, in washed platelets thromboxane-synthase inhibitors enhance the response to AA, while this phenomenon is abolished in PRP (Siess, 1989). In fact, isomerase activity in human serum albumin increases the production of the antiaggregating prostaglandin D_2 (PGD_2) from PGH_2 (Watanabe *et al*., 1982).

The present findings, associated with those on the presence of glutamate receptors in cardiac myocytes, suggest a possible role of excitatory amino acids in the physiology of the cardiocirculatory system. The functional differences between central and peripheral receptors could be important in the search for clinically useful compounds. Finally, the identification of cyclo-oxygenase inhibition as the common target of platelet NMDA receptor activation and ASA or indomethacin could provide a useful tool in the prevention and therapy of thrombosis-related disorders.

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