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THE ROLE OF EXTRACELLULAR Ca^{2+} AND Na^+ IN PAF-ACETHER-INDUCED HUMAN PLATELET ACTIVATION

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Abstract—The dependence of paf-acether (paf)-induced human platelet activation on extracellular Ca^{2+} and Na^+ was examined by quantitating aggregation, secretion and thromboxane (Tx) formation in the presence of physiological and/or low concentrations of Ca^{2+} and/or Na^+. In the presence of 2 mM Ca^{2+} and 140 mM Na^+, paf induced a dose-dependent reversible aggregation and less than 25% of [^{14}C]serotonin release. These responses were insensitive to aspirin or Tx antagonist SQ 29548 treatment and negligible amounts of Tx were formed. In low Ca^{2+} buffer, paf induced irreversible aggregation and the ^{14}C-serotonin release could exceed 60%. These increases in platelet response were associated with the formation of Tx and were suppressed by aspirin and SQ 29548 treatments, or by substituting NaCl with *N*-methylglucamine hydrochloride. Thus, in low Ca^{2+} medium, Tx synthesis is favored during platelet activation and is dependent on Na^+ concentrations. A decrease in extracellular Na^+ inhibited the paf-acether-induced Tx synthesis observed in low Ca^{2+} medium but not that induced by the Tx direct precursor, arachidonic acid (AA). Therefore, the increase observed in low Ca^{2+} medium, no longer seen when the Na^+ level is decreased is not related to an impairment of the cyclooxygenase activity but rather implicates an effect on the activity of phospholipase A_2. A decrease in extracellular Na^+ (2 mM Ca^{2+} present), inhibited [^{14}C]serotonin release induced by paf from platelets which had, or had not been, treated with aspirin. In this medium, the AA-induced release reaction was also affected whereas Tx formation was not altered, thus suggesting that other mechanisms involved in platelet response apart from Tx synthesis are dependent on extracellular Na^+.

Paf-acether (paf, formerly platelet-activating factor [1]) identified as a 1-*O*-hexa/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine [2, 3], is a potent activator of human platelets [4–21]. Numerous studies have shown that thromboxane synthesis is required for paf to evoke extensive human platelet aggregation and secretion [7–21]. In particular, the role of the thromboxane pathway is predominant in platelet activation induced by low doses of paf [7–14, 17, 18]. Indeed, aspirin-like drugs do inhibit the second phase of aggregation and/or the release reaction induced by threshold amounts of paf, whereas, when high concentrations of paf are used, usually only the release reaction is affected by treatment with cyclooxygenase blockers [7–14, 17, 18]. However, it is not clear whether this peculiar response of human platelets to paf is not influenced by experimental conditions such as the concentration of external ionized calcium that is known to modify human platelet response to agonists such as ADP and epinephrine [22–25]. Differences can indeed be observed in the response of human platelets to paf depending upon whether Ca^{2+} is present in a micromolar or millimolar range. In citrated plasma, where the concentration of Ca^{2+} is 40 μM [26], and in balanced salt medium without added Ca^{2+}, paf induces platelet activation (aggregation and/or release reaction) mainly through the cyclooxygenase pathway [8, 9, 11–20]. When Ca^{2+} is present at physiological range (1–2 mM) as in heparin- or hirudin-plasma [26], or citrated plasma to which Ca^{2+} and hirudin have been added, platelet release reaction in response to paf is usually lower compared to citrated plasma [5, 8, 10, 18], and contradictory results have been reported concerning its dependence upon the cyclooxygenase pathway [8, 9, 10, 18]. Interestingly, Kloprogge *et al.* [21] using gel-filtrated platelets suspended in buffer without added Ca^{2+}, observed that the release in response to paf can be impaired (by around 50%) by raising Ca^{2+} to a millimolar range, whereas aggregation is barely affected.

Extracellular Na^+ is also important for stimulus-provoked platelet release reaction. A decrease in extracellular Na^+ selectively inhibits platelet secretion that requires thromboxane synthesis to be elicited with, for example, ADP, epinephrine or low doses of thrombin [27, 28]. This control by Na^+ of the release reaction is exerted through the modulation of arachidonic acid mobilization [28, 29] necessary for thromboxane formation through the cyclooxygenase pathway. Decreasing the extracellular Na^+ did not suppress either the aggregation or the cytoplasmic Ca^{2+} rise of aspirin-treated platelet stimulated by paf [30]. However whether or not Na^+ could influence the paf-induced platelet release reaction, and whether or not this regulation is confined to an effect on the thromboxane pathway remain to be investigated.

The putative importance of paf and platelet secretion in inflammation and vascular diseases makes them currently pharmacological targets. The aim of the present study was to examine the dependence of human platelet aggregation and secretion on extracellular Ca^{2+} and Na^+, while attempting to clarify the

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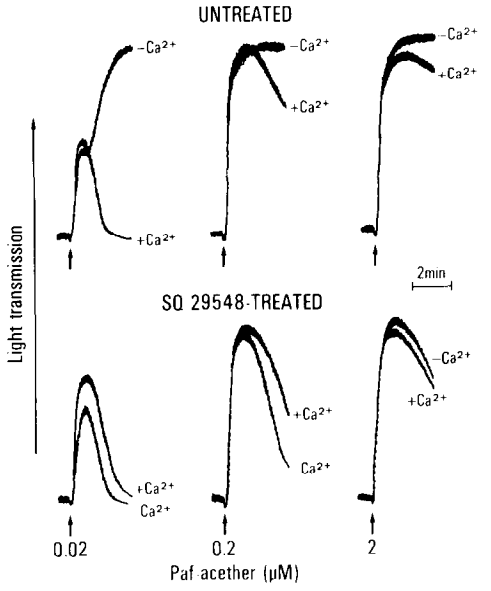

Fig. 1. Aggregation tracings obtained after addition of paf-acether (arrows) on washed human platelets untreated (top) or pretreated with 50 μM SQ 29548 (bottom), in Tyrode's Hepes albumin with no added Ca^{2+}, i.e. 30 μM Ca^{2+} present ($-Ca^{2+}$) or at physiological Ca^{2+} concentrations, i.e. 2 mM Ca^{2+} present ($+Ca^{2+}$). Figure is representative of five to seven experiments. Similar results were obtained when platelets are treated with aspirin (0.1 mM) instead of SQ 29548.

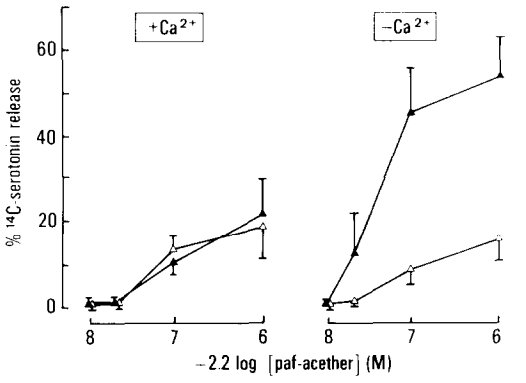

Fig. 2. Semi-log plot of the release of [^{14}C]serotonin obtained after addition of different concentration of paf-acether on washed human platelets in Tyrode's Hepes albumin with no added Ca^{2+} (right graph; $-Ca^{2+}$) or with 2 mM Ca^{2+} (left graphs; $+Ca^{2+}$). Platelets were pretreated (△) or not (▲) with 0.1 mM aspirin. Mean ± SE of five to seven experiments with platelet suspensions from different donors. Similar results were obtained when platelets are treated with SQ 29548 (50 μM) instead of aspirin.

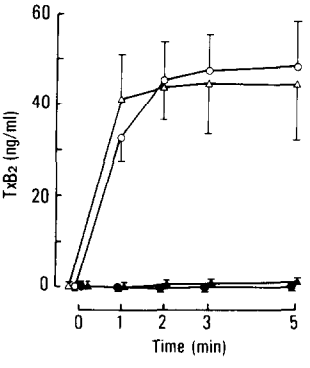

Fig. 3. Time-course of TxB_2 production after addition of 0.02 μM (○, ●) or 2 μM (△, ▲) paf-acether on human platelets in Tyrode's Hepes albumin with no added Ca^{2+} (○, △) or with 2 mM Ca^{2+} (●, ▲). Means ± SE of experiments with platelet suspensions from three different donors.

effectiveness of pharmacological inhibition of the thromboxane pathway on human platelet activation induced by paf.

MATERIALS AND METHODS

Materials were obtained from the following suppliers: arachidonic acid (AA), *N*-methylglucamine hydrochloride, imipramine and Hepes (*N*-2-hydroxyethyl-piperazine-*N*-ethanesulfonic acid) were from Sigma Chemical Co. (St Louis, MO). Paf (1.*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was from Bachem (Bubendorf, Switzerland); heparin was from Choay (Paris, France); bovine serum albumin (BSA) was from Armour Co. Phoenix, AZ) and aspirin as a lysin salt (Aspegic®) was from Egic (Amilly, France). Human fibrinogen (Kabi, Stockholm, Sweden) treated with diisopropylfluorophosphate (DFP) to remove coagulant contaminants, was a gift from Dr B. B. Vargaftig (Institut Pasteur, Paris, France). Apyrase, prepared from potatoes by the method of Molnar and Lorand [31] was a gift from Dr R. L. Kinlough-Rathbone (McMaster University, Hamilton, ONT, Canada). SQ 29548, a thromboxane A_2/PGH_2 receptor blocker [32], was a gift from Dr M. L. Ogletree (Squibb Institute for Medical Research, Princeton, NJ). [^{14}C]Serotonin (as 5-hydroxytryptamine-3'-[^{14}C]creatinine sulfate, 50 mCi/mmol) and scintillation counting liquid (ACS II) were from Amersham (Les Ulis, France).

Preparation of washed human platelets. Washed human platelets were prepared as previously described by Kinlough-Rathbone *et al.* [23, 33], with the addition of 10 mM Hepes (pH 7.35) in the washing fluids and the final suspending media. The platelet count was adjusted to 5×10^8/ml. The final suspending medium was a Tyrode's Hepes solution to which were added Mg^{2+} (1 mM $MgCl_2$), Ca^{2+} (2 mM $CaCl_2$), apyrase at a concentration that converted 0.25 μM ATP to adenosine monophosphate in 120 sec at 37°, glucose (1 g/l), and BSA 0.35% (w/v). Reactivity (aggregation and secretion or thromboxane formation) of platelets from the same donor was simultaneously compared in different media: to study the effect of "low-Ca^{2+} medium" on platelet response, $CaCl_2$ was omitted in the final suspending media; to study the effect of "low-Na^+ medium" on platelets response, NaCl was replaced by *N*-methylglucamine hydrochloride in the washing fluids and in

Table 1. Effect of low concentration of extracellular Ca^{2+} and/or Na^+ on the release of [^{14}C]serotonin from human platelets

Agonist (μM)	Percent release of [^{14}C]serotonin with Ca^{2+} (2 mM)		without added Ca^{2+}	
	$+Na^+$	$-Na^+$	$+Na^+$	$-Na^+$
paf 0.02	3.9 ± 1.4 (5)	1.9 ± 0.9	4.3 ± 1.4 (4)	1.6 ± 0.7
0.21	10.5 ± 3.9 (6)	5.2 ± 2.5	26.1 ± 9.4 (6)	4.0 ± 1.4
2.2	17.0 ± 4.1 (6)	7.1 ± 3.0**	39.2 ± 8.8 (6)	5.3 ± 2.4**
4.4	15.2 ± 5.6 (5)	6.2 ± 3.9**	41.9 ± 9.3 (5)	5.2 ± 3.0*

Values (means ± SE) represent percent radioactivity found in the supernatant of samples 3 min after stimulation of platelets with paf-acether (paf). Numbers in parenthesis indicate the number of experiments with platelet suspensions from different donors. Significance of difference from values obtained with platelet stimulation in buffer containing 140 mM NaCl ($+Na^+$) or 140 mM *N*-methylglucamine hydrochloride ($-Na^+$) *$P < 0.02$; **$P < 0.01$. (Student's *t*-test for paired data analysis).

Table 2. Effect of low concentration of extracellular Na^+ on the thromboxane production of human platelets in low Ca^{2+} medium

Agonist (μM)	TxB_2 (ng/ml)	
	$+Na^+$	$-Na^+$
paf 0.02	48.2 ± 10.8	0.27 ± 0.01
0.22	58.2 ± 20.2	0.38 ± 0.09
2.22	43.8 ± 12.3	1.70 ± 1.44
AA 160	703 ± 357	820 ± 243

Values (means ± SE) represent the amount of thromboxane B_2 (TxB_2) present in human platelet suspensions after 5 min stimulation with paf-acether (paf) or arachidonic acid (AA) in medium without added Ca^{2+}. $+Na^+$ = 140 mM NaCl present; $-Na^+$ = NaCl replaced by 140 mM *N*-methylglucamine hydrochloride. Experiments with platelet suspensions from three different donors.

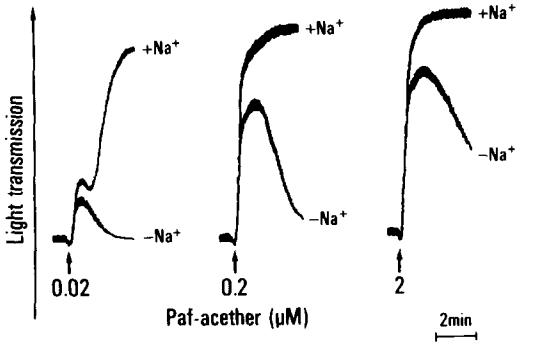

Fig. 4. Effect of low Na^+ medium ($-Na^+$) on aggregation tracings obtained after addition of paf-acether in the presence of low Ca^{2+} concentration. Figure is representative of four to ten experiments.

the final suspending media in order to obtain an extracellular Na^+ concentration lower than that of intraplatelet Na^+ that is 38 mM [34]. The concentrations of Ca^{2+} and Na^+ were approximately 30 μM and 15 mM for "low Ca^{2+} medium" and for "low-Na^+ medium" respectively as measured by atomic absorption spectrometry. Under these conditions, the concentration of Ca^{2+} in "low Ca^{2+} medium" was close to that in citrate-anticoagulated platelet-rich plasma [26]; the concentration of Na^+ in "low Na^+ medium" was sufficiently reduced to block arachidonic acid mobilization in response to "weak agonist" [28]. In some experiments, platelets were incubated with 0.1 mM aspirin in the first washing fluid for 20 min at 37°.

Platelet response study. Platelet aggregation, performed on 400 μl platelet suspension and in the presence of 0.35 mg/ml DFP-fibrinogen, was recorded as change in light transmission for 3 min at 37° in an aggregometer (Icare, France). In some experiments, platelets were preincubated for 1 min at 37° with 50 μM SQ 29548 before stimulation. Since Na^+ is required for platelets to uptake serotonin [35], platelets were labeled in plasma with [^{14}C]serotonin (1 μCi/10 ml platelet-rich plasma). Release of serotonin was measured as described previously [36] in the presence of imipramine (5 μM) to avoid reuptake of the released serotonin. Three min after addition of agonist, platelets samples were centrifuged for 1 min in an Eppendorff microcentrifuge, and 100 μl of supernatant counted for radioactivity. The release of [^{14}C]serotonin was calculated as per cent of the total radioactivity present in the platelet suspension and corrected for non-specific release from non-stimulated controls. The ability of platelets to synthesize thromboxane (Tx) A_2 was monitored by measuring its stable catabolite TxB_2, using a radioimmunoassay (RIA) kit from New England Nuclear (Boston, MA). Before addition of agonist (or solvent as control) and 1, 2, 3 and 5 min after, 20 μl aliquots from 400 μl platelet suspension were removed and quenched in 380 μl of ice-cold RIA buffer containing 0.5 mM indomethacin. The samples were stored at −20° until assay. Whatever the composition of the medium, negligible amounts of TxB_2 were found in non-stimulated platelets suspensions. Therefore, quantification of the TxB_2

present in the stimulated platelets suspensions were corrected for TxB_2 present in non-stimulated controls.

RESULTS

Effect of low concentration of extracellular Ca^{2+} *on human platelet response to paf*

The aggregating ability of paf was tested on washed platelet suspensions containing 2 mM Ca^{2+} from 41 donors. As described elsewhere [5, 37] the individual sensitivity to paf was highly variable. Weak platelet aggregation (less than 20% of increase in light transmission) was observed in suspensions from 18 of the subjects even when the paf concentration was increased up to 40 μM. These weak responders were therefore withdrawn from the present study.

In the presence of Ca^{2+} at physiological concentration (2 mM), paf induced a dose-dependent aggregation that for 19 of the 23 subjects kept for the trial was always reversible (Fig. 1) and associated with a release of [^{14}C]serotonin that did not exceed 25% (Fig. 2). In this condition, neither addition of the thromboxane/PGH_2 receptor blocker SQ 29548 nor aspirin-treatment modified the extent of aggregation and the release reaction (Figs 1 and 2), and less than 1 ng/ml of TxB_2 were detected (Fig. 3). Similar results were obtained with 1 mM Ca^{2+} present (data not shown).

In contrast, when Ca^{2+} was omitted from the suspending medium (i.e. in low Ca^{2+} medium that contains 30 μM Ca^{2+}), paf-induced aggregation became irreversible and the release reaction could exceed 60% (Figs 1 and 2). This increase in platelet response was associated with a TxB_2 synthesis that reached 45 ng/ml (Fig. 3) and was suppressed by aspirin-treatment or by addition of SQ 29548 (Figs 1 and 2). However, in the presence of physiological concentrations of Ca^{2+}, 4 out of the 23 subjects tested displayed a cyclooxygenase-dependent second wave of aggregation following stimulation with paf.

In conjunction with the results obtained with paf, in low Ca^{2+} medium [^{14}C]serotonin release induced by 0.16 mM AA is significantly decreased, as compared to the response in the presence of 2 mM Ca^{2+}, from 56.9 ± 3.4% to 43.8 ± 5.8% (means ± SE of experiments with platelets obtained from N = 7 different donors, $P < 0.05$ Student's test for paired data difference analysis). Whereas, no significant modification was observed in thromboxane synthesis in response to AA since 544 ± 202 ng/ml and 703 ± 357 ng/ml TxB_2 was detected in platelet suspension with and without added Ca^{2+} respectively (N = 3).

Effect of a low concentration of extracellular Na^+ *on human platelet response to paf*

Low concentrations of extracellular Na^+ inhibited the cyclooxygenase-dependent second wave of platelet aggregation and its associated [^{14}C]serotonin release that were observed in response to paf stimulation in low Ca^{2+} medium (Fig. 4 and Table 1). The thromboxane formation induced by paf in low Ca^{2+} medium was suppressed when extracellular Na^+ was reduced, whereas thromboxane synthesis that resulted from AA stimulation was not significantly modified (Table 2).

Table 3. Effect of low concentration of extracellular Na^+ on human platelet aggregation (2 mM Ca^{2+} present)

Agonist (μM)	Aggregation +Na^+	Aggregation −Na^+	% inhibition
paf 0.02	33.4 ± 5.7	18.3 ± 5.1*	46 (10)
0.22	51.9 ± 4.6	30.5 ± 4.8***	41 (14)
2.22	58.9 ± 3.7	37.4 ± 6.0**	36 (12)
4.44	54.4 ± 3.0	29.1 ± 4.9**	46 (7)
AA 160	75.4 ± 3.2	68.9 ± 4.4*	9 (12)

Values (means ± SE) represent aggregation heights (arbitrary units) of human platelets in medium with 2 mM Ca^{2+}. +Na^+ = 140 mM NaCl; −Na^+ = NaCl replaced by 140 mM *N*-methylglucamine hydrochloride. Numbers in parenthesis indicate the number of experiments with platelet suspensions from different donors. Significance of difference from values obtained with stimulation of platelets in buffer in presence of NaCl or *N*-methylglucamine hydrochloride: *$P < 0.02$; **$P < 0.01$; ***$P < 0.001$ (Student's *t*-test for paired data analysis).

Table 4. Effect of low concentration of extracellular Na^+ on the [^{14}C]serotonin release from aspirin-treated platelets in response to paf (2 mM Ca^{2+} present)

paf (μM)	Percent release of [^{14}C]serotonin +Na^+	Percent release of [^{14}C]serotonin −Na+
0.2	11.5 ± 2.4	2.5 ± 0.9
0.4	12.6 ± 2.3	3.1 ± 0.2
2.2	17.6 ± 4.9	6.7 ± 3.5

Values (means ± SE) represent percent radioactivity found in the supernatant of samples 3 min after stimulation with paf-acether (paf) in a medium with 2 mM Ca^{2+}. +Na^+ = 140 mM NaCl present; −Na^+ = NaCl replaced by 140 mM *N*-methylglucamine hydrochloride. Experiments with platelet suspensions from three different donors.

When Ca^{2+} was present at physiological levels, low concentrations of extracellular Na^+ reduced the extent of aggregation triggered by paf (up to 46% inhibition, Table 3). The release of [^{14}C]serotonin was also affected (up to 60% inhibition, Table 1). Similar inhibitory effect of low Na^+ medium on [^{14}C]serotonin release induced by paf was observed on aspirin-treated platelets (Table 4). Low extracellular Na^+ concentration (2 mM Ca^{2+} present) weakly affected platelet aggregation in response to 0.16 mM AA (Table 3). The [^{14}C]serotonin released from these platelets under the same experimental conditions, was also significantly reduced from 63.0 ± 3.0% to 54.1 ± 3.9% in high and low Na^+ medium respectively (N = 12 experiments with different donors, $P < 0.001$ Student's test for paired data difference analysis).

Thromboxane synthesis that resulted from AA

stimulation in high or low Na^+ medium (2 mM Ca^{2+} present) was not significantly different with 544 ± 202 ng/ml and 765 ± 336 ng/ml TxB_2 detected in medium containing high or low level of Na^+ respectively.

DISCUSSION

This comparative study shows the influence of extracellular Ca^{2+} and Na^+ on human platelet response to paf. Our results show an amplification of platelet response to paf in low Ca^{2+} medium. This potentiation was dependent upon thromboxane synthesis and regulated by extracellular Na^+. The present study further suggests that Na^+ controls other mechanisms involved in human platelet activation apart from thromboxane synthesis.

In the presence of physiological concentrations of Ca^{2+} (2 mM Ca^{2+}), cyclooxygenase-dependent metabolites do not participate in paf-induced platelet activation since neither aspirin nor the thromboxane/PGH_2 receptors antagonist SQ 29548 modified the extent of aggregation and the release reaction. By contrast, when platelets are aggregated by paf in a low Ca^{2+} medium 30 μM Ca^{2+}), the arachidonate pathway is activated, and it is the thromboxane and the prostaglandin endoperoxides thus formed that leads to further aggregation and the release reaction. Indeed, treatment with SQ 29548 or aspirin abolished this second phase of platelet response. The conditions leading to secondary aggregation in low-Ca^{2+} medium are similar to those in citrated platelet-rich plasma (PRP) which contains approximately 40 μM Ca^{2+} [26]. Our results could therefore explain the fact that paf induces secretion and secondary aggregation in citrated plasma almost exclusively via the thromboxane pathway [11–18]. However, in the presence of 2 mM Ca^{2+}, 4 out of 23 subjects displayed an aspirin-sensitive second wave of aggregation following paf stimulation. This suggests a predisposition of a few subjects to synthesize effective amounts of thromboxane during platelet activation by paf even though a physiological concentration of Ca^{2+} is present. This observation might also partly explain the contradictory results reported on the involvement of the cyclooxygenase pathway in platelet response to paf in the presence of millimolar Ca^{2+} concentration [5, 8–10].

This activation profile in low Ca^{2+} medium is not agonist specific since as shown in earlier studies, it can be brought about by ADP [22–24], epinephrine [25] or polylysine [24, 38]. None of these agonists induce thromboxane synthesis in the presence of 1 to 2 mM Ca^{2+} [22–25, 38]. However, the mechanism involved in the amplification of human platelets response observed in low Ca^{2+} medium is unknown. The present data show that the systematic thromboxane synthesis observed when platelets are exposed to paf in low Ca^{2+} medium is not attributable to the low Ca^{2+} concentration *per se*, since unstimulated platelets did not synthesize thromboxane either in high or low Ca^{2+} medium. This phenomenon could not be related either to a more sensitive state of human platelets in low Ca^{2+} medium. In fact, our results suggest that platelets are less responsive to thromboxane in low Ca^{2+} medium. Indeed, under AA stimulation, less [^{14}C]serotonin was released than in the presence of 2 mM Ca^{2+} even though similar amounts of thromboxane were formed in both media. These observations are in agreement with those of Packham *et al.* [24] who showed that platelets response to the thromboxane analogue U46619 was lower in the presence of a micromolar than a millimolar range of Ca^{2+} [24].

Low extracellular Na^+ concentration inhibited thromboxane synthesis and its subsequent potentiation of platelet response to paf when tested in low Ca^{2+} medium. This inhibition could be related to an impairment of arachidonic acid mobilization rather than to an alteration of the cyclooxygenase activity since the conversion of arachidonic acid to thromboxane still occurred. These results are in agreement with Limbird's group observations on the role of Na^+ and Na^+/H^+ exchange in thromboxane synthesis by platelets stimulated with ADP, epinephrine or paf [27, 28, 39]. However, it is noteworthy that this phenomenon was observed with platelets suspension with no added Ca^{2+} [27, 28], or in citrated plasma [39], i.e. in low Ca^{2+} medium.

The present data further suggest that the reduction in the platelet response observed in low Na^+ medium is not confined to an inhibitory effect on the cyclooxygenase pathway of platelet activation. Indeed, under conditions where paf trigger platelet activation independently of the cyclooxygenase pathway products, i.e. in the presence of 2 mM Ca^{2+} and/or after aspirin treatment, the presence of low extracellular Na^+ concentration inhibited both release reaction and aggregation evoked by paf. The reduction in the platelet response to paf in low Na^+ medium could not be ascribed to an impairment of intracellular Ca^{2+} mobilization since the rise of Ca^{2+} in aspirin-treated platelet cytosol was unaffected by Na^+ removal [30]. Neither could it be related to an alteration in the paf interaction with its receptor since Na^+ ions have been shown to reduce the paf receptor affinity [40].

Platelet activation involves sequential changes in morphology which starting from an indented disc moves to a sphere with short pseudopodia, and then progresses to aggregation, secretion and clot retraction. The cytoskeleton provides the structural basis for platelet morphology. Therefore, the observation of a role for Na^+ influx as well as for cytoplasmic alkalinization and Ca^{2+} rise, in the polymerization of platelet cytoskeleton to an activated state [41, 42] might account in part for the general decrease in the platelet response in low Na^+ medium.

In conclusion, the observation that the cyclooxygenase pathway is involved in human platelet activation triggered by paf is probably most often attributable to the presence of unphysiological low Ca^{2+} concentrations in the extracellular medium. It is unlikely that paf can trigger human platelets to generate thromboxane *in vivo* where Ca^{2+} is present in a millimolar range. In contrast, platelet to platelet close contact in low Ca^{2+} medium favored the activation of the thromboxane pathway, this phenomenon being regulated by extracellular Na^+. Our results further emphasize the critical role of Ca^{2+} in the *in vitro* pharmacological studies on platelet activation inhibitors.

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