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Influence of sodium conductances on platelet activation

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The effects of extracellular Na^+ and tetrodotoxin on resting membrane potential, cytosolic free Ca^{2+} levels and aggregation of human platelets have been studied. Neither the decrease in extracellular Na^+-concentration (from 140 mmol/l to 0 mmol/l) nor the addition of tetrodotoxin (10^{-7} to 10^{-5} mol/l) modified the platelet membrane potential. Zero extracellular Na^+ concentration or the presence of tetrodotoxin in the medium inhibited platelet aggregation; however, K^+-depolarized platelets showed an unchanged aggregation induced by ADP or thrombin in media with zero or low extracellular Na^+ concentrations or in the presence of tetrodotoxin. Moreover, zero extracellular Na^+ concentration or tetrodotoxin inhibited calcium mobilization in platelets during activation induced by thrombin. Hence, voltage-dependent activation linked to Na^+ influx appears to be necessary for ADP- and thrombin-induced platelet aggregation under control conditions. Mechanisms for the role of Na^+ conductances in platelet function are discussed.

Introduction

Human platelets suspended in physiological medium show a high resting membrane potential [1,2]. It has been suggested that this membrane potential could modulate platelet activity [1–4]. This influence could be explained by changes of ionic conductances. The platelet depolarization induced by ADP and thrombin [5–9] is probably mediated by changes in the Na^+-membrane conductances [6,10]. Moreover, Na^+ influx has been shown to be related to ADP-induced platelet activity [10,11] In fact, it has been suggested that Na^+ influx could be linked to Ca^{2+} mobilization which plays a key role in platelet activity [10]. Recently, Na^+/Ca^{2+} and a Na^+/H^+ exchange mechanisms have been shown to be related to platelet activity [12–14]. In order to study the role of Na^+ on platelet activation, we investigated the effects of different extracellular Na^+ concentrations ($[Na^+]_o$) or tetrodotoxin concentrations on: (i) platelet membrane potential; (ii) ADP or thrombin-induced platelet aggregation; and (iii) cytosolic free Ca^{2+} changes during platelet activation.

Materials and Methods

Preparation of platelets. Platelets were prepared from freshly drawn human blood anticoagulated with 1/6 vol. acid citrate/dextrose (ACD). Blood was centrifuged at $700 \times g$ for 10 min to obtain platelet-rich plasma (PRP). Platelets, obtained from PRP by centrifugation for 15 min at $2000 \times g$, were washed and resuspended in a standard medium containing (in mM): NaCl (140), KCl (5.4), $6H_2O \cdot MgCl_2$ (1), Dextrose (10) and Hepes (10) (37°C, pH 7.4). In some experiments, all or part of the NaCl was replaced by choline chloride in order to obtain different $[Na^+]_o$ (0 mmol/l, $0 \times [Na^+]$; 35 mmol/l, $0.25 \times [Na^+]$; 70 mmol/l, $0.50 \times [Na^+]$; and 105 mmol/l, $0.75 \times [Na^+]$). In K^+-induced depolarization experiments, platelet suspensions were performed substituting KCl for NaCl at the desired concentration (40 mmol/l). Isotonicity was maintained at 294–304 mosmol/kg H_2O and a pH value of 7.4. Platelets suspended in standard or high K^+ media were used after incubation with tetrodotoxin (Sigma Chemical Co., St. Louis, U.S.A.) at $3 \cdot 10^{-8}$, $3 \cdot 10^{-7}$ and $3 \cdot 10^{-6}$ mol/l for 30 min at room temperature.

Aggregation experiments. Aggregation was assessed at 37°C with platelets suspended in the different media

Abbreviations: PRP, platelet-rich plasma; BSA, bovine serum albumin.

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using Born's method [15] and constant stirring at 1100 rpm with a stirring bar (0.5 cm length). Aliquots of platelet suspension (500 µl) were placed in siliconized glass cuvettes (diameter 0.8 cm) for use in a Chronolog 400 Aggregometer equipped with a Yew Recorder. The platelet count was adjusted to $2.5 \cdot 10^8$/ml and 1 mmol/l $CaCl_2$ was added. In ADP-induced platelet aggregation, 0.5 mg/ml fibrinogen (Kabi, Sweden) was added. Final concentrations of 5 µmol/l ADP (Stago, Asnières, France) and thrombin 0.1 U/ml were used in saline solution in a volume of 10 µl. Platelet aggregation was evaluated by measuring in each case the maximal deflection obtained after 5 min of curve registration computed as a percentage of maximal aggregation. Statistical analysis was performed using the Student's *t*-test for paired or unpaired data as appropriate.

Measurement of membrane potential. Platelet membrane potential values were determined using the fluorescent potentiometric probe 3,3'-dipropylthiadicarbocyanine (diS-C_3(5)) Nippon Kankoh Kenkuysho Co. Ltd. Japan, according to the method described elsewhere [1,2]. Fluorescence measurements were made under the same conditions as those described above in a Kontron SFM-50 spectrofluorimeter equipped with a thermostated cell holder (37°C) and a stirring device.

Measurement of cytosolic free Ca^{2+}. The measurement of $[Ca^{2+}]$ was made using the fluorescent calcium indicator fura-2 according to Pollock et al. [16]. Washed platelets obtained as described above were loaded by incubation with 1 µM fura-2 acetoxymethyl ester (Molecular Probes, Eugene, OR, U.S.A.) for 45 min at 37°C. Aspirin 200 µM was added in the last 10 min. The platelets were then spun down at 350 × *g*, 20 min in the presence of 1 mg/ml bovine serum albumin (BSA) (Calbiochem) and 2% ACD. The supernatant was discarded and platelets were suspended in the corresponding medium with no ACD nor BSA. The cell count was adjusted to $2.5 \cdot 10^8$ platelet/ml. Fura-2 fluorescence was measured at 37°C in a Kontron SFM-50 spectrofluorimeter with 340 nm excitation and 500 nm emission in the presence of 1 mM Ca^{2+}. Fura-2 measurements were also made in the absence or in the presence of tetrodotoxin $3 \cdot 10^{-6}$ mol/l. $[Ca^{2+}]_i$ values monitored by the observed fluorescence (F) were calculated using the general formula

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$$

A value of 224 nm was used for K_d [16]. Maximal fluorescence (F_{max}) was obtained by adding 0.05% Triton X-100 to the cell suspension. Minimal fluorescence (F_{min}) was obtained by adding a sufficient quantity of EGTA to be in excess of the Ca^{2+} concentration, followed by adjustment of the pH with Tris base at approx. 8.5. The fluorescence recordings shown (Fig. 4) are representative of six replicate determinations within the same batch of cells repeated in five different experiments from different donors.

Results

Resting platelet membrane potential in physiological medium was estimated to be -63.4 ± 5.2 mV. This platelet membrane potential was not significantly modified when $[Na^+]_o$ was reduced by a factor of 0.75, 0.5,

TABLE I

Effects of different $[Na^+]_o$, tetrodotoxin (TTX) and 40 mmol/l K_o^+ on platelet membrane potential (V_m)

V_m values are expressed as mean ± S.D., $n = 10$ in all cases and were calculated as described in Refs. 2 and 3.

Experimental conditions				V_m (mV)
Na^+ (mmol)	K^+ (mmol)	TTX (mol/l)	Osmolarity * (mosmol/l)	
140	5.4	–	294–304	−63.4 ± 5.2
105	5.4	–	295	−62.3 ± 3.1
70	5.4	–	301	−60.2 ± 3.0
35	5.4	–	300	−64.3 ± 2.1
0	5.4	–	304	−64.2 ± 3.1
140	5.4	$3 \cdot 10^{-6}$	304	−60.2 ± 3.2
140	5.4	$3 \cdot 10^{-7}$	295	−61.3 ± 2.2
140	5.4	$3 \cdot 10^{-8}$	300	−59.3 ± 3.4
140	40.0	–	304	−40.6 ± 4.6

* Representative sample.

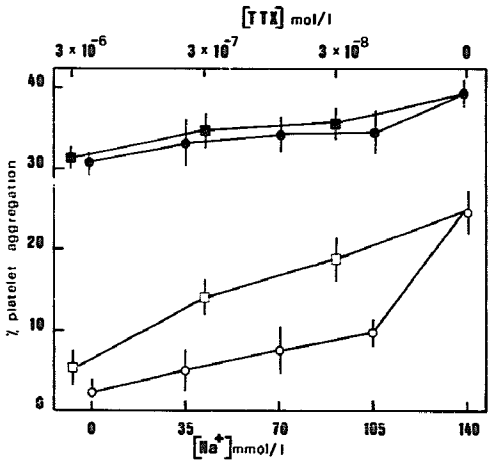

Fig. 1. Effects of $[Na^+]_o$ and tetrodotoxin on platelet aggregation induced by 5 µol ADP. Circles represent the percentage of aggregation versus different values of $[Na^+]_o$ in 5.4 mmol/l K^+-medium (○) and in 40 mmol/l K^+ medium (●). Squares represent the percentage of platelet aggregation versus tetrodotoxin (TTX) concentrations in 5.4 mmol/l K^+ medium (□) and 40 mmol/l K^+ medium (■). Each point represents the mean of five experiments ± S.D.

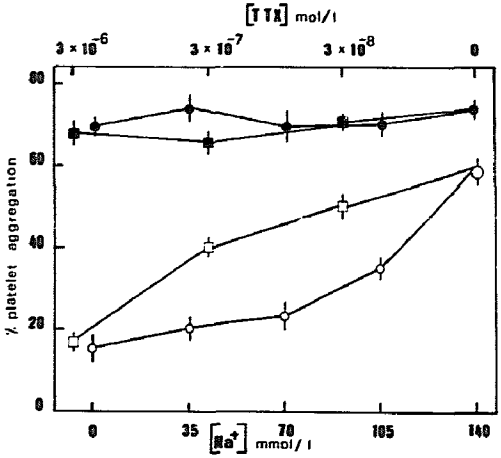

Fig. 2. Effects of $[Na^+]_o$ and tetrodotoxin on platelet aggregation induced by 0.1 U/ml thrombin. Circles represent the percentage of aggregation versus different values of $[Na^+]_o$ in 5.4 mmol/l K^+ medium (○) and in 40 mmol/l K^+ medium (●). Squares represent the percentage of platelet aggregation versus tetrodotoxin (TTX) concentrations in 5.4 mmol/l K^+ medium (□) and in 40 mmol/l K^+ medium (■). Each point represents the mean of five experiments ± S.D.

0.25 or 0. Also, the presence of tetrodotoxin, at every concentration used, did not modify the platelet membrane potential (Table I).

$0 \times [Na^+]_o$ medium elicited a significant inhibition ($P < 0.001$) of platelet aggregation induced by either ADP (90% inhibition) or thrombin (75% inhibition). The percentage of platelet aggregation induced by ADP

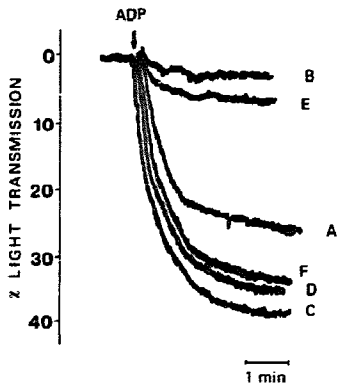

Fig. 3. Representative ADP-induced aggregation traces obtained from platelets suspended in: (A) standard medium (140 mmol/l Na^+, 5.4 mmol/l K^+); (B) Na^+ free medium (0 mmol/l Na^+, 5.4 mmol/l K^+); (C) high K^+ medium (140 mmol/l Na^+, 40 mmol/l K^+); (D) free Na^+ and high K^+ medium (0 mmol/l Na, 40 mmol/l K^+); (E) standard medium in the presence of $3 \cdot 10^{-6}$ mol/l tetrodotoxin and (F) high K^+ medium in the presence of $3 \cdot 10^{-6}$ mol/l tetrodotoxin. ADP was used at a final concentration of 5 μmol/l.

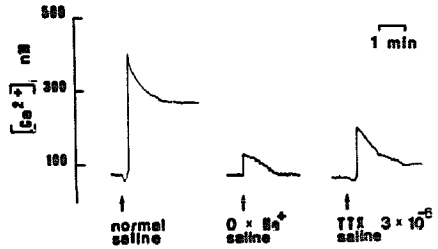

Fig. 4. Changes of intracellular Ca^{2+} concentration induced by thrombin (arrows) at final concentration of 0.1 U/ml in normal saline, $0 \times Na^+$ medium and in tetrodotoxin (TTX) $3 \cdot 10^{-6}$ mol/l medium. The traces shown are from the same batch of cells and are replicates of single representative recordings.

(Fig. 1) or thrombin (Fig. 2) versus $[Na^+]_o$, from $1 \times [Na^+]_o$ to $0 \times [Na^+]_o$ media are shown.

Platelet aggregation was completely blocked at control values of $[K^+]_o$ with $0 \times [Na^+]_o$ medium, but this inhibition was not observed in platelets previously depolarized by 40 mmol/l $[K^+]_o$ (Figs. 1 and 2). In fact ADP- or thrombin-induced platelet aggregation in 40 mmol/l $[K^+]_o$ medium was enhanced at any $[Na^+]_o$ used, although 40 mmol/l $[K^+]_o$ had no effect on platelet aggregation in the absence of agonists. Moreover, platelet aggregation in normal saline was inhibited by tetrodotoxin ($3 \cdot 10^{-8}$ to $3 \cdot 10^{-6}$ mol/l) in a dose-dependent manner, but tetrodotoxin failed to inhibit the enhanced aggregating response observed in K^+-depolarized platelets (Figs. 1 and 2). Typical ADP-induced aggregation traces are shown in Fig. 3. Similar traces were obtained using 0.1 U/ml thrombin.

In control saline, thrombin at a final concentration of 0.1 U/ml induced an increase of cytosolic free Ca^{2+} from a basal level of 95 ± 10 nM to 412 ± 35 nM (mean ± S.D., $n = 6$) ($P < 0.001$). This increase in cytosolic free Ca^{2+} was inhibited by 65.4 ± 8.3% (205 ± 15 nM, $P < 0.001$) in $0 \times [Na^+]_o$ medium and by 50.2 ± 10.3% (253 ± 21 nM, $P < 0.001$) in a medium containing tetrodotoxin ($3 \cdot 10^{-6}$ mol/l). A single representative recording from the same batch of cells is shown in Fig. 4.

Discussion

ADP and thrombin induce changes of membrane potential during platelet activation, probably due to Na^+ influx [5–8]. Changes of extracellular $[Na^+]$ or the presence of tetrodotoxin in the medium do not modify the resting platelet membrane potential. These results suggest that the resting platelet membrane potential is mainly dependent on extracellular $[K^+]$ and agree with those obtained by Friedhoff and Sonenberg [1].

It has been suggested that Na^+ fluxes could play a significant role in platelet activation [5,9,10]. Indeed, in

the experiments carried out under our experimental control conditions, the aggregation mechanism seems to require the presence of extracellular Na^+ and a Na^+ influx, since aggregation is inhibited by low Na^+ media or tetrodotoxin. In contrast, K^+-depolarized platelets, which do not aggregate spontaneously, show a significantly enhanced aggregating response to ADP, adrenalin, collagen and thrombin [2]; under these conditions, aggregation induced by ADP or thrombin is not inhibited by $0 \times Na^+$ medium or tetrodotoxin suggesting that a voltage-dependent mechanism could be involved in the early steps of platelet activation. Furthermore, the inhibition of the increase in cytosolic free Ca^{2+} during platelet activation in low $[Na^+]_o$ media or in the presence of tetrodotoxin suggests that under our control conditions, Na^+ conductances are linked to Ca^{2+} influx and/or mobilization.

Some mechanisms can be proposed to explain the relationship between Na^+ conductance and cytosolic Ca^{2+} mobilization reported herein, namely an involvement of a Ca^{2+} voltage-dependent conductance and/or a voltage sensitive Na^+/Ca^{2+} or Na^+/H^+ exchange. It has been shown that ADP and thrombin induce platelet membrane depolarization, probably due to a Na^+ influx [5–10]. Furthermore, the fact that in our experiments neither low $[Na^+]_o$ media nor tetrodotoxin inhibited K^+-depolarized platelet aggregation and that 40 mmol/l $[K^+]_o$ potentiates platelet aggregation could suggest that platelet activation is voltage-dependent through some voltage-sensitive Ca^{2+} conductance, as has been previously proposed [2]. The depolarization by a high K^+ concentration would contribute to the increase or the basal cytosolic free Ca^{2+} level or the Ca^{2+} influx induced by platelet agonists [17]. When platelet membrane depolarization is accomplished by high extracellular K^+ concentrations, it seems likely that the role of Na^+ influx to trigger platelet activation would be minor. Although some data [18] indicate that platelet depolarization induced by high $[K^+]_o$ does not increase cytosolic free Ca^{2+}, Sage and Rink [19] recognize that a high extracellular K^+ concentration appears to interfere with the Ca^{2+} influx mechanism.

Another possibility which would account for the observed relationship between Na^+ and Ca^{2+} through a Na^+/Ca^{2+} exchange [12,13], has been described – that platelet membrane depolarization seems to be linked to Na^+ influx [6,10,20], increasing $[Na^+]_i$. This increase would induce a rise in cytosolic Ca^{2+} by a Na^+/Ca^{2+} exchange mechanism. In favour of a functional role of Na^+/Ca^{2+} exchange in platelet activation, it has been described that ouabain, which increases intracellular Na^+ [21], is able to enhance ADP-induced platelet aggregation [2]. Furthermore, preliminary data obtained in our laboratory show that ouabain induces an increase of basal cytosolic Ca^{2+} in platelets. These data and the enhanced aggregation of K^+-depolarized platelets suspended in media with different $[Na^+]_o$ suggest that the proposed Na^+/Ca^{2+} exchange could be voltage-sensitive, as has been described in other cells [22].

On the other hand, Siffert and Akkerman have obtained inhibition of platelet activity by removal of extracellular Na^+ or by application of amiloride which blocks the electrogenic Na^+ channels [14]. Activation of a Na^+/H^+ exchange was postulated as an explanation of these results and was considered as a prerequisite for Ca^{2+} mobilization in human platelets.

The main conclusion from the present study is that under our control conditions Na^+ plays a role in platelet aggregation and Ca^{2+} mobilization. Although different mechanisms could mediate these Na^+ effects, our results provide a basis on which to speculate on the voltage dependence of the platelet activation and indicate the need to evaluate carefully the role of Na^+ conductances in that mechanism.

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