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Platelet membrane potential as a modulator of aggregating mechanisms

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The membrane potential of platelets suspended in physiological medium and membrane potential changes induced by high potassium concentrations, ouabain and cooling have been measured using a cyanine fluorescent dye (3,3′-dipropylthiodicarbocyanide). The membrane potential of platelets suspended in physiological medium was −63.8 mV. High potassium concentrations, ouabain and cooling induced depolarization of platelet membrane. Depolarization using the above procedures enhanced platelet aggregation induced by ADP, adrenaline and collagen. These results suggest that the membrane potential could modulate platelet activity.

Introduction

Membrane potential (V_m) changes are involved in some cellular mechanisms, such as coupling of contractile activity [1] or secretory activity [2,3] to stimulus. Calcium influx, which induces actomyosin activation is related to membrane depolarization in muscle cells [1]. In some secretory cells, such as adrenal and salivary ones, membrane depolarization has been proposed as a second messenger in the sense that it could act as a transduction mechanism. The action of secretagogues on these cells induce changes in V_m [2,3]. Human platelets are activated by several agents including ADP, adrenaline and collagen, which induce a contractile and/or secretory activity [4].

The information available on the possible effect of the V_m changes on platelet activity is controversial. Some authors have shown that depolarization sensitizes human platelets to aggregating agents [5], whereas others deny such as effect [6].

The participation of V_m on platelet activity elicited by ADP, adrenaline and collagen is studied in the present work. To study the influences of V_m on the mechanism of platelet aggregation, we have selected several procedures that change the action of V_m through different mechanisms, such as: (i) high extracellular $[K^+]$ and (ii) Na^+/K^+-ATPase blockade by ouabain or by cooling.

Materials and Methods

Preparation of platelets. Blood was obtained from healthy volunteers of both sexes, ranging in age from 19 to 38 years. They had not taken antiplatelet drugs for at least 2 weeks. Blood was added to 3.8% sodium citrate in a volume ratio of 9:1. and centrifuged for 10 min at $700 \times g$ at room temperature. Platelet-rich plasma was collected in plastic tubes and incubated for 30 min at

Abbreviations: diS-$C_3(5)$, 3,3′-dipropylthiodicarbocyanide; $[K^+]_o$, extracellular potassium concentration; $[K^+]_i$, intracellular potassium concentration.

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room temperature. Any tube containing red cells was discarded. Platelet-rich plasma was centrifuged for 15 min at 2000 × g, platelet-poor plasma was discarded and the platelet pellet as suspended in a standard medium containing (in mM): NaCl (137), KCl (5.4), $NaHCO_3$, (12), $NaH_2PO_4H_2O$ (0.42), $MgCl_2$ (1), $CaCl_2$ (2.9), dextrose (5), and bovine albumin (Calbiochem) 0.35%, 0.15 U/ml of apyrase (Sigma, St. Louis, MO, U.S.A.) and 50 U/ml of sodium heparin (Sigma) were also added to the buffer solution. The pH value was adjusted to 7.4 and the osmolality to 290 mosmol/kg H_2O; the platelets were washed in this medium twice and finally suspended in the same medium without heparin and without apyrase.

Platelet suspensions were used in: (i) K^+-induced depolarization experiments, replacing KCl for NaCl at the desired concentrations (5.4, 20, 40 and 80 mM); (i) ouabain-induced depolarization experiments, incubating the platelet suspension in standard medium with three ouabain (Sigma) doses (10^{-6}, 10^{-5} and 10^{-4} M) for 1 h at room temperature; and (iii) cooling-induced depolarization experiments, incubating samples at 0 °C for 1 h. All other reagents of analytical grade were purchased from Merck Darmstadt, F.R.G.

Measurement of membrane potential. Membrane potential was determined by the fluorescent potentiometric probe 3,3'-dipropylthiodicarbocyanine (diS-C_3(5)), Nippon Kankoh-Shikiso Kenkuysho, Japan). Fluorescence measurements were made on a Kontron SFM-25 spectrofluorimeter equipped with a thermostated cell holder and a stirring device. The samples were excited at 622 nm and the emission was monitored at 670 nm. The signal was first measured in the absence of the probe and after its addition at $2 \cdot 10^{-6}$ M final concentration in ethanol (10 µl in 1 ml of the sample). The fluorescence emission monitored at this moment was taken at initial fluorescence (F_0) and used as the reference value in each experimental design. The fluorescence was also monitored after each experimental maneuver until it reached a plateau. The value read at this moment was taken as final fluorescence (F). The results were reported as a ratio, F_0/F.

Fluorescence changes were studied under the following conditions: (i) platelet suspensions with 5.4, 20, 40 and 80 mM $[K^+]$ in the presence of valinomycin (Sigma) $2 \cdot 10^{-6}$ M; (ii) platelet samples with 5.4 mM $[K^+]$, immediately after 1 h incubation at 0 °C, in the presence of aspirin (Bayer) to avoid the interference of cold-induced reversible platelet aggregation with the fluorescence signal, and after rewarming at 37 °C for 10, 20 and 30 min after initial cooling; and (iii) platelet samples with 5.4 mM $[K^+]$, incubated with ouabain at 10^{-6}, 10^{-5} and 10^{-4} M concentrations. In all cases the final platelet count was adjusted to $6 \cdot 10^7$/ml.

To establish the relationship between fluorescence and V_m we plotted the fluorescence change in presence of valinomycin against $[K^+]_0/[K^+]$, ratio (semilog plot). Extracellular $[K^+]$ was determined with an Instrumentation Laboratory 143 Flame Photometer IL-143. To determine $[K^+]_0$, platelet supernatant was diluted 50 times with 15 mequiv./l LiCl. To determine $[K^+]_i$, platelet pellet was lysed with distilled water, sonicated and finally resuspended with 15 mequiv./l LiCl by stirring. From these data we determined the V_m, in each case, according to the method of Friedhoff and Sonnenberg [7].

Aggregation experiments. For aggregation experiments, platelets were obtained as above and incubated for 30 min at room temperature. Aggregation was assessed under the same experimental conditions as those described above. In the experiments with cold-depolarized platelets, aggregation was assessed in platelets rewarmed 10, 20 and 30 min at 37 °C after cooling, and in the absence of aspirin. Platelets incubated with 5.4 mmol KCl/l without ouabain at 37 °C were considered as controls. The platelet count was adjusted to $2.5 \cdot 10^8$/ml.

Platelet suspension aliquots, (600 µl) were placed in siliconized glass cuvettes (0.8 cm) for use in a Bryston Aggregometer equipped with an Omniscribe Tm Recorder. Aggregation experiments performed according to Born [8], were carried out at 37 °C with platelets being constantly stirred at 1100 rpm with a stirring bar (0.5 cm long). In all cases 5 mg/ml fibrinogen (Kabi, Sweden) were added. In each determination, the amount of light transmission was standardized so that buffer blank would indicate 100% light transmission and platelet samples 0% light transmission. Aggregation inducers, 5 µmol/l ADP and

adrenaline, and collagen, 10 μg/l final concentration (Diagnostica Stago, Asnières, France), were added in each case in saline isotonic solution to a volume of 100 μl. Platelet aggregation was evaluated by measuring the maximal deflection obtained in each case 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 for grouped comparison as appropriate.

Results and Discussion

The fluorescence change induced in platelet suspensions by addition of 2 μM valinomycin was dependent on the $[K^+]_0/[K^+]_i$ ratio (logarithmic scale), considering $[K^+]_i$, (132 mM) as a constant. This relationship was adjusted using linear regression analysis to a straight line $F = 21 \log[K^+]_0/132 + B(1)$ where F is the percent fluorescence change and B a constant ($r = 0.98$, $P < 0.001$). By combining this equation with the the Nernst equation, we calculated that a change of 1% in fluorescence was equivalent to a change of 2.92 mV in the membrane potential. According to this, the membrane potential of human platelets in physiological medium (5.4 mM $[K^+]$) was estimated at -63.8 ± 5.6 mV and the changes of V_m induced by high K^+, ouabain and cooling were determined.

Table I shows the effects of extracellular $[K^+]$, ouabain and cooling on the V_m value of platelets. Platelets incubated with four different extracellular $[K^+]$ showed a decrease in V_m value, from -63.8 mV in physiological medium to -16.7 ± 3.6 mV in 80 mM extracellular $[K^+]$. Incubation with ouabain also provoked a gradual decrease in V_m, reaching maximum depolarization at 10^{-4} M. Immediately after 60 min cooling at 0°C in the presence of aspirin. V_m decreased to -35 ± 6.2 mV. V_m measured in platelets rewarmed at 37°C for 10, 20 and 30 min after cooling were increasingly higher according to the length of the heating period, as showed in Table I. Longer periods of heating, 3 h, did not alter the value estimated after 30 min.

Since it has been described [2] that ouabain produces changes in platelet shape, it is possible that part of the change in fluorescence observed after ouabain exposure is, in fact, artifactual and produced by changes in light scattering associated with the shape change. In order to quantify the genuine change in V_m accurately it is necessary to correct for that potential source of error. Subsequently, in parallel control and ouabain-treated platelet suspensions, the difference in fluorescence signal was measured after equilibrium of the dye was reached (Fig. 1A). Immediately following this, platelets were centrifuged in complete darkness and the fluorescence in the suspension media was measured again. Fig. 1B shows the difference in

TABLE I

EFFECTS OF EXTRACELLULAR $[K^+]$, COOLING AND OUABAIN ON PLATELET MEMBRANE POTENTIAL

Vm was calculated by the method of Friedhoff and Sonnenberg [7] and is expressed as mean ± S.D.; $n = 10$ in all cases.

Depolarizing agents	V_m (mV)
K^+ (5.4 mmol/l)	-63.8 ± 5.6
K^+ (20 mmol/l)	-50.2 ± 5.8
K^+ (40 mmol/l)	-40.6 ± 4.6
K^+ (80 mmol/l)	-16.7 ± 3.6
0°C, 60 min	-35.0 ± 6.2
0°C, 60 min; 37°C, 10 min	-44.0 ± 4.6
0°C, 60 min; 37°C, 20 min	-57.0 ± 4.1
0°C, 60 min; 37°C, 30 min	-62.7 ± 5.3
Ouabain (10^{-6} mol/l)	-50.6 ± 6.2
Ouabain (10^{-5} mol/l)	-40.7 ± 4.2
Ouabain (10^{-4} mol/l)	-35.2 ± 4.8

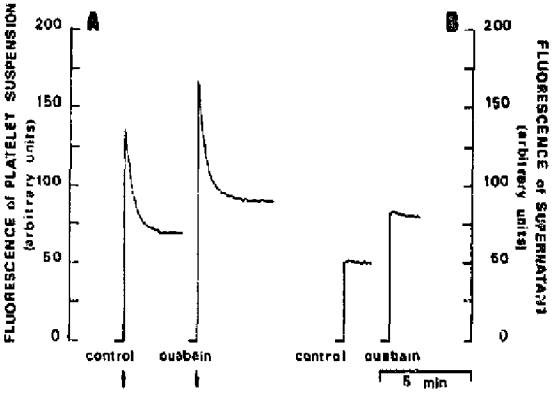

Fig. 1. Fluorescence signal in arbitrary units of (A) Control and ouabain-treated platelet suspensions (arrows indicated diS-C_3(5) addition) and (B) supernatant (platelet-free medium) obtained by centrifugation of samples from A.

TABLE II

EFFECTS ON PLATELET AGGREGATION OF CHANGES OF THE PLATELET MEMBRANE POTENTIAL INDUCED BY K^+, OUABAIN AND COOLING

Aggregation results are expressed as a percentage of mean platelet aggregation ± S.D.; V_m has calculated by the method of Friedhoff and Sonnenberg [7] and is expressed as mean ± S.D. (mV) and its values under each condition are shown in brackets. $n = 10$ in all cases. The K^+ concentration was 5.4 mmol/l and the temperature was 37°C where not indicated.

Conditions	Aggregation inducers		
	ADP (5 μl/l)	adrenaline (5 μmol/l)	collagen (10 μg/ml)
K^+ (5.4 mmol/l, 37°C) (−63.8 ± 5.6 mV)	21.3 ± 4.2	19.0 ± 3.1	48.2 ± 3.0
K^+ (20 mmol/l) (−50.2 ± 5.8 mV)	25.6 ± 3.0	26.4 ± 4.0	51.2 ± 3.2
K^+ (40 mmol/l) (−40.6 ± 4.6 mV)	38.2 ± 4.3 *	34.2 ± 5.1 *	84.0 ± 5.2 *
K^+ (80 mmol/l) (−16.7 ± 3.6 mV)	40.2 ± 3.3 *	38.2 ± 3.2 *	85.2 ± 3.4 *
Ouabain (10^{-6} mol/l) (−50.6 ± 6.2 mV)	22.2 ± 3.1	21.2 ± 4.6	50.2 ± 3.0
Ouabain (10^{-5} mol/l) (−40.7 ± 4.2 mV)	38.1 ± 2.0 *	32.2 ± 3.1 *	82.2 ± 4.3 *
Ouabain (10^{-4} mol/l) (−35.2 ± 4.8 mV)	45.2 ± 2.1 *	47.4 ± 4.2 *	82.4 ± 3.5 *
0°C, 1 h; 37°C, 10 min (−44.0 ± 4.6 mV)	41.2 ± 6.2 *	35.4 ± 4.1 *	96.1 ± 6.0 *
0°C, 1 h; 37°C, 20 min (−57.0 ± 4.1 mV)	27.2 ± 3.1	24.2 ± 4.1	76.0 ± 6.1 *
0°C, 1 h; 37°C, 30 min (−62.7 ± 5.3 mV)	22.4 ± 2.3	20.2 ± 4.0	51.2 ± 3.1

* $P < 0.001$ vs. control.

fluorescence signal in the platelet-free media which is identical to that observed with the platelets in suspension, indicating first a real difference in the distribution of the dye between control and ouabain-treated platelets and then a genuine difference in membrane potential.

Table II shows the effects of changes of $[K^+]_o$, ouabain and cooling with rewarming on platelet aggregation induced by ADP, adrenaline and collagen; the membrane potential values in each condition are also shown. In K^+-depolarized platelets, aggregation induced by ADP, adrenaline and collagen is significantly enhanced ($P < 0.001$). Platelets depolarized by 10^{-5} M ouabain, ($V_m = -40.7$ mV), also show a significant aggregation increase ($P < 0.001$). Platelets incubated for 60 min at 0°C showed a spontaneous, reversible aggregation, but these platelets rewarmed for 10, 20 and 30 min at 37°C after cooling disaggregates spontaneously. The sensitivity of platelets, warmed for 10 min after cooling, to ADP, adrenaline and collagen is significantly higher ($P < 0.001$) when compared to the control. After 20 min warming, the extent of platelet aggregation is not significantly different from that of the control.

Our estimation of −63.8 mV resting V_m for platelets does not differ from previously reported values obtained with potentiometric probes [7,10]. Horne et al. [11] have demonstrated that resting the V_m measured by fluorescent dyes correlates with the transmembrane K^+ redistribution following addition of valinomycin. Like these authors, we have observed that platelet membrane depolarization is proportional to extracellular $[K^+]$. The activity of plasma membrane Na^+/K^+-ATPase plays a major role in the maintenance of cell resting potential and, as in other cells, the platelet Na^+/K^+-ATPase is inhibited by the presence of ouabain. Our results agree with those of Wencel-Drake and Feinberg [10], who have measured the membrane potential changes by studying the distribution of a radiolabeled permeant ion, [^{14}C]thiocyanate and have shown that ouabain 10^{-5} M is able to induce platelet depolarization. The depolarization level observed by these authors is similar to our results obtained using diS-C_3(5). To ensure that the ouabain effects on platelet membrane are not due to a toxic effect on the platelets, we induced a Na^+/K^+-ATPase blockade by cooling [12–15], which induces a similar level of platelet depolarization.

The mechanism of the large potential-dependent fluorescence changes exhibited by cyanine dyes, such as diS-C_3(5), in cell suspensions was shown [16] to result from potential-dependent partition of dye molecules between the cell and the extracellular medium. Cell hyperpolarization results in uptake of the dye molecules by the cells, while depolarization results in release of the dye and in a fluorescence increase. In accordance with this, we measured the fluorescence signal in the presence of platelets and in platelet-free media after incubation with ouabain to ensure that the

fluorescence change corresponds with a genuine membrane-potential change. Our results indicate that the changes in fluorescence after ouabain exposure are due to a genuine membrane-potential change and not to a light-scattering changes.

Depolarized platelets with different $[K^+]_0$ exhibit an increase of aggregation in the presence of ADP, adrenaline and collagen. These observations agree, in part, with the results obtained by Friedhoff and Sonnenberg [7]. Platelets incubated with ouabain, a Na^+/K^+-ATPase blocker, show a significant increase in their aggregation. It is well known that platelet cooling induces spontaneous and reversible platelet aggregation [17]. This effect disappears after platelet warming for 10 min at 37°C, but platelets are still depolarized. This finding suggests that after this time platelets recover their stability, which enabled us to induce experimental aggregation in platelets already depolarized by cooling. Platelets warmed for 10 and 20 min at 37°C after cooling exhibit a significantly enhanced response to some aggregation inducers. This response is in accordance with the depolarization level of the platelets. When the platelet V_m reaches the resting potential after warming for 30 min, platelet aggregation is not significantly different from that of the control.

In normal platelets, ADP, adrenaline and collagen induce aggregation through Ca^{2+} mobilization [18–20]. It is likely that the observed sensitizing effect of depolarization could be explained by an increase in intracellular free Ca^{2+}. Further investigation is needed to ascertain whether Ca^{2+} conductances are modulated by a V_m action on Ca^{2+} inflow or on intracellular Ca^{2+} redistribution. In this context we should mention that preliminary results suggest that the high K^+ effect is independent of intracellular Ca^{2+} levels. Ishikawa and Sasakawa [21] have recently made the same observation on stored platelets. On the contrary, the effects of cooling and ouabain on aggregation are accompanied by an increase of $[Ca^{2+}]_i$, suggesting a possible effect of increased Ca^{2+} on the response.

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