The Membrane Potential of Human Platelets

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The membrane potential of the human platelet was investigated using the membrane potential probes 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide and tritiated triphenylmethylyphosphonium bromide. The membrane potential in physiological buffer was estimated to be 52–60 mV inside negative. The membrane was depolarized when extracellular potassium or hydrogen ion concentrations were increased. Changes in extracellular sodium, chloride, or calcium ion concentration had no measurable effect on membrane potential. Elevated extracellular potassium has been shown to increase platelet sensitivity to the aggregating agent, adenosine diphosphate. Our results show that changes in extracellular ion concentrations that depolarize platelets increase platelet sensitivity to aggregating agents. These results suggest that membrane potential changes may play a role in modulating the response of platelets to aggregating agents.

Platelet aggregation can be induced by a variety of agents. Many of these agents, including adenosine diphosphate (ADP), act by binding to surface receptors. Alterations of platelet sensitivity to these aggregating agents may play a role in the pathophysiology of arteriosclerosis. Because of this, various treatment regimens, which specifically alter platelet sensitivity to aggregating agents, have been used in attempts to prevent the progression of this disease. Some of these attempts appear to have been successful, while the evidence in other cases is less clear.

Membrane potential and/or extracellular potassium concentration have been shown to regulate cell sensitivity to agents that act via surface receptors in brain and parotid. Modulation of platelet function by membrane potential or extracellular potassium concentration could be important clinically since many of the drugs that have been used to treat the complications of arteriosclerosis (i.e., potassium, diuretics, cardiac glycosides, and glucose-insulin-potassium infusions) are known to affect extracellular potassium concentration and/or cellular membrane potential.

Because of these observations, we developed methods for measuring platelet membrane potential in an effort to determine whether platelet sensitivity to ADP is regulated by it. We used the fluorescent membrane potential probe 3,3'-dipropyl-2,2'-thiadicarbocyanine iodide (DiS) to examine the effect of ions on platelet membrane potential. Because of the possibility that these measurements could be subject to artifactual influences, we have repeated them using another probe, tritiated triphenylmethylyphosphonium bromide (TPMP), which is subject to different artifactual influences.

The fluorescent dye technique has been used in the past to measure the electrophysiologic properties of platelets. Horne and Simons have used the fluorescent dye, DiS, to measure changes in platelet membrane potential. They reported changes in response to thrombin. Their data analysis is based on the assumption that platelet membrane potential is not affected by extracellular potassium ion concentration in the absence of ionophores. Because of this, quantitative interpretation of their results is difficult. MacIntyre et al. have published a preliminary report showing changes in platelet membrane potential in response to changes in extracellular ion concentrations, also using DiS.

MATERIALS AND METHODS

Platelet Isolation

Platelets were obtained from normal human volunteers. No donor had used any medication during the preceding 2 wk. Blood was obtained by venipuncture and anticoagulated with 0.0129 M sodium citrate. The pH of the anticoagulated blood was 7.4. The citrated blood was centrifuged at 128 g for 10 min, and the supernatant platelet-rich plasma was removed. Plasma-free platelets were isolated using the gel filtration method of Tangen, Berman, and Marfey as modified by Horne and Simons. The column was equilibrated with a buffer that contained sodium chloride 126 mM, potassium chloride 5.4 mM, Tris hydrochloride 14.5 mM, magnesium chloride 1.0 mM, calcium chloride 0.05 mM, bovine serum albumin 0.5%, and potato apyrase 0.15 U/liter, pH 7.4 (normal buffer). The column had a diameter of 2.8 cm and the gel volume was at least 8 times the volume of the platelet-rich plasma applied. Platelets eluted in the calculated void volume. A volume of gel-filtered platelet suspension, approximately equal to the volume of platelet-rich plasma applied to the column, was collected and used for experiments. The fractions that were most turbid were pooled. Platelets were counted using an American Optical Bright-Line hemocytometer. All surfaces that came into contact with the platelet suspension were either plastic or siliconized glass.
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DiS Fluorescence Studies

A gel-filtered platelet suspension was diluted with appropriate buffer to obtain desired ionic concentrations of the buffer and a platelet count of 55,000/µl. Two milliliters of this platelet suspension were added to a quartz cuvette. Eight microliters of 0.5 mM DiS solution in absolute ethanol were added to the cuvette. Fluorescence was measured using a Perkin Elmer MFP-4 spectrophotometer modified for front surface fluorescence measurements, electrical stirring, and with the cuvette maintained at 37°C by a thermostated water bath. Excitation was at 620 nm and emission was measured at 670 nm. Band widths for both were 6 nm. Dye fluorescence decreased to a constant level within 5 min of addition of dye to the platelet suspension. The platelet suspensions were stirred using fluorescence measurements. All measurements were done using the same setting of the amplifier gain. Under these conditions, the fluorescence of the platelets in normal buffer was 97 U.

Platelet Aggregation

Platelet aggregation was measured using a Payton Dual Beam aggregometer set for a temperature of 37°C and stirred at 900 rpm. Platelet-rich plasma was diluted with either the normal buffer or normal buffer in which all the sodium had been replaced by potassium. One volume of platelet-rich plasma was diluted with 2 volumes of buffer. Aggregation was induced by adding 10 µl of ADP solution to the aggregometer cuvette. The aggregometer was standardized against a solution that contained 1 volume of platelet-poor plasma mixed with 2 volumes of normal buffer. The light transmittance of this solution was defined as 100% aggregation. Platelet-poor plasma was prepared by centrifuging platelet-rich plasma at 10,000 g for 30 min. In this system, suspensions of platelets prepared with normal buffer exhibited half maximal aggregation at ADP concentrations of 0.5–1.0 µM. Concentrations in this range were used in experiments to determine the effect of elevated extracellular potassium on aggregation. The transmittance of the low (4.9 mM) and high (89 mM) potassium platelet-poor plasma was the same.

TPMP, Water, and Methoxyinulin Uptake Studies

A gel-filtered platelet suspension was added to Falcon plastic test tubes (17 x 100 mm) and diluted with appropriate buffer to obtain desired ion concentrations, a platelet count of approximately 2 x 10^9/µl, and a final volume of 1.0 ml. Tritiated TPMP was added in 10 µl of 1 mM solution in water (specific activity 0.666 Ci/mM). Except where noted otherwise, platelets were incubated with TPMP for 90 min and then 200-µl aliquots were withdrawn and layered over a dinonyl phthalate, silicone oil mixture (1:1:v/v for all buffers except those with glucose where a 3:1 mixture was used) in a plastic microfuge tube and centrifuged at 10,000 g for 1 min. After centrifugation, the microfuge tube tip that contained the platelet pellet was sliced off and placed in a scintillation vial. Two-hundred microliters of distilled water were injected into the tip to remove and lyse the pellet. One milliliter of 10% sodium dodecyl sulfate was added to the vial, followed by 10 ml of ACS scintillation fluid (Amersham, Arlington Heights, Ill.) and radioactivity determined using a Packard Tricarb Scintillation Counter.

Intracellular water space and inulin trapping were measured using procedures that were identical to that for TPMP uptake except that tritiated water was incubated with the platelets for 20 min and tritiated methoxyinulin for approximately 1 min. Tritiated water was added as 10 µl of stock solution (2.5 mCi/g) to 1.0 ml of platelet suspension. Tritiated methoxyinulin was added as 10 µl of 1.6 mg/ml solution (419 mCi/g) per 1.0 ml of platelet suspension. All uptake measurements were corrected for extracellular trapping, except when only the kinetics of uptake were studied, in which case trapping was assumed to be the uptake at the first sampling time. All experiments were performed at 37°C. Counting efficiency for tritium was 44%.

Materials

DiS was a generous gift from Professor Alan Waggoner (Amherst College, Amherst, Mass.). Tritiated TPMP, water, and methoxyinulin were purchased from New England Nuclear (Boston, Mass.). ADP, valinomycin, potato apyrase, and bovine serum albumin (BSA) were purchased from the Sigma Chemical Corp. (St. Louis, Mo.). Best results were obtained when BSA cat. no. 9647 was used; thus, this type was used in all experiments. Nonradioactive TPMP was obtained from Pfaltz and Bauer (Stamford, Conn.). All other chemicals were from standard commercial sources. Silicone oil (specific gravity 1.05) was purchased from Aldrich Chemical Co. (Milwaukee, Wisc.).

RESULTS

Figure 1 shows the DiS fluorescence of platelets suspended in normal buffer. When the potassium ionophore, valinomycin, at 2 µM was added, the fluorescence decreased, implying a hyperpolarization. Maximal response to valinomycin was obtained at 2 µM. Therefore, this concentration was selected for the calibration curve. Figure 2 shows the fluorescence in the presence of 2 µM valinomycin as a function of extracellular potassium concentration. As can be seen, the fluorescence was strongly dependent on potassium concentration. Using linear regression analysis, the semilog plot was fit by the straight line,

\[ F = 64.7 \log \left( \frac{K_0}{1} \right) + c \]  

where F is the fluorescence, \( K_0 \) is the potassium concentration.
concentration outside the cell, \( (K)_0 \) is the potassium concentration inside the cell and \( c \) is a constant. Thus, the fluorescence increased by 64.7 U for each tenfold increase in potassium concentration. Since valinomycin is a powerful potassium ionophore, in its presence the membrane potential of the platelets is determined by the Nernst limits of the Goldman equation:15 which at 37°C is given by,

\[
E = 61.5 \log \left( \frac{(K)_0}{(K)_1} \right)
\]

where \( E \) is the platelet membrane potential. Assuming \( (K)_1 \) is constant and combining equations 1 and 2, we find that a change in fluorescence of 1 U is equivalent to a change of 0.951 mV of membrane potential.

Valinomycin induced a fall in fluorescence of 27.4 U in normal buffer (Fig. 1). Thus, we can say that the platelet membrane potential in normal buffer is 26.0 mV less than the potassium equilibrium potential. The latter is defined by equation 2. It has been shown that \( K_1/K_0 \) is approximately 25.16 Thus, the potassium equilibrium potential is \(-86 \) mV, and the platelet membrane potential in normal buffer is \(-60 \) mV. Figure 3 shows the membrane potential changes as measured by DiS fluorescence and TPMP of the platelet suspension when sodium ion was replaced with potassium ion in the absence of valinomycin. If the data are fit to a straight line, we find that the fluorescence increases 30.3 U for each tenfold increase in potassium concentration. This is equivalent to a depolarization of 28.8 mV for each tenfold increase in potassium concentration. Thus, the potential of the platelet was still significantly affected by changes in extracellular potassium even in the absence of valinomycin although, as would be expected, the dependence was less marked. We may conclude that potassium plays a large role in the maintenance of platelet membrane potential even in the absence of valinomycin.

DiS fluorescence was not significantly affected when choline (up to 112 mM) or lithium (up to 99 mM) was added in place of sodium or when gluconate (up to 96 mM) was added in place of chloride. Addition of calcium chloride up to 5 mM caused no significant changes in fluorescence.

Figure 4 shows the membrane potential changes, as measured by DiS fluorescence, that occurred when the extracellular pH was altered. There is a clear dependence of fluorescence on pH. It should be noted that DiS fluorescence is not affected by changes in pH in the absence of cells.11 If the data in Fig. 4 are fit to a straight line we find that the fluorescence increased 18.9 U for each 1 U decrease in pH. This is equivalent to a depolarization of 18.0 mV for each 1 U decrease in pH. These results show that in the absence of valinomycin, platelet membrane potential is determined primarily by extracellular potassium and pH.

Experiments done with DiS were repeated using the radioactive membrane potential probe, TPMP. Figure 5 shows the time dependence of TPMP uptake. It can be seen that the uptake consists of 2 parts, an initial rapid uptake that is complete at 90 min, and a slower
uptake that continues for a much longer time. Incubation with the mitochondrial inhibitor\(^{17}\) oligomycin (50 \(\mu M\)) blocked the uptake after 90 min without appreciably affecting it at earlier times (Fig. 5). This implies that uptake after 90 min is related to a different process than the early uptake. These data were interpreted as reflecting rapid initial uptake across the plasma membrane, which reflects plasma membrane potential, followed by a slower mitochondrial-depen-

\[ E = 61.5 \log \left( \frac{(TPMP)_0}{(TPMP)_i} \right) \]  

where \((TPMP)_0\) is the extracellular concentration of TPMP and \((TPMP)_i\) is the intracellular concentration.

The membrane potential in normal buffer was found to be \(-52 \pm 8.5\) mV (14 experiments). This value is in good agreement with the fluorescence data.

The effect of modification of the ionic composition of the buffer on platelet membrane potential was examined. Since intracellular volume was unaffected by any ionic alteration tested (see Table 1), the potential change caused by ionic alterations was found using the equation,

\[ E' = 61.5 \log \left( \frac{(TPMP)_o}{(TPMP)_i} \right) \]  

where \(E'\) is the change in membrane potential,

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where \(E'\) is the change in membrane potential,
(TPMP)$_N$ is the TPMP uptake in normal buffer, and (TPMP)$_A$ is the uptake in the altered buffer. Figure 3 shows the change in membrane potential, as measured by TPMP uptake, caused when extracellular sodium ion was replaced by potassium ion. It can be seen that there was a strong dependence. If the TPMP data are fit to a straight line, we find that the potential changed by 14 mV for each tenfold change in potassium concentration. There was no measurable change when sodium ion was replaced by lithium or choline. Figure 4 shows the dependence of membrane potential on extracellular pH. It is clear that platelet membrane potential is strongly dependent on extracellular pH. If the data are fit to a straight line, we find that the cells are depolarized by 15 mV for each 1 U decrease in pH. The potential was not measurably affected by addition of 5.0 mM calcium chloride or 37.4 mM gluconate added instead of chloride.

The effect of altered extracellular potassium on platelet aggregation was examined. Figure 6 shows the results obtained when platelets suspended in a mixture of buffer and platelet-rich plasma were exposed to ADP. It can be seen that when the extracellular potassium concentration was elevated (89 mM), the platelets were significantly more sensitive to the aggregating effects of ADP.

**DISCUSSION**

We have elucidated the basic electrophysiology of the human platelet. The membrane potential in physiological buffer is 52–60 mV, inside negative. The potential is most sensitive to alterations in extracellular potassium ion and hydrogen ion concentrations. The change in potential was in the direction predicted by the Goldman equation. Alterations of sodium ion, chloride ion, or calcium concentrations have no measurable effect on platelet resting potential. We have confirmed these observations using 2 probes of membrane potential. Results with both probes are in good agreement. This agreement is of particular importance in the case where extracellular pH was varied, since DiS fluorescence is known to respond to changes in intracellular pH as well as changes in membrane potential. TPMP uptake has not been reported to be affected by changes in intracellular pH. MacIntyre et al. in a preliminary report, showed that alterations of the extracellular concentrations of potassium ion had much greater effects on platelet membrane potential than alterations of sodium or chloride ions, using DiS as a membrane potential probe. Our results are in agreement with theirs. It is worthy of note that alteration of the extracellular chloride concentration had no effect on platelet membrane potential. Horne and Simons assumed that platelet membrane potential was primarily affected by extracellular chloride. This assumption was important in the analysis of their data. MacIntyre et al. estimated the resting potential of platelets at 60 mV, inside negative. Their estimate is in relatively good agreement with our results.

Rudnick has shown that substitution of potassium ion for sodium ion in the extracellular medium does not change the membrane potential of platelet membrane vesicles. Thus, there is a difference between the intact platelet and the platelet membrane vesicle.

Depolarization is not sufficient to trigger aggregation, since elevated extracellular potassium ion does not cause aggregation (Fig. 6). We have confirmed the report that elevated extracellular potassium ion potentiates the aggregating effects of ADP. We have shown that platelets are similar to brain and parotid. In all 3 tissues elevated extracellular potassium ion potentiates the effects of cell surface ligands, and in all 3 cells, elevated extracellular potassium is depolarizing. Since this effect is present in different tissues, it may represent some common mechanism that is important in the regulation of responsiveness to cell surface ligands.

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