Calcium-Activated Potassium Channels in Human Platelets (42963)

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Abstract. **The cationic fluorescent probe, $DiSC_3(5)$ was used to measure the membrane potential in human platelets. Hyperpolarization was induced by the addition of Ca^{2+} to the medium and also by the addition of the Ca^{2+} ionophore, A23187. In the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$) there was no response to A23187. The threshold concentration for $[Ca^{2+}]_o$ was 20 μM and for A23187 was 12 nM. The increase polarity induced by $[Ca^{2+}]_o$ was not affected by various K^+ channel blockers. However, the effect of A23187 was inhibited by quinine and charybdotoxin, while apamin, tetraethylammonium, and the calmodulin inhibitors trifluoperazine and compound R24571 were ineffective. The resting membrane potential was −66 ± 0.9 mV and was decreased by quinine. There are three conclusions from this study: (i) Ca^{2+}-activated K^+ channels exist in human platelets; (ii) they are the type that are apamin insensitive, charybdotoxin sensitive; and (iii) they may contribute to the resting membrane potential.** [P.S.E.B.M. 1989, Vol 192]

Animal cell plasma membranes possess K^+ channels, which, when opened, induce cytoplasmic voltage changes because of the large K^+ concentration gradient. These channels are involved in such dissimilar activities as repolarization of neuronal and cardiac pacemaker tissue (1), secretion by endocrine and exocrine cells (2, 3), and, possibly, responsiveness to mitogens (4). Potassium channels are classified into two broad, but not exclusive, categories, namely, voltage dependent and Ca^{2+} activated (5). These channels are further defined by the size of their conductance and by their response to various channel blockers.

Platelets maintain large ion gradients and a negative potential across the membrane comparable to other cells (6–8) and depolarize when activated by various agents (9, 10). The membrane potential (E_m) of platelets is determined primarily by the membrane permeability to K^+ while Na^+ permeability has little effect; the role of Cl^{+-} permeability in regulating platelet E_m is controversial (10, 11). The types and regulation of K^+ channels in these cells are undefined. Although reported data indirectly suggest the presence of Ca^{2+}-activated K^+ channels in human (9) and porcine platelets (8) this type of K^{pl} channel has not been demonstrated in platelets.

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This study was designed to determine the existence and type of Ca^{2+}-activated K^+ channels in human platelets by monitoring the E_m with a fluorescent probe while using a calcium ionophore and various blockers of these channels.

Materials and Methods

Preparation of Platelets. Eight milliliters of blood were drawn from six Caucasians (three male and three female) whose ages ranged from 24 to 54 years. They had taken no salicylates, nonsteroidal anti-inflammatory drugs, or any other medication for at least 7 days prior to phlebotomy. The blood was collected in siliconized glass tubes containing 1 ml of ACD (1.5 g of acid citrate, 2.5 g of sodium citrate, 2.0 g of dextrose/dl) and centrifuged at room temperature at 700g for 5 min (12). The platelet-rich plasma was separated and centrifuged at 350g for 20 min. The pellet was resuspended in Hepes buffer solution (HBS) of the following composition (in mM): NaCl, 145; KCl, 3; $MgSO_4$, 1, Hepes, 10, dextrose, 10, EGTA, 0.1 (pH 7.4). The platelets were counted in a Coulter Counter; contaminating leukocytes comprised fewer than 2% of the total number of cells.

***E_m* Measurement.** The E_m of platelets was measured by the membrane permeant, cationic, fluorescent probe, 3,3′-dipropylthiadicarbocyanine iodide [$DiSC_3(5)$] (Molecular Probes, Eugene, OR), which distributes across the membrane according to potential differences. The probe was dissolved in dimethyl sulfoxide at 10^{-3} M and stored in the dark at room temperature. Before use it was diluted 1/10 with dimethyl

sulfoxide and 7.5 μl were added to 3 ml of platelet suspension. Aggregation of the cationic probe within the cell quenches the fluorescence; therefore, changes in the extracellular concentration are measured. Eighteen million platelets were suspended in 3 ml of HBS and maintained at 37°C with constant stirring. $DiSC_3(5)$ was added to a final concentration of 250 nM—levels as low as 40 nM yielded qualitatively similar results. The fluorescence was measured at excitation and emission wavelengths of 643 and 666 nm with slits of 5 and 5 nm using a Perkin-Elmer LS-5 fluorospectrophotometer.

E_m standard curves were generated using fluorescence measurements at various concentrations of $[K^+]_o$ in the presence of valinomycin. The E_m, as calculated from the Nernst equation (see below), was linear with the fluorescence between $[K^+]_o$ 3 and 24 mM (in all subjects $r > 0.98$ and $P < 0.001$). These results were verified by the "null point" method in which K^+ was substituted for Na^+ until a concentration was found at which the addition of valinomycin caused neither depolarization nor hyperpolarization. In the presence of valinomycin,

$$E_K = E_m = 61.5 \text{ mV} * \log([K^+]_o/[K^+]_i)$$

where E_K is the equilibrium potential for K^+, and $[K^+]_o$, and $[K^+]_i$ are extra- and intracellular K^+ concentrations, respectively. $[K^+]_i$ was estimated by a null point method using the change in pH_i after addition of the K^+/H^+ ionophore, nigericin (13). The platelets were suspended in HBS at the pH of our platelets (6.90 ± .044) with 4 μM BCECF/AM (2′,7′-bis-(2-carboxyethyl)-5(and-6)carboxyfluorescein, acetoxymethyl ester) and incubated at 37°C for 30 min. They were then washed, centrifuged, and resuspended in Na^+-free HBS containing various K^+ concentrations between 10 and 150 mM. The osmolality was adjusted with LiCl. The $[K^+]_i$ was equal to the $[K^+]_o$ where no change in pH_i was noted after the addition of the K^+/H^+ ionophore, nigericin (0.8 μM). The measured platelet $[K^+]_i$ was 142 ± 3.7 mM. This method has two advantages: submembrane ionized $[K^+]_i$, which is the value used in the equation, is measured, and the necessity to determine cellular water content is eliminated.

$[Ca^{2+}]_i$ Measurement. The platelet suspension in HBS without $[Ca^{2+}]_o$ was incubated with 1 μM fura-2/AM at 37°C. After 30 min the sample was divided, centrifuged at 1000g for 6 min, washed, and resuspended in Ca^{2+}-free HBS. Fluorescence readings at excitation 340 and 380 nm and emission at 505 nm were recorded. A23187 to a final concentration of 12 nM was added to one sample without $[Ca^{2+}]_o$ and to the other after the addition of Ca^{2+} to 1 mM. Fluorescence was read after 2 min when no further change in readings occurred. The equation to convert ratio illumination to concentration is:

$$[Ca^{2+}]_i = 225 * [(R - R_{min})/(R_{max} - R)] * (Sf/Sb)$$

where 225 nM is the K_d for Ca^{2+}-fura-2 interaction, R = ratio at 340/380, R_{max} = ratio after addition of 20 μM digitonin and 1 mM Ca^{2+}, R_{min} = ratio after addition of 10 mM EGTA at pH 8.8, Sb is reading at 380 nm after digitonin plus Ca^{2+}, and Sf is the fluorescence reading at 380 nm in the presence of excess EGTA. The leakage of fura-2 from the cells was estimated by the change in fluorescence before and after the addition of Ca^{2+} to the Ca^{2+}-free suspension; this value was subtracted from the readings after the addition of A23187.

Determination of Effect of $[Ca^{2+}]_o$ on E_m. Platelets in Ca^{2+}-free HBS containing 0.1 mM EGTA were titrated with $CaCl_2$ until maximum hyperpolarization was attained. Valinomycin (2 μM) was then added to determine whether complete polarization had been achieved. The concentration of $[Ca^{2+}]_o$ was calculated using a computer program adapted from Perrin and Sayce (14) using the hydrogen and metal ligand association constants from Stout and Diecke (15). The response to $[Ca^{2+}]_o$ was tested in separate experiments using the K^+ channel blockers: quinine, 50 μM; tetraethylammonium (TEA), 10 mM; apamin, 25 nM; *Leiurus quinquestriatus* venom (LQV), 28 μg/ml and its purified toxin, charybdotoxin (CTX), 20 nM; and by the calmodulin antagonists, trifluoperazine, 3.3 μM; and compound R24571, 500 nM. (All chemicals were purchased from Sigma, St. Louis, MO except charybdotoxin which was a gift from Merck Sharp & Dohme, Rahway, NJ.) These compounds were added in separate experiments 2 min prior to the addition of calcium. At the end of each experiment, valinomycin, and then K^+, was added to verify that the K^+ gradient was still present.

Determination of the Effect of $[Ca^{2+}]_i$ on E_m. A platelet suspension in HBS containing 1 mM Ca^{2+} was titrated with the Ca^{2+} ionophore, A23187, until maximal hyperpolarization was achieved at 12 nM in all cases. This concentration was subsequently used to study the K^+ channels. The various K^+ channel blockers at the doses stated above were added to the platelet suspension prior to the addition of A23187.

Statistics. The data were first evaluated by two-way analysis of variance measuring the effects of sex and the drug. Neither the effects of sex nor the interactions were significant in any instance. Therefore, the data from both sexes were combined, and the drug effects were subsequently tested by paired comparisons. Computations were performed by SAS statistical package for ANOVA and for paired comparisons (TTEST) (16). Multiple comparisons with a control were tested by the method of Dunnet (17). The two-tailed level of significance used was 0.05.

Results

Effect of $[Ca^{2+}]_o$ on E_m. The threshold of $[Ca^{2+}]_o$ to elicit a change in E_m was 20 μM in all six subjects.

There was no additional response upon titrating to concentrations of 2 mM. Maximal hyperpolarization was 34% of that caused by valinomycin. This effect of $[Ca^{2+}]_o$ was not blocked by any of the agents used in this study (data not shown).

Effect of $[Ca^{2+}]_i$ on E_m. In the absence of Ca^{2+} in the medium the $[Ca^{2+}]_i$ increased 5-fold after the administration of 12 nM A23187 (Fig. 1, B_2), whereas in the presence of 1 mM $[Ca^{2+}]_o$ the increase after addition of A23187 was almost 30 times baseline (Fig. 1, A_2). In the presence of 1 mM $[Ca^{2+}]_o$—but not in its absence—the addition of A23187 also caused hyperpolarization (Fig. 1, A_1 and B_1). The threshold concentration of A23187 was 12 nM. This change of polarization was 56% of the difference from resting E_m to E_K. Higher concentrations of the ionophore did not produce further hyperpolarization.

The hyperpolarization induced by A23187 was completed blocked by LQV, CTX, and quinine (Figs. 2 and 3); TEA, apamin, and the calmodulin inhibitors did not affect the hyperpolarization.

Resting E_m. The resting E_m in platelets bathed in Ca^{2+}-containing HBS was -66 ± 0.9 mV vs -54 ± 2.2 mV (mean ± SE, $n = 6$) in Ca^{2+}-free HBS ($P = 0.0025$). Various channel blockers were employed to test the role of Ca^{2+}-activated K^+ channels on the resting E_m. In the presence of $[Ca^{2+}]_o$ the platelets were slightly, but significantly, depolarized by quinine (3.2 ± 0.7 mV, $P = 0.0064$). The fluorescence artifact caused by quinine was measured in disrupted platelets and could account for only a small portion of the apparent effect. TEA, apamin, and CTX had no effect on resting E_m when Ca^{2+} was present in the medium. Without $[Ca^{2+}]_o$ the cells were again depolarized by quinine (22 ± 1.7 mV, $P < 0.0001$); under these conditions CTX and LQV also caused significant depolarization. TEA had no effect on resting E_m, whereas, apamin caused a slight—but significant—increase in polarity.

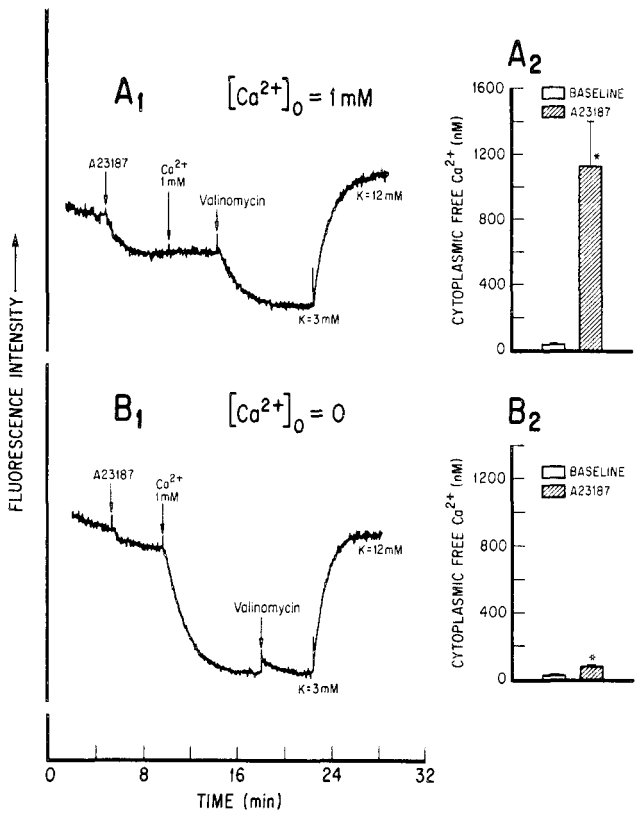

Figure 1. Changes in E_m after administration of A23187 in the presence (A_1) and absence (B_1) of $[Ca^{2+}]_o$. The response to valinomycin (E_K) and to $[K^+]_o$ demonstrates the preservation of the potential gradient. The mean cytoplasmic free Ca^{2+} is depicted before (baseline) and after the addition of A23187 in the presence (A_2) and absence (B_2) of $[Ca^{2+}]_o$. Mean ± SE, $n = 6$; $^*P < 0.01$ as compared with the baseline value.

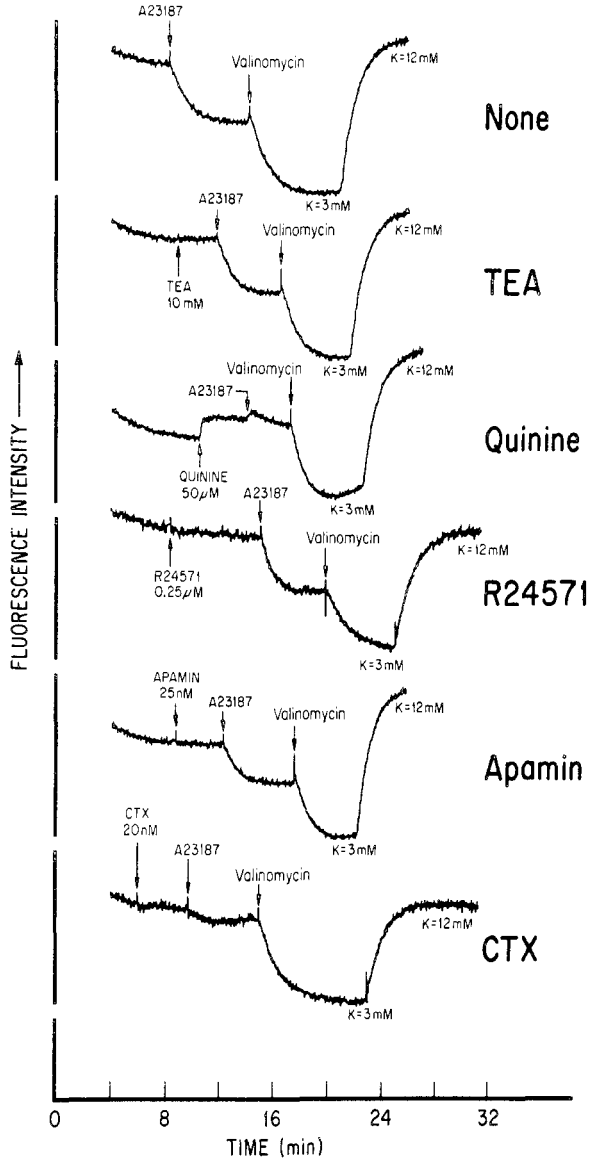

Figure 2. Changes in platelet membrane potential after addition of A23187 to a final concentration of 12 nM. The platelets were suspended in HBS ($[K^+] = 3$ and $[Ca^{2+}] = 1$ mM) containing various Ca^{2+}-activated K^+ channel blockers. Valinomycin (2 μM) and K^+ were added at the end of each experiment to verify the response of the cells to K^+ gradients. These studies are from the same subject and are representative of the other five subjects (as summarized in Fig. 3).

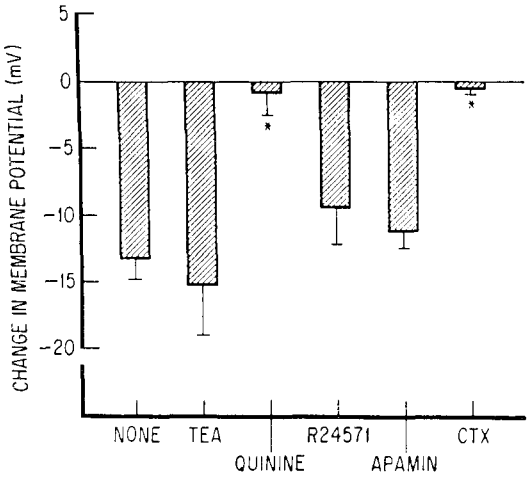

Figure 3. Average E_m changes in platelet suspensions from the six subjects after addition of A23187 in the presence of Ca^{2+}-activated K^+ channel blockers. Mean ± SE, $n = 6$; $^*P < 0.01$ as compared with "none" group (control) by the Dunnet test (17).

Discussion

The cationic fluorescent dye $DiSC_3(5)$ was used as an indicator of platelet E_m. Results obtained with this probe in platelets have been validated by other using titrated triphenylmethylphosphonium bromide (11). The cyanine dyes do have some membrane toxicity, although the effect is small at the low probe concentrations and high $[K^+]_o$ we used (18, 19). The advantage of the cyanine dyes is that standard curves to quantitate E_m can be constructed using valinomycin. This is not possible with the anionic oxonol dyes which aggregate with valinomycin-K^+ complexes (20).

The results from this study suggest that human platelets possess Ca^{2+}-activated K^+ channels that can be opened by both $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$. The opening of these channels by $[Ca^{2+}]_o$ has also been shown in other cell types (21–23), although MacIntyre and Rink (10) were unable to alter E_m of human platelets by the addition or removal of Ca^{2+} from the bathing solution. In our study the threshold concentration of $[Ca^{2+}]_o$ needed to repolarize the platelets was 20 μM. A recent study has suggested that in vascular smooth muscle cells the active level of $[Ca^{2+}]_o$ is in the mM range and may affect resting E_m (22). Inoue *et al.* (24) have recently described Ca^{2+}-activated K^+ channels in portal vein cells that are opened by $[Ca^{2+}]_o$ at a threshold of 0.2–7.5 μM; a maximal response was reached at a concentration of greater than 600 μM. In their patch clamp experiments, these channels (K_{Ca} channels) were blocked by TEA. However, we were unable to block the polarizing effects of $[Ca^{2+}]_o$ by TEA concentrations as high as 10 mM or by quinine, apamin, CTX, or the calmodulin antagonists.

The opening of Ca^{2+}-activated K^+ channels by A23187 has been demonstrated in diverse cell types and by various techniques. This study shows that in platelets in the presence of $[Ca^{2+}]_o$, but not in its absence, A23187 causes hyperpolarization presumably by transporting Ca^{2+} into the cell and, thereby, activating the K^+ channels. The ionophore threshold was 12 nM; this concentration raised the $[Ca^{2+}]_i$ to μM levels and appeared to give the maximal E_m response. The highest degree of hyperpolarization after activation by A23187 was 56% of the difference between resting E_m and E_K. The failure of complete hyperpolarization after A23187 could indicate a low number of Ca^{2+}-activated K^+ channels or partial inhibition of these channels, possibly by the E_m indicator dye (18).

Quinine, in low concentrations (50 μM) that only slightly reduced the resting E_m, completely blocked the response to increased $[Ca^{2+}]_i$. Quinine is generally accepted as an inhibition of Ca^{2+}-activated K^+ channels but has been reported to inhibit Ca^{2+}-independent K^+ channels in insulin-secreting cells (25). Our results indicate that platelets possess K^+ channels that can be blocked by quinine.

The Ca^{2+}-activated K^+ channels in platelets in this study were shown to be the type that are insensitive to apamin. The venom from the Israeli scorpion *Leiurus quinquestriatus* contains a peptide, charybdotoxin, that blocks the apamin-insensitive, large conductance Ca^{2+}-activated K^+ channels (26, 27). Human platelets in our study appear to possess these channels—they are insensitive to apamin and are completely inhibited by LQV and the purified protein (CTX) in doses previously reported to block these channels (26, 28).

Platelet suspensions in Ca^{2+}-containing medium had an average resting E_m equal to −66 mV, which agrees closely with reported results of −52 to −60 mV (6, 11). The variability between subjects in our study was small with a coefficient of variation of 3.3%. However, when bathed in a Ca^{2+}-free medium—a common practice when using platelets—the resting E_m was decreased significantly to −54 mV. The resting E_m was reduced also by quinine, an indication that the resting potential in human platelets is partially dependent on this channel. We can explain neither the slight hyperpolarization produced by apamin nor the finding that CTX decreases the resting E_m only in the absence of extracellular Ca^{2+}.

In this study we have identified Ca^{2+}-activated K^+ channels in human platelets that are blocked by quinine and CTX and are insensitive to TEA, apamin, and calmodulin blockers. These channels appear to play a role in setting the resting membrane potential.

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