Membrane depolarization inhibits thrombin-induced calcium influx and aggregation in human platelets

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The relationship between thrombin-evoked changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and aggregation was examined in Indo-1-loaded human platelets. The stimulus-induced intracellular calcium release and external calcium influx, as well as platelet aggregation, were studied in the same cell preparation. A close correlation between the sustained high $[Ca^{2+}]_i$ level, depending on calcium entry, and the aggregation response was found. Gramicidin, at a concentration high enough to induce membrane depolarization, strongly inhibited the calcium influx and aggregation, but did not influence the thrombin-induced intracellular calcium release. We conclude that calcium influx through depolarization-inhibited calcium channels is a prerequisite of thrombin-induced platelet aggregation.

Human platelet; Aggregation; Thrombin; Calcium flux; Receptor-operated calcium channel; Membrane potential

1. INTRODUCTION

Human platelets react to different stimuli with shape change, aggregation and degranulation, which are correlated with a rise in $[Ca^{2+}]_i$ [1]. Agonists induce different calcium fluxes such as the discharge of internal stores, calcium influx, and extrusion through the plasma membrane and the membrane of intracellular organelles [2-4]. In thrombin-activated platelets, the mechanism of calcium liberation from internal stores has been characterized [5-7]. On the other hand, the nature and regulation of the receptor-mediated calcium influx pathways are not completely understood [8-10]. Recently three different receptor-mediated calcium entry mechanisms have been proposed in human platelets [11]. It has been suggested that these calcium channels are different from the voltage-gated channels of excitable cells [12-15]. As to the influence of the membrane potential changes on the calcium influx pathway or the cell functions, depending on the sustained increase of $[Ca^{2+}]_i$ in different cell types, contradictory results have been published [16-20].

In the present paper we report a correlation between the thrombin-induced receptor-operated calcium movements and platelet aggregation, as well as on the membrane potential sensitivity of the thrombin-mediated calcium influx pathway. By using Indo-1-loaded intact platelets the two components of the stimulus-evoked calcium signal (see also [10]), as well as the partial and full-scale aggregation could be studied.

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Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular free Ca^{2+} concentration; diS-C_3-(5), 3,3'-dipropylthiodicarbocyanine; EGTA, ethyleneglycol-bis-(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)- 1-piperazine-ethanesulfonic acid; Indo-1/AM, acetoxy-methylester of Indo-1

2. MATERIALS AND METHODS

2.1. *Reagents and cells*

BSA, EGTA, Hepes, gramicidin, indomethacin, PGE_1 and valinomycin were purchased from Sigma. Bovine thrombin was obtained from Hoffmann LaRoche, fibrinogen was from Kabi. Indo-1/AM was from Calbiochem, wherease diS-C_3-(5) was a gift from Dr A. Waggoner. All chemicals used were of reagent grade.

The standard incubation medium (Na^+-Tyrode) contained 128 mM NaCl, 2.56 mM KCl, 0.5 mM NaH_2PO_4, 10 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 8.5 mM Hepes-Na and 1 g/l glucose at pH 7.25. In choline$^+$-Tyrode the Na^+ ions were replaced by choline-Cl, except for NaH_2PO_4 and $NaHCO_3$; thus the remaining Na^+ concentration was 10.5 mM.

Human platelets were isolated from freshly drawn blood of healthy volunteers as described previously [10]. The final concentration of platelets was set at 3×10^8 cells/ml.

For the *$[Ca^{2+}]_i$ measurements* platelets were loaded in Na^+-Tyrode, supplemented with 50 nM final concentration of PGE_1, 50 μM of indomethacin and 3.5 g/l BSA, at 37°C for 30 min with 0.2 μM final concentration of Indo-1/AM. Before each fluorescence measurement an aliquot of the cell suspension was rapidly centrifuged (12 000 × *g*, 10 s), the pellet was rinsed 4 times with the appropriate medium and then the cells were resuspended in 2 ml of the same medium (1.5×10^8 cells/ml). Fluorescence was measured in a Hitachi F-4000 fluorescence spectrophotometer at 37°C with continuous stirring. For Indo-1 the excitation wavelength was set to 331 nm, emission was measured at 410 nm (bandwidth 5 nm). $[Ca^{2+}]_i$ was calculated according to the method of Grynkiewicz et al. [21] (see also [10]).

Membrane potential in intact Indo-1-loaded platelets was estimated by using the fluorescent dye diS-C_3-(5) based on the method described

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in [22,23]. For calibration the cells were treated with valinomycin (1 μM), the extracellular K^+ concentration was raised gradually and the changes of the fluorescence were followed (620 nm excitation and 670 nm emission wavelengths).

Platelet aggregation was measured in Indo-1-loaded platelets (1.5×10^8 cells/ml final concentration in a Lumi-aggregometer (Chronolog, Model 460, Pica) at 37°C with gentle stirring. The media were supplemented with 25 μg/ml fibrinogen.

The experiments were repeated at least 5 times with platelets from different donors. The intraassay variability was less than 5–10%.

3. RESULTS AND DISCUSSION

Consistent with our previous observations [10], we detected a partial aggregation and a transient $[Ca^{2+}]_i$ elevation in the presence of 0.5 mM EGTA following the addition of thrombin (0.03–0.05 U/ml) in human platelets (Fig. 1, traces a). If $[Ca^{2+}]_o$ was adjusted to 0.5 mM free Ca^{2+} after the thrombin-stimulus, a sustained elevation of $[Ca^{2+}]_i$ and a parallel full-scale aggregation were observed (traces d). The thrombin-induced Ca^{2+} entry and the full-scale aggregation did not considerably change if $[Ca^{2+}]_o$ was varied between 10 μM and 2 mM (data not shown). When excess EGTA (2 mM final concentration was added to the medium, $[Ca^{2+}]_i$ strongly decreased, and partial inhibition of the aggregation was detected. The extent of this inhibition showed a close correlation with the duration of the Ca^{2+} entry. If the addition of excess EGTA followed the Ca^{2+} repletion by 15 s, a 35–45% inhibition was observed, and if this time was 45 s, a 15–25% inhibition of the full-scale aggregation was observed (Fig. 1, traces b and c). To obtain a maximum aggregation at least 1–1.5 min sustained $[Ca^{2+}]_i$ elevation was necessary.

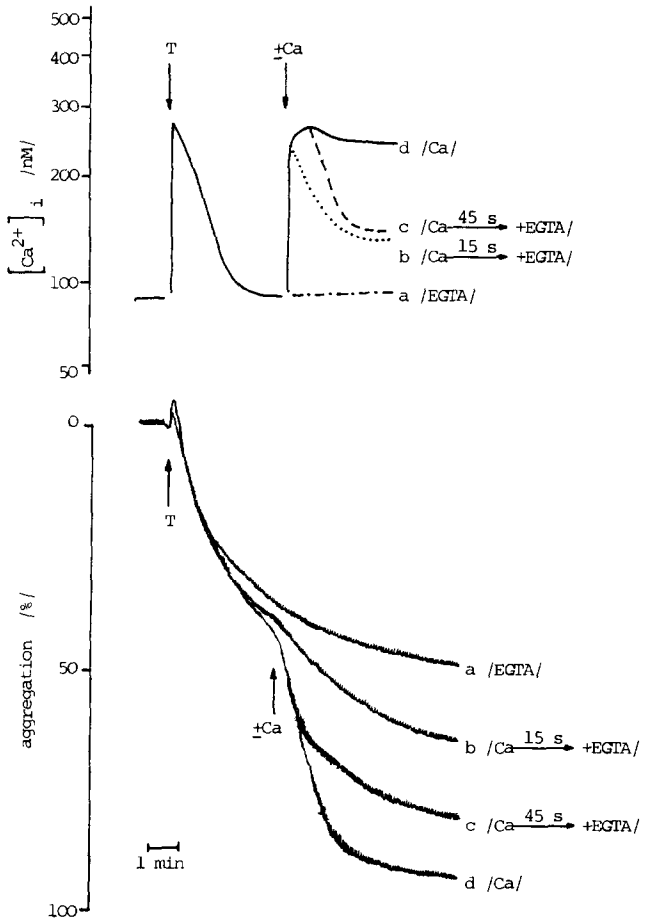


Fig. 1. Parallel recording of the thrombin-induced changes in $[Ca^{2+}]_i$ and aggregation of Indo-1-loaded platelets. The cells were suspended in Na^+-Tyrode containing 0.5 mM EGTA. (Traces a) Control, stimulation in the presence of 0.5 mM EGTA by 0.04 U/ml thrombin (T). (Traces b, c and d) 3.5 min after the addition of thrombin 1 mM $CaCl_2$ (Ca) was added. (Traces b and c) Excess EGTA (2 mM final concentration) was added 15 s (b) and 45 s (c) after the addition of calcium, respectively.

As demonstrated in Fig. 2, gramicidin selectively inhibited the thrombin-activated Ca^{2+} entry. In Na^+-Tyrode, containing 0.5 mM free Ca^{2+}, gramicidin (125 nM) significantly lowered the sustained $[Ca^{2+}]_i$ elevation and inhibited the aggregation by approximately 50% (Fig. 2A). In the virtual absence of extracellular free Ca^{2+}, the ionophore dit not modify the extent and the time-course of either the Ca^{2+} release from internal stores or the partial aggregation (in the presence of 0.5 mM EGTA - panel B). In contrast, gramicidin strongly inhibited the Ca^{2+} influx and the second phase of the full-scale aggregation (aggregation following the addition of 1 mM $CaCl_2$), independently of the sequence of thrombin and gramicidin additions (panels B and C).

As shown in Fig. 3, in choline$^+$-Tyrode (that is, in a medium containing extremely low (10.5 mM) extracellular Na^+) the calcium signal and the aggregation response were similar to that seen in Na^+-Tyrode. In choline-Cl based medium, gramicidin was not effective; bot curves were practically identical to those in the control cells.

When measuring the changes in membrane potential of Indo-1-loaded platelets with diS-C_3-(5), we found that in Na^+-Tyrode the resting value was -75 ± 3 mV. The addition of gramicidin (125 nM) caused fast depolarization to approximately 0 mV. In cholin$^+$-Tyrode in which this ionophore does not allow the permeation of choline$^+$ [24], it caused only a slight depolarization from -72 ± 3 mV to -54 ± 2 mV due to the presence of 10.5 mM Na^+ in the medium.

The data presented here indicate that gramicidin does not directly inhibit the thrombin-induced calcium movements and platelet aggregation, but selectively abolishes the receptor-operated calcium influx by depolarizing the cells. Similar findings were recently published by our group for Jurkat lymphoblasts [20]. The profound effect of changes in intracellular Na^+ and K^+ concentrations, wich probably occurs after gramicidin addition, on platelet aggregation can be ruled out by the finding that the addition of a calcium ionophore (A 23187) plus Ca^{2+} caused aggregation even in the presence of gramicidin (data not shown here).

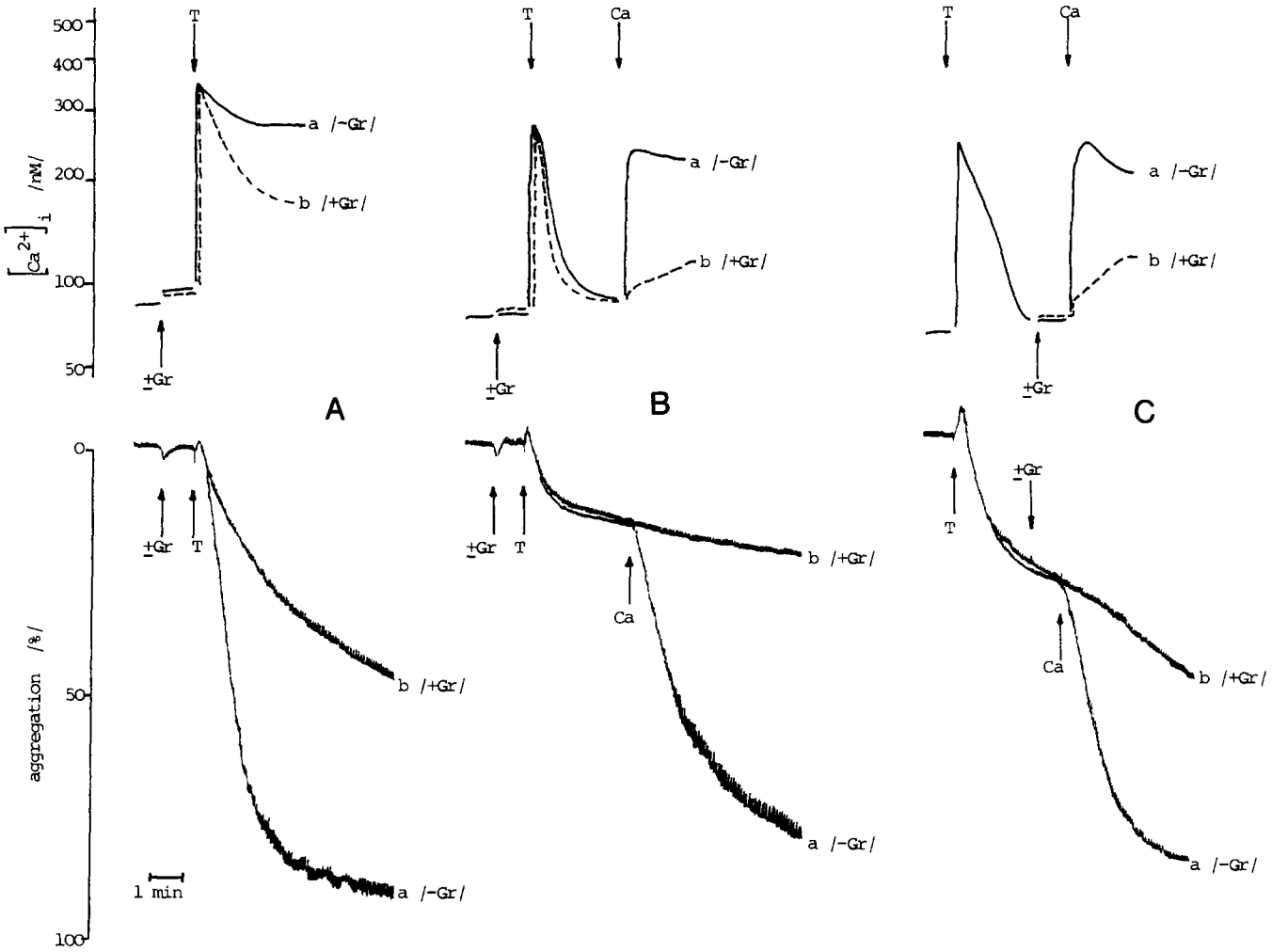


Fig. 2. Effect of gramicidin on the changes in $[Ca^{2+}]_i$ and aggregation of Indo-1-loaded platelets in Na^+-Tyrode. (A) The medium contained 0.5 mM EGTA and 1 mM $CaCl_2$. (B and C) The medium contained 0.5 mM EGTA. Thereafter 125 nM gramicidin (Gr), 0.04 U/ml thrombin (T) and 1 mM $CaCl_2$ (Ca) were added where indicated by the arrows.

From the results described here we conclude: (i) there is a strong correlation between the sustained $[Ca^{2+}]_i$ elevation, depending on the calcium entry, and the development of full-scale aggregation; (ii) the thrombin-induced calcium influx can be inhibited by membrane depolarization in human platelets.

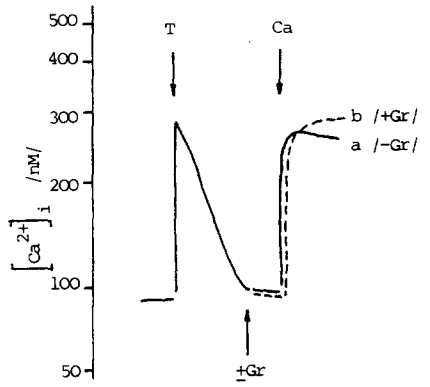
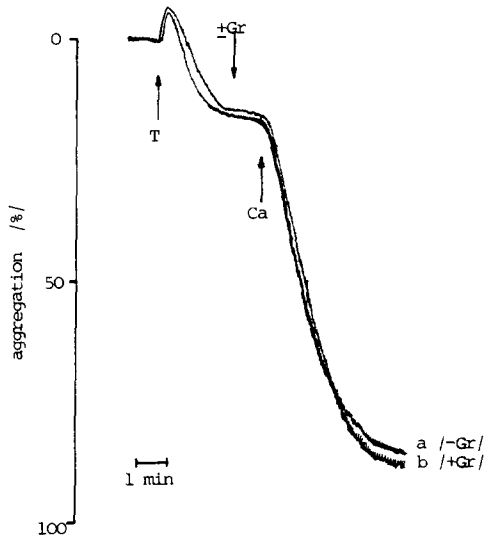


Fig. 3. Effect of gramicidin on the changes in $[Ca^{2+}]_i$ and aggregation of Indo-1-loaded platelets in choline$^+$-Tyrode. 0.04 U/ml thrombin (T), 125 nM gramicidin (Gr) and 1 mM $CaCl_2$ (Ca) were added where indicated by the arrows.



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