Receptor occupancy regulates Ca^{2+} entry and intracellular Ca^{2+} redistribution in activated human platelets

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Fura-2-loaded human platelets were used to study Ca^{2+} release from intracellular compartments, as well as Ca^{2+} influx from the extracellular space. We investigated the response towards the endoperoxide/thromboxane-receptor agonist, U46619, and the inhibitor of the endoplasmic-reticulum Ca^{2+} -ATPase, thapsigargin. U46619 dose-dependently depleted intracellular Ca^{2+} stores, followed by active sequestration of released Ca^{2+} . Ca^{2+} influx induced by U46619 largely relies on receptor occupancy. Removing the thromboxane analogue from its receptor by using the endoperoxide/thromboxane-receptor antagonist BM13177 largely blunted U46619-mediated Ca^{2+} influx. The Ca^{2+} -ATPase inhibitor thapsigargin evoked a gradual rise in intracellular Ca^{2+} , which was potentiated by a preceding activation of platelets with the receptor agonist U46619. This agonist-sensitizing effect also depends on receptor occupancy. Removing U46619 from its receptor by addition of the endoperoxide/thromboxane-receptor antagonist BM13177 suppressed the sensitizing effect completely.

Furthermore, interrupting downstream receptor signalling events by raising intracellular levels of cyclic nucleotides (cyclic AMP, cyclic GMP) again suppressed the U46619-sensitizing effect on thapsigargin-induced Ca^{2+} release. This study indicates that the process of Ca^{2+} release followed by resequestration in response to a platelet agonist by its own is not sufficient to produce the sensitizing effect. Rather, a continuously occupied receptor triggering sustained downstream signalling events seems to be required for sensitization. The presence of a receptor agonist may induce an increased cycling of Ca^{2+} between the agonist-responsive and the thapsigargin-dischargeable compartment, leading to faster and more intense accumulation of Ca^{2+} in the cytosolic compartment after inhibition of the Ca^{2+} -ATPase. Suggestively, receptor occupancy increases the Ca^{2+} -releasing potency of thapsigargin by coupling the thapsigargin-sensitive Ca^{2+} -storing compartments with an agonist-responsive compartment that exhibits a high leakage rate in stimulated platelets.

INTRODUCTION

Activation of human platelets initiates an ordered sequence of events, associated with phospholipase C activation and a rapid elevation of $[\text{Ca}^{2+}]_i$ as a major component of signal transduction [1,2]. Ca^{2+} increase is accompanied by hydrolysis of phosphatidylinositol 4,5-bisphosphate, IP_3 formation, 1,2-diacylglycerol production, and activation of protein kinase C [3,4]. The concerted action of these signalling events induces subsequent platelet responses such as aggregation, secretion and thromboxane A_2 formation (for reviews, see [5,6]). Platelets show an elaborate mechanism of Ca^{2+} signalling, involving IP_3 -induced mobilization of stored Ca^{2+} and receptor-mediated Ca^{2+} entry across the cell membrane (for reviews, see [7,8]). Although receptor-induced Ca^{2+} influx has attracted ample attention regarding the channels involved, their mechanisms of activation are not fully understood (for detailed information, see [9–13]). In this context the following mechanisms were suggested: first, activation of the channels by IP_3 and/or higher phosphorylation products of this initial messenger; second, a G-protein-mediated coupling between the receptor and the channel; third, a ‘capacitative’ link between intracellular stored Ca^{2+} and transmembrane influx [10]. In this model a messenger termed CIF (Ca^{2+} -influx factor) was proposed [14], and the involvement of GTP seems likely [15,16].

IP_3 [17,18] and inhibitors of endoplasmic-reticulum ATPases were shown to increase $[\text{Ca}^{2+}]_i$ in permeabilized and intact cells, respectively. TG [19] and tBuBHQ [20] cause Ca^{2+} -store depletion, resulting from prevention of re-uptake. Therefore, both

compounds are useful tools with which to study intracellular Ca^{2+} release without involving receptor-coupled activation mechanisms.

TG and tBuBHQ have been shown to evoke Ca^{2+} mobilization [21] and Ca^{2+} entry in human platelets [22]. Along with our initial observation, further reports [23] demonstrated that platelet aggregation, thromboxane formation and secretion are significantly attenuated when the platelet self-amplifying eicosanoid formation is prevented. At least in human platelets, TG partially acts as an indirect receptor agonist, as long as endoperoxide/thromboxane formation is allowed. Similar observations account for TG-elicited Ca^{2+} responses. TG produces an elevation of $[\text{Ca}^{2+}]_i$ indistinguishable from that of a thrombin response. However, blocking the platelet self-amplification system [24] by not allowing the formation of endoperoxides/thromboxane (cyclo-oxygenase inhibition) or by inhibiting the endoperoxide/thromboxane receptor dramatically decreases the Ca^{2+} traces after TG addition, but not after thrombin (cyclo-oxygenase-independent concentrations) stimulation [25]. These experiments suggest the presence of at least two distinguishable types of intracellular Ca^{2+} -storing compartments in platelets, a TG-sensitive and a TG-insensitive Ca^{2+} compartment. The latter most probably is identical with the IP_3 -sensitive store. In contrast, studies with other cells have elucidated a certain degree of overlap between the TG-sensitive and IP_3 -sensitive intracellular stores, although cell-specific differences were reported (for references, see [8]). Interestingly, in platelets the prior redistribution of Ca^{2+} by receptor agonists potentiates subsequent

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic intracellular Ca^{2+} concentration; TG, thapsigargin; tBuBHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone; TxB_2 , thromboxane B_2 ; IP_3 , inositol 1,4,5-trisphosphate; cGMP, cyclic GMP; cAMP, cyclic AMP; U46619, 15S-hydroxy-11,9-(epoxymethano)prosta-5,13-dienoic acid.

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Ca^{2+} mobilization induced by TG [25]. An explanation would be that the sub-population of TG-depletable Ca^{2+} stores is enlarged by prior platelet activation, by diverse receptor agonists. Confirmatory results were reported by Heemskerk et al. [26], suggesting an agonist-induced shift of Ca^{2+} from an agonist-responsive to a TG-sensitive intracellular store. We now extended these studies, using the stable thromboxane-receptor agonist U46619 in the absence and presence of its receptor antagonist BM13177. Focusing on intracellular Ca^{2+} release, our results demonstrate a strict correlation between agonist-receptor occupancy and the ability of TG to promote enhanced Ca^{2+} accumulation in the cytosol. Displacement of the agonist from its receptor or interruption of the receptor signalling event prevents a subsequent enlarged TG-induced $[\text{Ca}^{2+}]_i$ increase, although the agonist-responsive store had been discharged beforehand. We suggest an intracellular Ca^{2+} -cycling mechanism as a result of receptor occupancy. Interference with the membrane signalling event induced by U46619 decrease the ability of TG to promote significant Ca^{2+} accumulation in the cytoplasmic space.

EXPERIMENTAL

Materials

U46619 was purchased from Paesel, Frankfurt, Germany, and thrombin was bought from Hoffmann-La Roche, Basel, Switzerland. Prostacyclin, sodium nitroprusside, ibuprofen and BW 755C were purchased from Sigma Chemie, Deisenhofen, Germany. BM13177 (sulotroban) was delivered by Boehringer Mannheim, Mannheim, Germany. Tg was obtained from Gibco, Eggenstein, Germany. All other materials were as previously described [25] or were obtained in the highest grade of purity available from local commercial sources.

Preparation of platelet-rich plasma and washed human platelets

Platelet-rich plasma (PRP) and suspensions of washed human platelets were mainly prepared as previously outlined [25]. Briefly, fresh human blood was anticoagulated by using trisodium citrate (0.38 %, w/v) and PRP was obtained by centrifugation at 200 g for 20 min. PRP was removed, prostacyclin (50 ng/ml) was added and platelets were separated by centrifugation (1200 g, 10 min), washed once, and resuspended in a HEPES-buffered Tyrode's solution (138 mM NaCl, 0.36 mM NaH_2PO_4 , 2.9 mM KCl, 1 mM MgCl_2 , 5 mM glucose, 20 mM HEPES, pH 7.4). Incubations $[(5-8) \times 10^8$ platelets/ml] were performed at 37 °C for the times indicated. Platelet cyclo-oxygenase and lipoxygenase were inhibited by preincubation with 100 μM BW 755C or 150 μM ibuprofen for 2 min before starting each individual experiment. Platelet endoperoxide/thromboxane receptor was blocked with 200 μM BM13177.

$[\text{Ca}^{2+}]_i$ measurements

These were carried out in intact platelets as stated in [25]. When necessary, external Ca^{2+} was removed by adding 0.5 mM EGTA. For the Ca^{2+} -overshoot experiments, the indicated concentration of free Ca^{2+} was added at the end of the experiment as described in the individual Figures. In parallel experiments, the small $[\text{Ca}^{2+}]_i$ changes occurring in non-stimulated cells as a consequence of chelation of Ca^{2+} in the medium and its re-introduction were carefully evaluated. One example is presented in Figure 1 (lower trace). Corresponding values were subtracted graphically, in correct proportion to the logarithmic scale, from the experimental traces of stimulated cells. Briefly, platelets were incubated with 2 μM fura-2 acetoxymethyl ester at 37 °C for 40 min, washed and

resuspended in the buffer described above. Changes in fluorescence of the fura-2-loaded platelets at the excitation wavelengths of 335 and 362 nm and emission wavelengths above 450 nm were determined after addition of antagonists and agonists by using a Sigma ZWS-II dual-wavelength spectrofluorimeter (Biochem, Puchheim, Germany). The ratio of the fluorescence is a measure of platelet $[\text{Ca}^{2+}]_i$ [27]. The ratios of maximum and minimum fluorescence were determined by addition of 250 μM digitonin in the presence of 1 mM free Ca^{2+} and in the presence of 10 mM EGTA (pH > 8.5), respectively. Changes in $[\text{Ca}^{2+}]_i$ are recorded as relative variations, giving arbitrary fluorescence units on a logarithmic scale. For comparison, resting and maximal $[\text{Ca}^{2+}]_i$ values (i.e. nM concentrations) after individual agonists are given in the legend of each Figure. Platelets suspended in a nominally Ca^{2+} -free buffer showed resting levels in the range 70–110 nM, whereas the basal $[\text{Ca}^{2+}]_i$ of platelets in the presence of EGTA ranged from 40 to 90 nM. Platelets not showing basal Ca^{2+} values within the given range were eliminated from further studies. Small variations in the Ca^{2+} response as well as solvent carriers or inhibitors affected neither the method nor the overall observations.

Statistical methods

Ca^{2+} traces show one typical experiment out of a minimum of five different experiments. Variability did not exceed 10 % for the effect observed.

RESULTS

Ca^{2+} release and influx in response to U46619

U46619, a commonly used stable thromboxane-receptor agonist, is known to activate human thrombocytes by binding to the endoperoxide/thromboxane receptor. Human platelets resuspended in the presence of EGTA (Figure 1) responded dose-

Figure 1 Ca^{2+} mobilization and Ca^{2+} entry induced by U46619

Fura-2-loaded human platelets suspended in the presence of 0.5 mM EGTA were used to measure Ca^{2+} release from intracellular stores, followed by determination of Ca^{2+} influx as outlined in the Experimental section, in response to various U46619 concentrations. None of the agents or their carrier solvents had a direct effect on fura-2 fluorescence. Resting $[\text{Ca}^{2+}]_i$ was 87 nM; $[\text{Ca}^{2+}]_i$ after 4 μM U46619 was 342 nM. The lower trace illustrates the changes in fluorescence observed in resting cells investigated in parallel after EGTA and Ca^{2+} application.

Figure 2 Ca^{2+} release and Ca^{2+} influx in response to U46619

Human platelets were used to measure intra-platelet Ca^{2+} mobilizations and Ca^{2+} influx as described in Figure 1. The endoperoxide/thromboxane-receptor agonist (U46619), the receptor antagonist (BM13177) and Ca^{2+} were added as indicated. Resting $[\text{Ca}^{2+}]_i$ was 84 nM; $[\text{Ca}^{2+}]_i$ after 2 μM U46619 was 337 nM. In this and subsequent Figures, a dash in explanations of traces indicates no addition.

Figure 3 Intracellular Ca^{2+} release by U46619 and thapsigargin

$[\text{Ca}^{2+}]_i$ was measured in human platelets by using the fluorescent Ca^{2+} indicator fura-2 as described in the Experimental section. Extracellular Ca^{2+} was chelated by addition of 0.5 mM EGTA. U46619 (U46; 1 μM) and TG (1 μM) were applied according to the time schedule indicated, at the concentration given. The cyclo-oxygenase inhibitor ibuprofen (Ibu; 150 μM) was used as shown. Resting $[\text{Ca}^{2+}]_i$ was 90 nM; $[\text{Ca}^{2+}]_i$ after 1 μM TG was 385 nM.

dependently, showing an increase in $[\text{Ca}^{2+}]_i$ after addition of U46619. The transient Ca^{2+} increase is followed by a re-uptake phase, allowing Ca^{2+} to reach basal values after several minutes.

Addition of external Ca^{2+} after the U46619-induced $[\text{Ca}^{2+}]_i$ transient resulted in a rise in $[\text{Ca}^{2+}]_i$ which involved stimulation of Ca^{2+} entry, since it was effectively prevented by Ni^{2+} (results not shown). Ni^{2+} is considered to be a blocker of receptor-mediated Ca^{2+} entry [28]. The magnitude of Ca^{2+} entry was found to be proportional to the concentration of U46619 and to the extent of Ca^{2+} mobilized by concentrations from roughly 0.12 μM up to 4 μM of the agonist. Use of BM13177 as the specific thromboxane-receptor antagonist inhibited a $[\text{Ca}^{2+}]_i$ response when administered before U46619 (results not shown). However, when BM13177 was introduced to platelets 2–3 min after U46619, $[\text{Ca}^{2+}]_i$ returned immediately to resting values (Figure 2). Moreover, the extent of subsequent $[\text{Ca}^{2+}]_i$ influx was

Figure 4 Ca^{2+} release in response to TG after pre-activation with U46619

Intracellular Ca^{2+} release in response to U46619 (U46; 2 μM) and TG (5 nM) with 150 μM ibuprofen and 0.5 mM EGTA outside was measured as described in Figure 3. The time-lapse between the end of the U46619 signal and addition of TG was 2 and 10 min. Resting $[\text{Ca}^{2+}]_i$ was 69 nM; $[\text{Ca}^{2+}]_i$ after 2 μM U46619 was 324 nM.

decreased considerably compared with cells treated with U46619 alone.

Control determinations revealed no significant influence of BM13177 on thrombin-induced Ca^{2+} release or thrombin-induced Ca^{2+} influx when the Ca^{2+} -overshoot protocol was used, as long as thrombin was employed at concentrations where a cyclo-oxygenase-independent response is elicited, i.e. at thrombin concentrations above 0.1 unit/ml. At lower concentrations of the agonist, when thrombin (0.01–0.05 unit/ml) relies on the cyclo-oxygenase-amplification mechanism for a maximal Ca^{2+} response, BM13177 inhibited Ca^{2+} release and Ca^{2+} influx.

U46619 modulated the Ca^{2+} -release properties of TG

In platelet suspensions TG evoked a fast and large rise in $[\text{Ca}^{2+}]_i$. However, in human platelets that had been incubated with ibuprofen in order to inhibit cyclo-oxygenase activity, not allowing the generation of self-amplifying eicosanoids, TG only elicited a gradual rise in $[\text{Ca}^{2+}]_i$ (Figure 3). This response was dramatically changed when an agonist such as U46619 had transiently increased $[\text{Ca}^{2+}]_i$ before the addition of TG.

Addition of TG to platelets preactivated with U46619 at a time point when Ca^{2+} had reached basal values again evoked larger rises in $[\text{Ca}^{2+}]_i$, making the response comparable with the TG signal observed in the absence of cyclo-oxygenase inhibition. The preceding discharge of the agonist-responsive Ca^{2+} store resulted in an increased potency of TG to cause intracellular Ca^{2+} accumulation. Similar measurements were recorded when ibuprofen was replaced by the dual cyclo-oxygenase/lipoxygenase inhibitor BW 755C.

The agonist-sensitizing effect was still observed when the concentration of TG was lowered from 1 μM to 5 nM (Figure 4). A concentration of 5 nM TG was unable to promote any significant Ca^{2+} release from intracellular stores. Preactivation of platelets with U46619 still potentiated the Ca^{2+} -release properties of TG. Moreover, this response was independent of either cyclo-oxygenase inhibition or thromboxane-receptor blockade.

To gain insight into a possible time-dependency concerning

Figure 5 Ca^{2+} movements in response to U46619, TG and BM13177

Measurements of Ca^{2+} release and Ca^{2+} influx were carried out as outlined in the Experimental section. Compounds at the concentrations given (U46, 2 μM U46619; TG, 5 nM TG; BM, 200 μM BM13177; Ca^{2+} , 500 μM Ca^{2+}) were added at the time points indicated. Further details are as in Figure 3. Extracellular Ca^{2+} was chelated with EGTA, and cyclo-oxygenase was inhibited by addition of ibuprofen. Resting $[\text{Ca}^{2+}]_i$ was 51 nM; $[\text{Ca}^{2+}]_i$ after 2 μM U46619 was 289 nM.

U46619 stimulation and the enhanced response towards TG, we changed the activation protocol as follows: the time-lapse between U46619-induced $[\text{Ca}^{2+}]_i$ transients and the subsequent addition of TG varied from 2 to 10 min. As indicated in Figure 4 the U46619-potentiating effect measured in the presence of 150 μM ibuprofen was virtually time-independent. Again, similar Ca^{2+} traces were recorded without cyclo-oxygenase inhibition (results not shown).

An enhanced TG response required receptor occupancy

After establishing the lack of a certain time frame for the potentiating effect of the receptor agonist on TG-induced increase in $[\text{Ca}^{2+}]_i$, we focused on the potential role of receptor occupancy. The best experimental approach in this respect is to use a stable thromboxane analogue in combination with its specific receptor antagonist. Results presented in Figure 5 were obtained in the presence of ibuprofen and external EGTA. Experiments refer to TG-induced Ca^{2+} release as well as Ca^{2+} influx and their modulation by U46619 in the absence and presence of BM13177.

With EGTA outside, preincubation of platelets with U46619 potentiated the TG-induced elevation of $[\text{Ca}^{2+}]_i$ (Figure 5, trace 1). This effect is similar to that shown in Figure 4 (trace 1 or 2).

Figure 6 Ca^{2+} movements induced by U46619, TG and BM13177

Calcium measurements were done as outlined in Figure 5. Compounds (U46, 2 μM U46619; TG, 5 nM TG; BM, 200 μM BM13177; Ca^{2+} , 500 μM Ca^{2+}) were used at the concentration given, according to the protocol shown. Measurements were performed in the presence of ibuprofen and EGTA. Resting $[\text{Ca}^{2+}]_i$ was 69 nM; $[\text{Ca}^{2+}]_i$ after 2 μM U46619 was 321 nM.

Figure 7 Ca^{2+} release evoked by U46619, TG and BM13177 in a nominally Ca^{2+} -free medium

Fura-2-loaded platelets were used for Ca^{2+} measurements as described in the Experimental section in a nominally Ca^{2+} -free solution. The action of U46619 (1 μM ; U46), TG (5 nM) and BM13177 (200 μM ; BM) is indicated. Concentrations and sequential addition of the agents were as indicated. Resting $[\text{Ca}^{2+}]_i$ was 103 nM; $[\text{Ca}^{2+}]_i$ after 1 μM U46619 was 378 nM.

As shown, addition of 500 μM free Ca^{2+} at the end of the experiment resulted in a marked Ca^{2+} influx. Addition of the U46619-receptor antagonist BM13177 after previous stimulation with the agonist U46619 altered the TG-induced Ca^{2+} release dramatically (Figure 5, trace 2). When U46619 was displaced from its receptor, the potentiating effect on TG-induced $[\text{Ca}^{2+}]_i$ transients disappeared. Under the same conditions, Ca^{2+} influx was decreased significantly. Similar results (not shown) were obtained with concentrations of TG up to 1 μM . Figure 6 describes the situation when platelets were stimulated by the protocol employed in Figure 5, although BM13177 was added at a later time point.

Using the combination of U46619 and TG in the presence of EGTA outside, we observed the established potentiating effect on Ca^{2+} release. In the absence of BM13177, addition of Ca^{2+} at the end of the experiment resulted in a marked increase in $[\text{Ca}^{2+}]_i$. Addition of BM13177 shortly before introducing Ca^{2+} dramatically blunted the Ca^{2+} overshoot. The action of BM13177 presented in Figures 5 and 6 concerning the Ca^{2+} influx is comparable. Previous experiments had been carried out in the presence of EGTA. Therefore, Ca^{2+} liberation by U46619 followed by subsequent TG addition resulted from intracellular compartments only. However, performing experiments similar to those described in Figures 5 and 6 in a nominally Ca^{2+} -free solution gave identical results (Figure 7). Addition of the receptor agonist U46619 resulted in a $[\text{Ca}^{2+}]_i$ transient. Ca^{2+} stayed slightly elevated, whereas a further application of TG produced an elevated Ca^{2+} plateau. Introducing BM13177 at a later time point instantly lowered $[\text{Ca}^{2+}]_i$ (Figure 7, trace 1). The new steady state reached after BM13177 addition was equivalent to the level observed in the absence of TG.

In the converse experiment, adding BM13177 after the $[\text{Ca}^{2+}]_i$ transient elicited by U46619 largely suppressed the TG response (Figure 7, trace 2). The Ca^{2+} levels at the end of these manipulations were nearly identical. Although TG-induced Ca^{2+} release was transient in platelets treated in the presence of EGTA, compared with a more sustained response when measured in a nominally Ca^{2+} -free solution, the overall effect of BM13177 remained the same.

To exclude non-specific effects of BM13177, we investigated its action in similar experiments, using thrombin instead of U46619 as the sensitizing agonist. BM13177 had no effect on thrombin-

Figure 8 Ca^{2+} release by U46619 in combination with TG and its modulation by sodium nitroprusside (SNP) and prostacyclin (PGI_2)

Human platelets in the presence of 0.5 mM EGTA were used as described in the Experimental section to measure $[\text{Ca}^{2+}]_i$ and Ca^{2+} influx. SNP (100 μM), PGI_2 (1 $\mu\text{g/ml}$), U46619 (U46; 4 μM), TG (5 nM) and Ca^{2+} (500 μM) were specifically added at time points and concentrations indicated. Resting $[\text{Ca}^{2+}]_i$ was 48 nM; $[\text{Ca}^{2+}]_i$ after 4 μM U46619 was 280 nM.

evoked Ca^{2+} signals, nor did it interfere with the sensitizing potency of thrombin concerning subsequent TG-induced Ca^{2+} accumulation, when thrombin concentrations above 0.1 unit/ml were used; i.e. cyclo-oxygenase-independent activation mechanism (results not shown). Thus its effects were considered specific to modulation of thromboxane/endoperoxide-receptor function.

Intervention by cyclic nucleotides

The experiments described above demonstrated that sustained receptor occupancy seems to be required for potentiation of TG-evoked Ca^{2+} accumulation. Besides receptor antagonists, cyclic nucleotides are known to inhibit receptor-mediated signalling events in human platelets (see [8] for references). Sodium nitroprusside causes an intra-platelet cGMP increase, whereas prostacyclin stimulates cAMP production in thrombocytes. Inhibition of the phosphatidylinositol response after receptor occupancy is a common target of both cGMP as well as cAMP. Taking this action into consideration, we also used either of the above-mentioned cyclic-nucleotide-increasing compounds instead of BM13177. We were interested to see whether they interfered with the signalling events that cause potentiated TG-induced Ca^{2+} accumulation (Figure 8). Activation of platelets by U46619 produced a $[\text{Ca}^{2+}]_i$ transient, followed by the enhanced TG response in combination with a marked Ca^{2+} influx.

Addition of either sodium nitroprusside or prostacyclin after the U46619-induced $[\text{Ca}^{2+}]_i$ transient totally abolished the subsequent TG-induced increase in $[\text{Ca}^{2+}]_i$. Furthermore, Ca^{2+} influx was decreased substantially. It is noteworthy that the small decrease in $[\text{Ca}^{2+}]_i$ immediately after the addition of sodium nitroprusside or prostacyclin was observed in all experiments. The action of compounds increasing either cGMP or cAMP (Figure 8) was identical with the action of BM13177 (Figure 5) in modulating a TG response followed by receptor stimulation.

We expected increased levels of cyclic nucleotides to be effective also in preventing thrombin-evoked potentiation of TG-induced Ca^{2+} accumulation, in contrast with the thromboxane/endoperoxide-receptor antagonist. Indeed, in an experimental set-up similar to that described in Figure 8, increased cyclic nucleotide levels partially suppressed the sensitizing effect of

thrombin (0.07 unit/ml) when given beforehand in place of U46619 (results not shown).

DISCUSSION

Human platelets, while activated, liberate Ca^{2+} from intracellular stores and show Ca^{2+} influx from the extracellular space [1,7]. Weak agonists rely on the formation of thromboxane in order to cause full aggregation [6]. Therefore, the stable thromboxane-receptor agonist U46619, binding to the endoperoxide/thromboxane receptor, can substitute for thromboxane A_2 to elicit platelet activation. Application of U46619 resulted in an immediate and substantial elevation of $[\text{Ca}^{2+}]_i$. In another experimental approach we studied Ca^{2+} entry by measuring the $[\text{Ca}^{2+}]_i$ response upon restoration of extracellular Ca^{2+} in platelets that were depleted of the agonist-sensitive Ca^{2+} store by a preceding stimulation with U46619 (Figures 1 and 2). Interestingly, for U46619 to cause considerable Ca^{2+} influx a sustained receptor occupancy seems necessary. Replacement of U46619 from its receptor before restoration of extracellular $[\text{Ca}^{2+}]$ or elevating intracellular levels of cyclic nucleotides blunted Ca^{2+} entry. This leads into the ongoing discussion of how Ca^{2+} entry is regulated. For platelets, both store-dependent 'capacitative' Ca^{2+} influx and receptor-dependent mechanisms have been revealed [29–31]. Similar mechanisms operate in other cells, for example in PC-12 cells [32] or Jurkat T cells [33]. Since it was reported that both regulatory mechanisms may control distinct channels with differential cation selectivity [34,24], we decided to rely on Ca^{2+} -overshoot studies rather than on Mn^{2+} influx, for correct measurement of Ca^{2+} entry.

Our results indicated that Ca^{2+} entry in response to U46619 mainly demands receptor occupancy. In contrast with the 'capacitative model', where parotid cells stimulated with methacholine followed by the addition of the receptor antagonist atropine still show substantial Ca^{2+} entry [10], in human platelets the signal for Ca^{2+} entry was blunted after replacement of the agonist U46619 by BM13177. Thus the signal for Ca^{2+} entry in human platelets in response to U46619 must be somehow controlled by the occupied receptor. The existence of such a receptor-regulated Ca^{2+} -entry pathway became evident from other studies using angiotensin II-stimulated adrenal glomerulosa cells, in which Ca^{2+} pools had been emptied beforehand. After re-addition of Ca^{2+} , a significant increase in the amplitude and the rate of the rise in $[\text{Ca}^{2+}]_i$ was seen in agonist-stimulated compared with unstimulated controls [35]. Evidently, Ca^{2+} entry by receptor-operated channels would be one explanation for the observed effect in platelets. However, if there is a role of store depletion in control of Ca^{2+} entry, then this regulatory link is again gated by the activated receptor. For exact evaluation of the role of store depletion in regulation of Ca^{2+} entry, an experimental set-up would be necessary in which Ca^{2+} release from the regulating store is blocked without interference by other receptor-mediated signalling events. Such a system is currently not available for human platelets.

Thapsigargin, a Ca^{2+} -ATPase inhibitor, completely inhibits all known isoforms of the sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPases [36]. Treatment of intact cells with TG leads to depletion of certain intracellular Ca^{2+} stores. For many cell types TG has been considered also to empty IP_3 -sensitive intracellular Ca^{2+} stores. However, attention should be paid to response-amplifying autocrine receptor stimulation of TG-treated cells, such as platelets. Treatment of thrombocytes with TG, under conditions where thromboxane A_2 production or binding of thromboxane A_2 to its receptor had been prevented, resulted in emptying of the TG-sensitive Ca^{2+} pool [22,25,26], and sub-

sequent stimulation with a receptor agonist still triggered substantial Ca^{2+} release. Thus, under this condition, TG did not cause effective depletion of an agonist-sensitive Ca^{2+} pool. In line with this are observations that TG alone, without the platelet self-amplification feedback mechanism, did not fully activate platelets [21,37]. Moreover, TG-evoked responses were potentiated by pre-activation of platelets with thrombin, ADP, ionomycin and thimerosal [25,26]. The magnitude and rapidity of this effect suggests that the Ca^{2+} signals produced by agonists and TG synergize, in such a way that TG acts more efficiently when agonist-sensitive stores were depleted before TG application (Figure 3). The data point to a mechanism involving Ca^{2+} transport from TG-insensitive to TG-sensitive Ca^{2+} stores. A functional heterogeneity within intracellular Ca^{2+} pools [38] might also be explained by distinct sequestering and releasing compartments within the IP_3 -sensitive Ca^{2+} pool. For RINm5F cells it has been proposed that TG quickly mobilized Ca^{2+} from the sequestering compartment, whereas Ca^{2+} from the releasing compartment could be mobilized only very slowly, in the absence of IP_3 [39]. Other studies using platelet mixed membranes revealed a complete overlap of the TG-sensitive and the IP_3 -sensitive Ca^{2+} pool, although in intact cells a functional heterogeneity became evident, indicating agonist utilization of a Ca^{2+} -ATPase-inhibitor-insensitive pool [40]. With this study we intend to suggest an explanation of these contradictory findings that involves a fast cycling of large quantities of intracellular Ca^{2+} between sequestering and releasing Ca^{2+} compartments. As previously shown by different laboratories using thrombin [25,26], we now suggest a functional heterogeneity of U46619-sensitive and TG-responsive Ca^{2+} compartments as well. Moreover, comparably with thrombin, U46619 sensitizes TG-evoked $[\text{Ca}^{2+}]_i$ transients. Surprisingly, addition of BM13177, in order to remove U46619 from its receptor, completely blunted the subsequent TG-induced $[\text{Ca}^{2+}]_i$ transient as well as the Ca^{2+} influx although the agonist-sensitive store had been allowed to discharge normally. Likewise, elevation of intracellular cyclic nucleotide levels, which are known to terminate agonist receptor signalling (for references, see [8]) reversed the U46619-sensitizing effect. Therefore, a simple transfer of Ca^{2+} from one agonist-sensitive pool to another, TG-sensitive, pool seems unlikely. Otherwise the addition of BM13177 or the elevation of cyclic nucleotides after the U46619-evoked $[\text{Ca}^{2+}]_i$ transient should not have suppressed the TG-elicited Ca^{2+} release.

The present results fit best to a model assuming cycling of Ca^{2+} between a TG-sensitive and a virtually TG-unresponsive but agonist-sensitive Ca^{2+} compartment. The TG-unresponsive compartment either may lack the TG-sensitive pump or, alternatively, may show no Ca^{2+} leakage in the case of pump inhibition as long as the cells are not activated. In resting cells, whose ability of autocrine stimulation is prevented, the gradual TG-evoked $[\text{Ca}^{2+}]_i$ increase may reflect the inherent leakage of Ca^{2+} from the thus TG-sensitive subset of intracellular Ca^{2+} stores. The agonist-sensitive store exhibits a much lower basal leakage rate, and is therefore not efficiently depleted by TG, as seen by the ability of agonists to release substantial amounts of stored Ca^{2+} also after preincubation with TG. A preceding stimulation with agonists may sensitize the TG response by influencing the permeability of the TG-insensitive compartment. Thus U46619 causes an increase in $[\text{Ca}^{2+}]_i$, followed by resequestration, which decreases the steady state of $[\text{Ca}^{2+}]_i$, although an increased cycling, not detectable by using fura-2, may be established. Under these conditions inhibition of the Ca^{2+} pump by TG leads to a potentiated apparent Ca^{2+} increase compared with unstimulated cells. This potentiation therefore may reflect accumulation of cycling Ca^{2+} upstream of the blocked Ca^{2+} -ATPase.

Our time-lapse studies (Figure 4) argue against a role for transiently formed second messengers, i.e. IP_3 , in potentiation of TG-induced Ca^{2+} accumulation. Rather, the BM13177 and cyclic nucleotide experiments show clearly the requirement for sustained receptor occupancy and signalling for both potentiation of the TG-evoked Ca^{2+} liberation as well as for Ca^{2+} influx after U46619 addition.

As a model of how this Ca^{2+} cycling may be achieved, we suggest a coupling between two Ca^{2+} -storing compartments, by analogy with coupling of the depleted agonist-sensitive store with plasma-membrane Ca^{2+} entry. Intriguingly, Ca^{2+} cycling and Ca^{2+} entry were found to be regulated exactly in parallel. After IP_3 -mediated release of its stored Ca^{2+} , the agonist-sensitive store may couple not only with the extracellular Ca^{2+} compartment, but also in a similar way with the TG-sensitive intracellular Ca^{2+} -storing compartment. This coupling then enables Ca^{2+} entry and cycling of intracellular Ca^{2+} . To maintain this coupling, a so-far unknown signalling event from the activated receptor is required. One can speculate about the involvement of GTP or perhaps small G-proteins. Conceivably, the termination of this signal causes closing of Ca^{2+} channels in the agonist-sensitive compartment, leading to its refilling and uncoupling from the other compartments.

The study was supported by the Deutsche Forschungsgemeinschaft. The expert technical assistance of B. Diewald is gratefully acknowledged.

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Received 13 April 1994/11 July 1994; accepted 26 July 1994