Ouabain Increases the Calcium Concentration in Intracellular Stores Involved in Stimulus–Response Coupling in Human Platelets

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The effect of ouabain on Ca\(^{2+}\) homeostasis in human platelets was studied using both quin 2 and chlorotetracycline to monitor changes in cytosolic Ca\(^{2+}\) as well as changes in the amount of Ca\(^{2+}\) accumulated in intracellular storage sites. In resting platelets, ouabain induces a concentration- and time-dependent increase in cytosolic Ca\(^{2+}\) concentration and a marked elevation of Ca\(^{2+}\) in the intracellular stores. The amount of Ca\(^{2+}\) mobilized from these stores upon stimulation with thrombin, as well as thrombin–induced secretion of platelet 5-hydroxytryptamine, was increased after preincubation with the glycoside (3×10\(^{-6}\) M). These data show that ouabain induces an elevation of intracellular Ca\(^{2+}\) levels, most likely mediated via Na\(^+\)-Ca\(^{2+}\) exchange, and that this incremental amount of Ca\(^{2+}\) is accumulated in an intracellular store involved in stimulus–response coupling. This may explain the enhanced functional responses of platelets to agonists in the presence of ouabain and suggests a role for Na\(^+\)-Ca\(^{2+}\) exchange in Ca\(^{2+}\) homeostasis of the human platelet. (Circulation Research 1990;67:1494–1502)

Cardiac glycosides are known to inhibit the plasma membrane–associated Na\(^+\),K\(^+\)-ATPase, resulting in an increase in intracellular Na\(^+\) and in the inhibition of K\(^+\) uptake. The increased intracellular Na\(^+\) has been suggested to reduce the gradient across the cell membrane and in turn to reduce the magnitude of the Na\(^+\)-Ca\(^{2+}\) exchange–coupled calcium influx.\(^1\) The consequent net increase in intracellular Ca\(^{2+}\) could then account for the increased contractility induced by cardiac glycosides in cardiac and smooth muscle.\(^2\)–\(^4\) Ouabain, a typical cardiac glycoside, also enhances functional responses in the platelet,\(^5\)–\(^7\) and both the rate and the amplitude of the aggregatory response to agonists are increased.\(^2\) Recently Na\(^+\)-Ca\(^{2+}\) exchange activity has been demonstrated in human platelet plasma membrane vesicles.\(^8\) In the unstimulated platelet, this Ca\(^{2+}\) transport mechanism may well contribute to the low cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{cyt}\)) against the steep Ca\(^{2+}\) gradient. If this is the case, it should be expected that lowering of the transmembrane Na\(^+\) gradient decreases the driving force for outward Ca\(^{2+}\) flux by the exchanger and that, as a consequence, [Ca\(^{2+}\)]\(_{cyt}\) increases. Indeed, studies in which extracellular Na\(^+\) was replaced by choline described a small increase in [Ca\(^{2+}\)]\(_{cyt}\) that was measured using quin 2, but the results depended on the kind and/or the purity of the substitute for Na\(^+\) that was used.\(^9\),\(^10\) The use of ouabain to increase intracellular Na\(^+\) has the advantage that the Na\(^+\) gradient is lowered at extracellular Na\(^+\) in the physiological range. In smooth muscle cells, an ouabain-induced increase in [Ca\(^{2+}\)]\(_{cyt}\) has been evidenced using fluorescent probes.\(^3\),\(^11\) However, in the platelet, quin 2 studies failed to demonstrate an effect of ouabain on basal [Ca\(^{2+}\)]\(_{cyt}\).\(^3\),\(^12\),\(^13\) These puzzling data and the lack of effect of Na\(^+\) on total platelet Ca\(^{2+}\) make the role of Na\(^+\)-Ca\(^{2+}\) exchange in platelet Ca\(^{2+}\) homeostasis controversial,\(^9\),\(^14\)–\(^17\) in spite of the reported effects of Na\(^+\) on platelet intracellular Ca\(^{2+}\) pools, assessed by \(^{45}\)Ca\(^{2+}\) flux experiments.\(^9\) Nevertheless, it has been generally accepted that Ca\(^{2+}\) plays a pivotal role as an intracellular messenger for platelet excitatory agonists. Its release from intracellular stores is made possible by the breakdown of inositol-containing phospholipids.\(^17\)

Although there is much circumstantial evidence indicating that in muscle cells ouabain increases Ca\(^{2+}\) in intracellular stores that are involved in excitation–response coupling, direct proof on the working mechanism is still absent. We have taken the human blood platelet as a model to test this hypothesis. In platelets, the Ca\(^{2+}\)-sensitive fluorescent probes quin 2 and,
to a lesser extent, chlorotetracycline (CTC) have been used to measure respectively changes in [Ca\(^{2+}\)]\(_{ci}\) and changes in intracellular Ca\(^{2+}\) stores.\(^{18-20}\) We used both methods to study the effect of ouabain on platelet Ca\(^{2+}\) handling. We found that inhibition of Na\(^+\),K\(^+\)-ATPase activity leads to a concentration- and time-dependent increase in [Ca\(^{2+}\)]\(_{ci}\) and in the amount of Ca\(^{2+}\) accumulated in an intracellular pool that is involved in stimulus–response coupling. The results can be explained by ascribing a role to Na\(^+\)-Ca\(^{2+}\) exchange in platelet Ca\(^{2+}\) homeostasis.

**Materials and Methods**

CTC was obtained from Sigma Chemical Co., St. Louis, ouabain from Janssen Chimica, Beerse, Belgium, and thrombin (Topostasin) from Roche Laboratories, Basel, Switzerland. They were all dissolved in 0.9% NaCl. Quin 2-AM came from Calbiochem, La Jolla, Calif., and was dissolved in dimethyl sulfoxide. Pure grade bovine serum albumin was obtained from Serva, Heidelberg, FRG. Sepharose-CL2B came from Pharmacia Laboratories, Uppsala, Sweden. [\(^{32}\)P]Orthophosphate and [\(^{3}H\)]5-hydroxytryptamine (5-HT) were purchased from Amersham, Buckinghamshire, England.

**Platelet Isolation and Handling**

Blood collection from healthy human volunteers and preparation of platelet-rich plasma were carried out as described previously.\(^{21}\) Except for quin 2 experiments, platelets were isolated by repeated centrifugation\(^{21}\) and suspended in buffer A containing (mM) HEPES 25, NaCl 125, KCl 2.7, MgSO\(_4\) 1, glucose 10, EGTA 0.1, Na\(_2\)HPO\(_4\) 0.5, and 0.1% bovine serum albumin at pH 7.4. In CTC experiments, MgSO\(_4\) and bovine serum albumin were omitted. In all experiments, except for those shown in Figure 5, platelets were preincubated for 15 minutes in buffer with 1 mM CaCl\(_2\) to allow them to recover from the normal Ca\(^{2+}\) homeostasis before ouabain was added. All incubations were at 37° C.

**Fluorescence Measurements Using Quin 2 as a Probe**

Platelet-rich plasma was incubated with 15 \(\mu\)M quin 2 acetoxymethyl ester at 37° C for 12 minutes. Platelets were separated from plasma and extracellular dye by gel filtration on a Sepharose-CL2B column (2.5 x 18 cm) equilibrated with buffer A. The platelet concentration was adjusted to 2 x 10\(^8\)/ml, and 1.1 \(\mu\)mol CaCl\(_2\)/ml was added. Fluorescence was measured at 37° C using a thermostated cuvette in a purpose-modified fluoronephelometer (excitation at 340 nm, emission at 490 nm; Technicon, Tarrytown, N.Y.). Platelets (1 ml) were placed in the cuvette, and fluorescence in the resting state was recorded. Subsequently, 2 mM EGTA was added, and 1 minute later, platelets were triggered with thrombin. Quantification of [Ca\(^{2+}\)]\(_{cy}\) was performed as described by Rink and Pozzan.\(^{22}\) The measurements were corrected for dye leakage in each sample using 100 \(\mu\)M Mn\(^{2+}\) and 1 mM diethylentriaminepentaacetic acid (DTPA). Unstimulated platelets were exposed to free Mn\(^{2+}\) ions (100 \(\mu\)M) for maximally 20 seconds before they were chelated by an excess of DTPA (1 mM). Preliminary experiments had shown that neither this short exposure to free Mn\(^{2+}\) ions nor the presence of free DTPA and the Mn-DTPA complex had any effect on platelet Ca\(^{2+}\) response to stimulation by thrombin, in agreement with Rink and Pozzan.\(^{22}\)

Calibration was also carried out on every platelet sample studied. One measurement took 5 minutes. The sensitivity of platelets to thrombin slightly decreased within the time of the experiment. Therefore, measurements on ouabain-treated platelets and control platelets were performed alternately and evaluated as paired samples.

**Fluorescence Measurements Using CTC as a Probe**

The mechanism by which platelet-associated CTC responds to changes in Ca\(^{2+}\) in intracellular stores has been described in detail by others.\(^{18}\) We used the following procedure: 10 \(\mu\)M CTC was added to washed platelet suspension (2 ml, 2 x 10\(^8\) platelets/ml) in the cuvette at 37° C in the presence of 1 mM external Ca\(^{2+}\). Fluorescence was measured at 390 nm excitation and 530 nm emission in a fluorometer (model 3C, Jobin-Yvon, Longjumeau, France) equipped with a thermostatically controlled cell holder (four cuvettes). The addition of CTC gives rise to an instantaneous increase of fluorescence (fast phase), followed by a slow increase of fluorescence, which we monitored for 15 minutes (slow phase). Other authors\(^{18}\) have shown that the rapid change is due to fluorescence of the Ca-CTC complex in the aqueous phase and binding of the Ca-CTC complex on the outer surface of the plasma membrane. The slow fluorescence increase has been shown to arise primarily from the binding of the Ca-CTC complex to the inner surfaces of Ca\(^{2+}\)-sequestering organelles and is proportional to the free Ca\(^{2+}\) concentration in these organelles.\(^{18,23}\)

We calculated the CTC ratio as the amplitude of the slow phase (A\(_{slow}\)) divided by the amplitude of the fast phase (A\(_{fast}\)) taken 15 minutes after CTC addition. It represents a quantitative index of net Ca\(^{2+}\) accumulated into intracellular Ca\(^{2+}\)-sequestering pools per unit surface area of platelets.\(^{24}\)

**Measurement of 5-HT Secretion**

Platelet amine storage granules were loaded with [\(^{3}H\)]5-HT by incubating platelet-rich plasma for 20 minutes at 37° C with 0.3 \(\mu\)Ci/ml [\(^{3}H\)]5-HT. The loaded platelets were washed as described above. Secretion of [\(^{3}H\)]5-HT after stimulation was assessed by removing 200 \(\mu\)l platelet suspension (2 x 10\(^8\)/ml) and adding it to 20 \(\mu\)l of 13.5% (wt/vol) formaldehyde in 0.9% NaCl on ice. After centrifugation at 10,000g for 2 minutes, aliquots (100 \(\mu\)l) of the supernatant were removed for liquid scintillation counting.
Results are expressed as a percentage of total tritium in an equivalent volume of the platelet suspension lysed with 0.1% Triton X-100.


Isolated platelets (2×10^9/ml) were incubated for 70 minutes with 50 μCi/ml[^2]P]orthophosphate. Phospholipid analyses were performed exactly as described previously.25


Platelet isolation, labeling, and [^3]H]inositol phosphate analyses were performed exactly as described previously.25

Data Presentation

In each test, ouabain-treated platelets were compared with control platelets. Data are presented as mean±SEM. Unless indicated otherwise, the asterisks in the figures denote the statistical evaluation of data performed by Student’s t test for paired observations. Values of p<0.05 were considered significant. Dose–effect data were also evaluated by analysis of variance.

For quin 2 experiments, each measurement on ouabain-treated platelets was compared with either the preceding control measurement or (only in Figure 6) the succeeding control measurement. In Figure 2, control values corresponding with different ouabain concentrations were not significantly different by analysis of variance and thus were pooled in the presentation of the results. Because the variance of [Ca^{2+}]_{cyt} data increased with the mean value, analysis was performed on the logarithmic transformation.26

Where error bars do not appear on figures, errors are within the symbol size.

Results

Ouabain-Induced Changes in Ca^{2+} Homeostasis

When quin 2 was used as an intracellular probe, [Ca^{2+}]_{cyt} was 112±9 nM (mean±SEM; n=25). Incubation of platelets with ouabain during 75 minutes, in the presence of extracellular Ca^{2+} (Ca^{2+}_o), increased the resting [Ca^{2+}]_{cyt} in a concentration-dependent manner (Figure 1).

Addition of excitatory platelet agonists induces an elevation of [Ca^{2+}]_{cyt} by mobilization from intracellular stores and by influx from the extracellular space.27 We investigated whether ouabain had any effect on the mobilization of Ca^{2+} from the intracellular stores. Therefore, platelets were stimulated with thrombin in the presence of an excess of extracellular EGTA over Ca^{2+}. Exposure to the chelator was brief in order to avoid a time-dependent EGTA-induced modification of Ca^{2+} homeostasis. Because of the virtual absence of Ca^{2+}_o at the moment of triggering, the rise in fluorescence can be attributed to mobilization from intracellular stores.20 In the absence of ouabain, the increase in [Ca^{2+}]_{cyt} after the addition of thrombin was 214±16 nM (mean±SEM; n=25) (Figure 2).

Preincubation with ouabain increased the amount of Ca^{2+} mobilized from intracellular stores in a concentration-dependent manner (Figure 2). Maximal elevation was obtained at 10^{-5} M of the glycoside. These data indicate that more Ca^{2+} was accumulated in intracellular pools, due to ouabain treatment.

CTC is a probe that has been shown to monitor changes in free Ca^{2+} concentration in intracellular storage sites, in which this concentration approaches the millimolar range.18 We determined the platelet CTC ratio after 75 minutes incubation, according to quin 2 measurements. In the presence of ouabain (3×10^{-6} M), A_{slow} was markedly higher than in its absence (Figure 3), whereas pretreatment with ouabain did not significantly change the magnitude of A_{fast}. A_{fast} as well as A_{slow} depend on platelet concentration, the concentration of Ca^{2+}_o, and the amount of CTC added. A_{slow}, however, has also been shown to be proportional to the free Ca^{2+} concentration in Ca^{2+}-sequestering organelles.18 The CTC ratio (A_{slow}/ A_{fast}) can therefore be considered as an index of the amount of Ca^{2+} that is accumulated in these organelles. The CTC ratio increased from 0.68±0.07 in the control to 1.12±0.06 (mean±SEM; n=8) after 60 minutes of treatment with the glycoside. The relevance of the CTC ratio to measure the amount of Ca^{2+} accumulated in intracellular storage sites was validated by the use of ionomycin. In the absence of Ca^{2+}_o, ionomycin has been shown to discharge Ca^{2+} from intracellular stores.14,18 If the increased A_{slow} in ouabain-treated platelets indicates an increased
Effects of ouabain on platelet calcium homeostasis.

**Figure 2.** Graph showing the effect of ouabain on the increase in cytosolic free Ca^{2+} concentration ([Ca^{2+}]_{cyt}) after stimulation with thrombin in the absence of extracellular Ca^{2+}. Quin 2–loaded platelets were preincubated for 75 minutes with different concentrations of ouabain (●) or its solvent (○) in the presence of 1 mM extracellular Ca^{2+}. Basal [Ca^{2+}]_{cyt} was determined and presented in Figure 1. Subsequently, 1 mM extracellular Ca^{2+} was chelated by the addition of 2 mM EGTA, and 1 minute later, platelets were stimulated with thrombin (0.02 units/ml). Peak [Ca^{2+}]_{cyt} was determined, and the increase in [Ca^{2+}]_{cyt} was calculated. The data shown are mean ± SEM from five experiments and from all the controls (○; n=25). NS, not significant; *p<0.01 and **p<0.001 by analysis of variance for repeated measures using a Bonferroni correction for the significance level.

The amount of Ca^{2+} in intracellular pools, complete discharge by ionomycin should result in a drop of CTC fluorescence to a same level, independent of ouabain treatment. Figure 3 shows that, upon the addition of ionomycin (3 μM) together with EGTA to complex Ca^{2+}_{extr}, the fluorescence of control platelets, as well as ouabain–treated platelets, indeed falls to a common steady-state level. Apparently, the increased amount of Ca^{2+} in intracellular stores of ouabain–treated platelets, which we measured as an increase of the CTC ratio, could be released by the ionophore.

The Ca^{2+} accumulated in these stores could also be mobilized on stimulation with thrombin. This is shown in Figure 4. After 15 minutes of pretreatment with CTC (10 μM), platelets were triggered with thrombin in the presence of 2 mM EGTA, as was the case in quin 2 experiments. Upon the addition of thrombin (0.5 units/ml), CTC fluorescence decreased to a larger extent in ouabain–treated platelets than in control platelets, indicating that thrombin indeed could release more Ca^{2+} out of the intracellular pool in the presence of ouabain. The threshold level for

**Figure 4.** Tracings showing thrombin-induced changes in platelet chlorotetracycline (CTC) fluorescence in the absence of extracellular Ca^{2+}. Washed platelets were preincubated for 60 minutes with solvent (panel A) or ouabain (3×10^{-6} M) (panel B) in the presence of 1 mM Ca^{2+}; 10 μM CTC was added, and the incubation was continued for 15 minutes. Then extracellular Ca^{2+} was chelated by the addition of 2 mM EGTA, and 3 minutes later, fluorescence was recorded. Zero fluorescence was set with platelet suspension without CTC. The data represent 24 measurements.

**Figure 3.** Graphs showing the effect of ouabain on the chlorotetracycline (CTC) ratio. A_{slow} and A_{fast} amplitude of the slow and fast phases of fluorescence increase, respectively. Washed platelets were preincubated for 60 minutes with solvent (panel A) or ouabain (3×10^{-6} M) (panel B) in the presence of 1 mM Ca^{2+}. CTC was added, and fluorescence was recorded as described in “Materials and Methods.” The CTC ratio is calculated as A_{slow}/A_{fast}. At indicated time periods, ionomycin (3 μM) together with 2 mM EGTA was added. The data represent 30 measurements.
measuring thrombin-induced release of Ca$^{2+}$ from this intracellular store, using CTC as a probe, was as high as 0.25 units/ml (results not shown).

**Dependence on Ca$^{2+}_{ex}$**

To determine the contribution of Ca$^{2+}_{ex}$ on [Ca$^{2+}$]$_{cyt}$ and on the thrombin-induced mobilization from intracellular stores, Ca$^{2+}$ was omitted during pretreatment of quin 2–loaded platelets. Because buffer A contained 0.1 mM EGTA, Ca$^{2+}_{ex}$ was virtually absent under these conditions. The data presented in Figure 5 show that after 75 minutes of incubation in the virtual absence of Ca$^{2+}_{ex}$ platelet [Ca$^{2+}$]$_{cyt}$ was markedly lower than after incubation with Ca$^{2+}_{ex}$ in the physiological range. Treatment with ouabain, in the presence as well as in the absence of Ca$^{2+}_{ex}$, slightly augmented the resting [Ca$^{2+}$]$_{cyt}$ and markedly amplified the mobilization of intracellular Ca$^{2+}$ induced by thrombin. Furthermore, Figure 5 illustrates that the amount of Ca$^{2+}$ mobilized from intracellular sources on stimulation with thrombin increased with increasing resting [Ca$^{2+}$]$_{cyt}$.

**Time Dependence**

In resting platelets, ouabain (3×10$^{-6}$ M) increased [Ca$^{2+}$]$_{cyt}$ in a time-dependent manner, and changes were significant within 10 minutes (Figure 6). After 35 minutes of contact with the glycoside, thrombin-induced mobilization of intracellular Ca$^{2+}$ was significantly higher than in the controls. The most pronounced effect appeared only after 60 minutes contact with ouabain. The sensitivity of control platelets to thrombin notably decreased as a function of time, resulting in a 50% loss of the response after 85 minutes. The same experiments were performed using CTC as a probe. Figure 7 illustrates that the drop of thrombin–induced increase in [Ca$^{2+}$]$_{cyt}$ in control platelets, as shown in Figure 6, coincided with a decrease in CTC ratio. Conversely, the CTC ratio increased in the presence of ouabain and was obvious after 30 minutes of contact with the glycoside. There appeared to be no significant differences between A$_{top}$ of control platelets and ouabain-treated platelets within the course of the experiment (not shown).
**Figure 7.** Graph showing the time course of the effect of ouabain on the chlorotetracycline (CTC) ratio. Washed platelets preincubated in the presence of 1 mM Ca\(^{2+}\) for 15 minutes were divided into two batches. At zero time, ouabain (3×10^{-6} M, ■) was added to one batch while the control (○) received solvent, and incubation was continued. At indicated time periods, samples were taken, and CTC ratio was determined. Results represent mean±SEM of four experiments with duplicate determinations. Four cuvettes were measured at the same time; two were filled with control platelet suspension, and two were filled with ouabain-treated suspension. The means of the duplicate determinations (n=4) were compared using Student's t test for paired data. NS, not significant; *p<0.05; **p<0.01; ***p<0.001.

**Figure 8.** Graph showing the effect of ouabain on the increase in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]_c) on stimulation with different thrombin concentrations in the absence of extracellular Ca\(^{2+}\). Quin 2–loaded platelets were preincubated for 75 minutes with ouabain (3×10^{-6} M) (●) or its solvent (○) in the presence of 1 mM Ca\(^{2+}\). [Ca\(^{2+}\)]_c was determined in the resting state (not shown) and after stimulation with indicated concentrations of thrombin in the presence of 2 mM EGTA. Results shown are mean±SEM of the increase in [Ca\(^{2+}\)]_c from three to six measurements in three experiments. *p<0.05 by Student’s t test. The upper curve differs significantly from the lower curve (p=0.0028 by one-way analysis of variance).

**Thrombin Concentration Dependence**

Thrombin induced a concentration-dependent mobilization of intracellular Ca\(^{2+}\) (Figure 8). Even an agonist concentration as low as 0.0025 units/ml was able to release more Ca\(^{2+}\) from the intracellular stores, in the presence of ouabain (3×10^{-6} M).

We verified whether ouabain also increased the functional response of the platelet to stimulation in our experimental conditions. Therefore, we measured the thrombin-induced secretion of dense granular 5-HT. In the presence of the glycoside, the concentration–response relation for thrombin-induced 5-HT release was shifted to the left (Figure 9), which substantiates that platelets are more sensitive to thrombin in the presence of ouabain.

**Effect on the Signal Transducing System**

As in other cell types, mobilization of intracellular Ca\(^{2+}\) in platelets depends on the generation of second messengers. Thrombin activates an inositol phospholipid-specific phospholipase C. A stimulatory effect of ouabain on the activation of this phospholipase C might thus be the underlying mechanism for the thrombin-induced increase in [Ca\(^{2+}\)]_c, and secretion. Therefore, we measured activation of the phospholipase C by the thrombin-induced formation of [\(^{32}\)P]phosphatidic acid ([\(^{32}\)P]PA) in [\(^{32}\)P]orthophosphate-prelabeled cells. Table 1 shows that the thrombin-induced [\(^{32}\)P]PA formation was not increased by ouabain (3×10^{-6} M). However, in resting platelets, ouabain significantly decreased the [\(^{32}\)P]PA incorporation in this phospholipid during the prelabeling period (Table 1).

**Discussion**

**Ouabain-Induced Changes in Ca\(^{2+}\) Homeostasis**

In the present communication, changes in platelet Ca\(^{2+}\) homeostasis were assessed with quin 2. Although fura 2 has several properties that are advantageous compared with those of quin 2, its accuracy is strongly hampered after long incubation times, due to dye leakage, especially at physiological temperature levels. In contrast, using quin 2, we were able to quantify minor changes in cytosolic Ca\(^{2+}\) even after 75 minutes of incubation at 37°C (Figure 1). In the light of specific limitations that have been reported on the use of cytosolic Ca\(^{2+}\) indicators, we used...
Schaefffer and Blaustein\textsuperscript{16} though, using fura\textsuperscript{2} demonstrated an ouabain-induced increase in [Ca\textsuperscript{2+}]\textsubscript{cyt} at low extracellular Na\textsuperscript{+}, and very recently, an ouabain-induced enhancement of basal and stimulus-induced platelet [Ca\textsuperscript{2+}]\textsubscript{ex} at physiological levels of extracellular Na\textsuperscript{+} was reported.\textsuperscript{13} However, the authors were unable to decide on which aspect of Ca\textsuperscript{2+} homeostasis the ouabain-enhanced functional response is ultimately dependent. Since in their experiments platelets were only triggered in the presence of Ca\textsuperscript{2+}\textsubscript{ex}, the release of Ca\textsuperscript{2+} from intracellular stores upon stimulation with thrombin was, most likely, masked by influx of Ca\textsuperscript{2+}.

The use of CTC enabled us to demonstrate that ouabain induced a marked elevation of Ca\textsuperscript{2+} in intracellular stores (Figures 3 and 7). The importance of this store in stimulus–response coupling became apparent on stimulation with thrombin in the absence of Ca\textsuperscript{2+}\textsubscript{ex}. Thrombin released Ca\textsuperscript{2+} from this store (Figure 4) into the cytosol (Figure 5). This phenomenon occurred in an ouabain concentration- and time-dependent manner (Figures 2 and 6) and was quantitatively of much more importance than the small ouabain-induced increases in resting [Ca\textsuperscript{2+}]\textsubscript{cyt} (Figure 1).

When prolonged incubation of control platelets was performed in the absence of Ca\textsuperscript{2+}\textsubscript{ex}, basal [Ca\textsuperscript{2+}]\textsubscript{cyt} was markedly lower (Figure 5). This phenomenon has been reported before.\textsuperscript{32,33} In these conditions, both Na\textsuperscript{+} and Ca\textsuperscript{2+} can flow along their chemical gradients. As a consequence, Ca\textsuperscript{2+} efflux via Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange is most likely facilitated and reaches a new equilibrium at the lower level in resting [Ca\textsuperscript{2+}]\textsubscript{cyt}. In the absence as well as in the presence of Ca\textsuperscript{2+}\textsubscript{ex}, the ouabain-induced decrease of the transmembrane Na\textsuperscript{+} gradient resulted in an increase in basal [Ca\textsuperscript{2+}]\textsubscript{cyt} compared with the control level. Jy and Haynes\textsuperscript{18} demonstrated, by the use of CTC, that the resting [Ca\textsuperscript{2+}]\textsubscript{cyt} determines the Ca\textsuperscript{2+} level in intracellular stores. This view was supported by our finding that, as [Ca\textsuperscript{2+}]\textsubscript{cyt} is elevated, more Ca\textsuperscript{2+} can be mobilized by thrombin from the intracellular stores to the cytosol (Figure 5).

Using CTC as a probe, the thrombin-induced release of Ca\textsuperscript{2+} from intracellular stores, which is measured as a decrease in fluorescence, could only be made evident at higher concentrations of the agonist when compared with the quin 2 method; this occurred in control as well as in ouabain-treated platelets. Most likely, the thrombin releasable pool is only a minor part of the total intracellular Ca\textsuperscript{2+} store. Indeed, the release of a minor part of the intracellular pool that contains a millimolar concentration of Ca\textsuperscript{2+} can produce relatively major elevations of Ca\textsuperscript{2+} in the cytosol, where it is present at submicromolar concentrations. Furthermore, the Ca\textsuperscript{2+}-buffering capacity of quin 2 may induce an exaggerated mobilization of Ca\textsuperscript{2+} out of intracellular stores. It has been reported that, when CTC is used in combination with the nonfluorescent intracellular Ca\textsuperscript{2+} chelator bis(\textsuperscript{o}-aminophenox)-ethane-N,N,N',N'-tetraacetic acid, which mimics this Ca\textsuperscript{2+}-buffering capacity,
the CTC method becomes more sensitive to lower concentrations of thrombin.20

**Time-Dependent Aspects of the Ouabain Effect**

The time course experiments (Figures 6 and 7) indicate that prolonged incubations are needed to demonstrate a marked effect of ouabain on platelet Ca\(^{2+}\) homeostasis. This is not surprising since the influx of Na\(^{+}\) down its concentration gradient by passive diffusion is the most likely rate-limiting step in this phenomenon. Influx of Na\(^{+}\) via Na\(^{+}\)-Ca\(^{2+}\) exchange seems unlikely by the strong Ca\(^{2+}\) gradient that is directed inward, and influx via Na\(^{+}\)-H\(^{+}\) exchange has been reported to be nearly quiescent in the nonactivated platelet.34

The time-dependent fall of the CTC ratio (Figure 7) and of the thrombin-induced increase of [Ca\(^{2+}\)]\text{int}\ in control platelets (Figure 6) indicate that there is a gradual loss in the amount of Ca\(^{2+}\) that is accumulated in the intracellular stores. The release of Ca\(^{2+}\) from intracellular stores into the cytosol is thought to be one of the earliest steps in platelet activation.17,35 Decreased accumulation of Ca\(^{2+}\) in these stores may thus be a part of the molecular mechanism behind the previously reported time-dependent decline of platelet reactivity during long-term experiments.7,16

The amplification of the platelets' functional responses to agonists after ouabain pretreatment, as found by others8-13 as well as by ourselves (Figure 9), is also in line with the idea that CTC-detectable Ca\(^{2+}\) stores play an important role in the platelet response. Taken together, these data point to the increased intracellular accumulation of Ca\(^{2+}\) as a likely explanation for the enhancement of platelet reactivity after ouabain treatment.

**Ouabain Does Not Affect the Signal-Transducing System for Thrombin**

The mobilization of Ca\(^{2+}\) from intracellular stores in control platelets, as well as in ouabain-treated platelets, was dependent on the agonist concentration (Figure 8), indicating the particular role of receptor-induced second messenger formation in the Ca\(^{2+}\)-mobilizing process. The increased amount of Ca\(^{2+}\) mobilized in ouabain-treated platelets could therefore alternatively be explained by an ouabain-induced increase in the amount of second messenger formed. In this context, our finding that the thrombin-induced activation of the phospholipase C (the enzyme that generates the second messengers) was not increased in the presence of ouabain (Table 1) is of major importance. Although not conclusive, since we were unable to measure inositol 1,4,5-trisphosphate (IP\(_3\)) formation, these results indicate a lack of effect of ouabain on the primary enzyme involved in the signal transducing system in which Ca\(^{2+}\) functions as an intracellular messenger.

Platelet activation by thrombin and ADP has been shown to induce a rapid increase in Na\(^{+}\) influx, which could imply that Na\(^{+}\) plays a role in stimulus-response coupling.5,36 Although the effect of ouabain on platelet Ca\(^{2+}\) homeostasis is evident, a separate role for intracellular Na\(^{+}\) in the ouabain-induced effects on stimulus-response coupling cannot be excluded. The increase of the cytosolic Na\(^{+}\) concentration in the resting platelet most likely causes acidification of the cytosol via Na\(^{+}\)-H\(^{+}\) exchange.11 By increasing the sensitivity of the dense tubular system to IP\(_3\), these changes in cytosolic pH and/or cytosolic Na\(^{+}\) concentration may also explain the ouabain-induced enhancement of the platelet response to agonists. This possibility has been addressed by Brass and Joseph,35 who studied the effect of pH and Na\(^{+}\) on IP\(_3\)-induced \(^{45}\)Ca\(^{2+}\) release in permeabilized platelets. They demonstrated that Na\(^{+}\) concentration (10-90 mM) was without effect and that a decrease of pH from 7.1 to 6.7 resulted in a marked decrease of IP\(_3\)-induced \(^{45}\)Ca\(^{2+}\) release. Neither of these observations is likely to explain the ouabain-induced enhancement of the physiological response nor the increased mobilization of Ca\(^{2+}\) from intracellular stores in ouabain-treated platelets.

Our finding that the \(^{32}\)P incorporation in PA (Table 1) was significantly lower when platelets were prelabeled in the presence of ouabain when compared with the control is noteworthy. After 70 minutes incubation with \(^{32}\)Porthophosphate, PA is labeled near the isotopic equilibrium37; therefore, changes in \(^{32}\)P incorporation reflect changes in mass. Phillipon and Nishimoto38 demonstrated a positive correlation between Na\(^{+}\)-Ca\(^{2+}\) exchange activity and the amount of PA present in sarcolemmal vesicles. Thus, PA metabolism could be involved in Na\(^{+}\)-Ca\(^{2+}\) exchange activity in the intact cell. Although we have no direct evidence, the observation that the change in Na\(^{+}\)-Ca\(^{2+}\) exchange activity, which presumably occurs during ouabain treatment, coincides with a change in the amount of PA in the resting platelet tends to favor this view.

**Concluding Remarks**

As far as we know, our data demonstrate for the first time the ouabain-induced accumulation of Ca\(^{2+}\) into intracellular stores and its importance in stimulus-response coupling. In cardiac and smooth muscle cells, as well as in platelets, the same biochemical entities regulate Ca\(^{2+}\) homeostasis (ATPase-driven ion pumps and Na\(^{+}\)-Ca\(^{2+}\) exchanger and mitochondrial ion transport mechanisms). In all these cell types, ouabain amplifies the Ca\(^{2+}\)-mediated functional responses. Therefore, the increased contractility provoked by cardiac glycosides in cardiac and smooth muscle cells is probably based on the same working mechanism as the one we identified in platelets. We have to admit that the weakest link in explaining the effect of ouabain is the role of Na\(^{+}\)-Ca\(^{2+}\) exchange in elevating cytosolic Ca\(^{2+}\). Conclusive evidence on the importance of this transport mechanism in intact cells will have to await development of selective inhibitors.

Elevation of cell Na\(^{+}\), increased Ca\(^{2+}\) storage, and increased aggregation of platelets were found in
untreated mildly hypertensive patients. Furthermore, similar abnormal Ca\textsuperscript{2+} handling and hyperaggregability were reported for platelets from patients with a thrombotic disorder.\textsuperscript{24,40} In view of striking analogies between observations that were made in these pathological conditions and in our in vitro data from platelets upon treatment with ouabain, the use of cardiac glycosides in this type of patient needs particular care since it might aggravate the already existing acute risk of thrombosis.

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