Altered calcium signalling in platelets from bile-duct-ligated rats

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ABSTRACT

In the present study, we have analysed the mechanisms of Ca\(^{2+}\) entry and release in platelets obtained from BDL (bile-duct-ligated) rats, 11–13 days and 4 weeks after surgery. Platelets were washed and loaded with fura-2, and [Ca\(^{2+}\)]\(_i\) (cytosolic Ca\(^{2+}\) concentration) was determined in cell suspensions by means of fluorescence spectroscopy. Basal [Ca\(^{2+}\)]\(_i\) was similar in platelets from BDL rats compared with those from their respective controls, both in the absence and presence of extracellular Ca\(^{2+}\). Platelet stimulation with thrombin in the absence and presence of extracellular Ca\(^{2+}\) induced a rapid rise in [Ca\(^{2+}\)]\(_i\), that was of greater magnitude in platelets from BDL rats than in controls. Ca\(^{2+}\) storage was significantly elevated in platelets from BDL rats, as well as the activity of SERCA (sarcoplasmic/endoplasmic-reticulum Ca\(^{2+}\)-ATPase). Capacitative Ca\(^{2+}\) entry, as evaluated by inhibition of SERCA with thapsigargin, was also altered in platelets from BDL rats, having lower rates of Ca\(^{2+}\) entry. In conclusion, chronic BDL alters intracellular Ca\(^{2+}\) homoeostasis in platelets, such that an enhanced Ca\(^{2+}\) release is evoked by thrombin, which may be due to an increased amount of Ca\(^{2+}\) stored in the intracellular organelles and secondary to an enhanced activity of SERCA. These alterations are already evident before cirrhosis has completely developed and occurs during the cholestasis phase.

INTRODUCTION

Previous studies have suggested that patients with cholestatic liver diseases have less bleeding complications than patients at similar stages of viral or alcoholic liver cirrhosis. These disorders are associated with a better outcome of variceal bleeding and less blood loss at transplantation [1–3]. Although the origin of this alteration is not clear, a previous study [4] has suggested the existence of a hypercoagulable state in patients with primary biliary cirrhosis, due to a more effective platelet function in these patients than in those suffering from other liver disorders. In fact, membrane density of the platelet receptor CD42b was higher in cholestatic than in non-cholestatic patients [5]. This antigen is part of the platelet adhesion receptor gpIb/V/IX which binds subendothelial von-Willebrand factor and is internalized after platelet activation. The presence of these binding sites allows platelets to stop their forward movement in the arterial circulation long enough to become activated and fully adherent [6].

A rise in [Ca\(^{2+}\)]\(_i\), (cytosolic Ca\(^{2+}\) concentration) is a major signal underlying platelet activation [7–10]. Physiological agonists such as ADP and thrombin stimulate both
the release of Ca$^{2+}$ from intracellular stores and the entry of Ca$^{2+}$ across the plasma membrane, which contributes to full platelet activation. When agonist stimulation ceases, a number of mechanisms remove Ca$^{2+}$ from the cytosol to restore the resting [Ca$^{2+}$]. Among these, the most relevant are Ca$^{2+}$ re-uptake into the intracellular stores by SERCA (sarcoplasmic/endoplasmic-reticulum Ca$^{2+}$-ATPase) and Ca$^{2+}$ extrusion carried out by two different transporters, PMCA (plasma membrane Ca$^{2+}$-ATPase) and the Na$^+$/Ca$^{2+}$ exchanger [7–10].

Thus, in order to characterize the molecular basis of the activation of platelets in biliary cirrhosis further, we have studied the mechanisms involved in Ca$^{2+}$ signalling in platelets obtained from an experimental model of liver cirrhosis and cholestasis, the BDL (bile-duct-ligated) rat.

**MATERIALS AND METHODS**

**Drugs**

Fura-2/AM (fura 2 acetoxymethyl ester) was dissolved in DMSO. All the products used were from Sigma, except where indicated. Stock solutions were prepared in distilled water, except thapsigargin and ionomycin which were dissolved in DMSO, and maintained frozen (−20 °C). Appropriate dilutions were prepared freshly every day in measurement buffer.

**Animals**

Male Sprague–Dawley rats born and raised in the Animal House of the Universidad de Murcia were used in the present study. All the experiments were performed according to the ethical rules for the treatment of laboratory animals of the European Union and were approved by the Ethics Committee of the Universidad de Murcia.

**Experimental groups**

Animals weighing approx. 200 g were subjected to bile-duct ligation and excision or sham operation, as described previously [11–13]. Normal rat chow and tap water were offered ad libitum. Experiments were performed after 11–13 days (cholestasis group) and at 4 weeks (BDL group; cirrhosis group) after surgery.

**Isolation of platelets, fura-2 loading and determination of [Ca$^{2+}$]$_i$**

Isolation of platelets, fura-2 loading and determination of [Ca$^{2+}$]$_i$ were performed as described previously [14]. Briefly, animals were anesthetized with thiobutabarbital (Inactin; 100 mg/kg of body weight, intraperitoneally; RBI) and blood was obtained from the abdominal aorta in a plastic tube containing an anticoagulant solution (80 mmol/l sodium citrate, 52 mmol/l citric acid and 180 mmol/l glucose). After obtaining platelet-rich plasma, platelets were washed and incubated with 2.5 µmol/l fura-2/AM (Molecular Probes) for 45 min at room temperature (19 °C). Then, after washing out fura-2, platelets were stored at room temperature in the dark until Ca$^{2+}$ measurements were performed. Liver biochemistry was analysed in plasma samples by an automated method (Hitachi 737; Boehringer Mannheim).

Platelets were placed in fluorescence-free cuvettes (Sigma) in the optical field of a fluorescence spectrometer (Aminco Bowman 2; Microbeam), and excited alternatively with light at 340 and 380 nm and the light emitted at 510 nm was collected. Changes in [Ca$^{2+}$]$_i$ were obtained by using the fura-2 340/380 fluorescence ratio and calibrated as described previously [14,15]. Only one concentration of each drug was tested on every platelet suspension. The calibration procedure was done in every experiment to take into account differences in the number of platelets between animals. After obtaining baseline values for 30 s, the appropriate drug concentration was added and the fluorescence recorded. Three protocols were performed. (i) The response to thrombin (0.03, 0.1, 0.3 and 1 units/ml) was studied in the absence (no Ca$^{2+}$ added plus 0.5 mmol/l EGTA) and presence (1 mmol/l CaCl$_2$) of extracellular Ca$^{2+}$. In separate experiments, 100 µmol/l 2-APB (2-aminoethoxydiphenyl borate) was used to block the IP$_3$ (inositol 1,4,5-trisphosphate) receptor. (ii) CCE (capacitative Ca$^{2+}$ entry) was determined by stimulating platelets in Ca$^{2+}$-free medium with thapsigargin (1 µmol/l). After 180 s, Ca$^{2+}$ (1 mmol/l) was added and changes in [Ca$^{2+}$]$_i$ were monitored for another 180 s. In a second set of experiments, CCE was evaluated as the rate of Mn$^{2+}$ influx [16]. MnCl$_2$ (100 µmol/l) was added and fura-2 fluorescence, monitored at 360 nm, was observed for 180 s. (iii) Ca$^{2+}$ accumulation into intracellular stores was determined by suspending platelets in a Ca$^{2+}$-free medium (+100 µmol/l EGTA) and challenging with ionomycin (5 µmol/l) and thapsigargin (1 µmol/l). In additional experiments, platelets were incubated with 0.5 mmol/l CaCl$_2$ for 5 min prior to the experiment, to ensure that they were not depleted of Ca$^{2+}$, and then stimulated as mentioned above after the Ca$^{2+}$ added was chelated with 1 mmol/l EGTA.

**Determination of Ca$^{2+}$-ATPase activity in microsomes**

Microsomes were prepared according to the method described by Eletr and Inesi [17], as modified for its use in platelets [18]. Protein concentration was measured by the bicinchoninic acid method [19]. The initial rate of P$_i$ release was measured in microsomes by the colorimetric method described by Lanzetta et al. [20]. Experiments were also performed in the presence of 1 µmol/l thapsigargin to inhibit SERCA.

**Statistical analysis**

Results are expressed as means ± S.E.M. In order to compare the responses between groups, the AUC (area
under the curve) of the individual Ca\(^{2+}\) responses was calculated as the integral of the rise in [Ca\(^{2+}\)], for 180 s above basal, taking a sample every second. The resulting values, as well as baseline values, were compared by ANOVA, followed by a Student–Newman–Keuls post-hoc test. A probability level of \(P < 0.05\) was considered to be a significant difference.

**RESULTS**

All of the BDL rats (cholestasis and BDL groups) used in the present study had typical features of this model: jaundice, enlarged liver and spleen, and mesenteric oedema. Ascites was not present in any animal. Sham-operated animals did not have any of these alterations. Plasma total bilirubin was elevated at day 12 (12.8 ± 0.8 mg/dl in the cholestasis group compared with 0.02 ± 0.01 mg/dl in the control group) and decreased slightly in the BDL group (9.8 ± 0.4 mg/dl). A similar pattern was observed with \(\gamma\)-glutamyl transpeptidase (97.4 ± 26.4, 1.2 ± 0.3 and 55.6 ± 5.6 units/l in the cholestasis, control and BDL groups respectively), alkaline phosphatase (154.4 ± 37.5, 10.5 ± 3.9 and 96.5 ± 14.8 units/l in the cholestasis, control and BDL groups respectively) and alanine aminotransferase (174.7 ± 24.7, 89.5 ± 15.7 and 86.4 ± 8.2 in the cholestasis, control and BDL groups respectively).

Resting [Ca\(^{2+}\)], in platelets was similar in all groups both in the presence of Ca\(^{2+}\) (43.1 ± 3.4, 39.4 ± 4.1 and 47.0 ± 3.4 nmol/l in the BDL, cholestasis and control groups respectively) and in a Ca\(^{2+}\)-free medium (35.1 ± 3.4, 32.4 ± 2.9 and 32.1 ± 2.0 nmol/l in the BDL, cholestasis and control groups respectively).

In Ca\(^{2+}\)-free medium, thrombin induced a transient and concentration-dependent elevation of [Ca\(^{2+}\)], in both the cholestasis and BDL groups (Figure 1), and the response in platelets from the cholestasis and BDL groups was greater than in controls, as confirmed by the AUC values (Figure 2, top panel).

Platelet stimulation with thrombin in the presence of 1 mmol/l extracellular Ca\(^{2+}\) induced a greater and more sustained increase in [Ca\(^{2+}\)], than in Ca\(^{2+}\)-free medium. These responses were of greater magnitude in platelets from the cholestasis and BDL groups than in controls, but only at lower concentrations, as shown in Figure 2 (middle panel).

The estimation of thrombin-evoked net Ca\(^{2+}\) entry (Figure 2, lower panel) was significantly greater at the two lower concentrations of thrombin in platelets from the cholestasis and BDL groups; however, this finding should be taken with some caution, as these results do not take into account the contribution of Ca\(^{2+}\) entering the cell, but only Ca\(^{2+}\) that can be pumped out of the cell and/or stored by Ca\(^{2+}\)-ATPases.

2-APB, used at 100 \(\mu\)mol/l to block IP\(_3\) receptors (Figure 3), almost abrogated the response to 0.1 unit/ml thrombin in Ca\(^{2+}\)-free medium (90% compared with experiments performed in the absence of 2-APB), although a significant elevation of [Ca\(^{2+}\)], still remained after stimulation with a higher concentration of thrombin (20–25% of the response was still evident after treatment with 0.3 unit/ml thrombin). Again, the response observed in platelets from the BDL group was greater than that in
controls. Similar results were obtained in the cholestasis group (results not shown).

To estimate the amount of Ca\(^{2+}\) accumulated into intracellular stores, we used thapsigargin, an inhibitor of SERCA, plus ionomycin; both drugs are needed for extensive depletion of the Ca\(^{2+}\) compartments in platelets where two types of stores, with high- and low-Ca\(^{2+}\) leakage rates, have been reported [21–23]. Figure 4 shows that this produced a significantly greater increase in [Ca\(^{2+}\)], in the platelets obtained from the BDL group (Figure 4, left-hand panel). The maintenance of platelets in Ca\(^{2+}\)-free medium did not deplete intracellular Ca\(^{2+}\) as a similar result was obtained when platelets were equilibrated with sufficient Ca\(^{2+}\) (Figure 4, right-hand panel), indicative of a greater amount of Ca\(^{2+}\) stored in the intracellular organelles in platelets from the BDL group. Similar results were obtained in the cholestasis group (results not shown).

With regard to CCE, thapsigargin induced a slow and sustained increase in [Ca\(^{2+}\)] that was greater in platelets from the cholestasis and BDL groups compared with controls (Figures 5 and 6, left-hand panel). The addition of Ca\(^{2+}\) produced a rapid increase in [Ca\(^{2+}\)], indicative of Ca\(^{2+}\) entry (Figure 5). However, the amount of Ca\(^{2+}\) that entered the cells after thapsigargin treatment was significantly lower in platelets from the BDL group (Figure 6, right-hand panel). A tendency to lower Ca\(^{2+}\) entry was also observed in the cholestasis group, but it did not reach statistical significance. These results were confirmed by measuring the rate of Mn\(^{2+}\) influx.

In unstimulated platelets (Figure 7, top panel), there was a small decay in fluorescence, probably due to the presence of extracellular fura-2, leakage of the indicator and to some non-specific entry of Mn\(^{2+}\). However, after thapsigargin administration, the decay in fluorescence was much greater, due to the entry of Mn\(^{2+}\) through store-operated channels (Figure 7, middle panel). When the non-capacitative entry (basal) was subtracted from that induced by thapsigargin, net capacitative entry of calcium into the cells was significantly lower in platelets from the BDL group (Figure 7, right-hand panel). A tendency to lower Ca\(^{2+}\) entry was also observed in the cholestasis group, but it did not reach statistical significance. These results were confirmed by measuring the rate of Mn\(^{2+}\) influx.
Platelet calcium in cholestatic and cirrhotic rats

Figure 4 Changes in \([\text{Ca}^{2+}]_i\) in response to ionomycin and thapsigargin in the control and BDL group in the absence of extracellular \(\text{Ca}^{2+}\)

Results are the means of five experiments.

Figure 5 CCE in response to thapsigargin in platelets from the control, cholestasis and BDL groups

Results are the means of five experiments.

Figure 6 AUC of the integrated \(\text{Ca}^{2+}\) responses after thapsigargin (left-hand panel) and \(\text{Ca}^{2+}\) (right-hand panel) administration

Results are mean ± S.E.M.

Mn\(^{2+}\) was obtained (Figure 7, lower panel). As observed, there was a lower entry of Mn\(^{2+}\) in platelets from BDL rats, also indicative of a lower CCE.

\(\text{Ca}^{2+}\) removal from the cytosol by SERCA and PMCA was investigated by assessing the enzymatic activity of these \(\text{Ca}^{2+}\)-ATPases in platelet microsomes (Figure 8). The activity of the \(\text{Ca}^{2+}\)-ATPases was significantly increased in the BDL group, as shown by the slope of the relationship (11.4 ± 0.4 compared with 8.1 ± 0.3 nmol · min\(^{-1}\) · mg\(^{-1}\) of protein in the BDL and control groups respectively). Activity of the pumps in the cholestasis group was also similar to that observed in the BDL group (10.7 ± 0.3 nmol · min\(^{-1}\) · mg\(^{-1}\) of protein).

After inhibition of SERCA with thapsigargin, the activity of PMCA still remained, and it was significantly greater in platelets from the BDL group compared with the controls (slopes, 2.8 ± 0.2 compared with 3.8 ± 0.2 nmol · min\(^{-1}\) · mg\(^{-1}\) of protein respectively). These results also suggest that the greater difference observed when both ATPases are active, in the absence of thapsigargin, is mainly mediated by the enhanced activity of SERCA in BDL rats.

DISCUSSION

Several studies indicate that cholestatic diseases are associated with alterations in platelet function [1–5]. These results point to alterations that may be related to abnormalities in \(\text{Ca}^{2+}\) homoeostasis. Therefore, in the present study, we have analysed \(\text{Ca}^{2+}\) signalling in platelets obtained from a rat model frequently used for the study of cholestatic and cirrhotic diseases, the BDL model.

As shown in the present study, platelets obtained from the cholestasis and BDL groups exhibited similar \([\text{Ca}^{2+}]_i\); in the resting state. Although platelet \(\text{Ca}^{2+}\) levels have been widely measured in other experimental rat models [24,25], we believe this is the first time that platelet \([\text{Ca}^{2+}]_i\) has been determined in an experimental model of liver cirrhosis and cholestasis.
Figure 7 Percentage decay in fluorescence, collected at 360 nm after administration of 100 µmol/l MnCl₂ in unstimulated platelets (top panel), after addition of thapsigargin (middle panel) and the difference between the two (lower panel).

Results are the means of five experiments per group.

Figure 8 Changes in SERCA enzymatic activity as a function of time in microsomes obtained from platelets from the control and BDL group

Results are means ± S.E.M. (n = 6).

The responses to thrombin were significantly enhanced in platelets from BDL rats, both in the absence and presence of extracellular Ca²⁺. When platelets were stimulated with thrombin in the absence of external Ca²⁺, the elevation in [Ca²⁺], was the result of Ca²⁺ release from the internal stores, mainly the endoplasmic reticulum [7–10]. Our results indicate that thrombin-stimulated release of Ca²⁺ from the internal stores was clearly elevated in platelets from the cholestasis and BDL groups. The mechanisms responsible for this alteration are unknown, but it may be due to enlarged Ca²⁺ stores, enhanced IP₃ generation, and/or enhanced affinity of IP₃ receptors for IP₃ or an increased resistance of these receptors to desensitization. Although we have not measured IP₃ production itself in the present study, we have used 2-APB, a cell-permeant IP₃ receptor blocker [26,27], and found that it reduced thrombin-induced Ca²⁺ release; however, under these conditions, a significantly greater thrombin-evoked response was still observed in platelets from BDL rats, which suggests that this alteration is at least partly independent of the IP₃-dependent pathway. Another explanation for the increased release of Ca²⁺ in platelets from BDL rats is that the amount of Ca²⁺ stored could be greater. Indeed, when we induced extensive depletion of the intracellular stores with thapsigargin and ionomycin, we found that the amount of Ca²⁺ accumulated into the intracellular compartments by platelets from BDL rats was significantly greater than that of controls. These results suggest that the increased Ca²⁺ release induced by thrombin in platelets from BDL rats may be related to an elevated accumulation of Ca²⁺ in the intracellular stores.

When platelets were stimulated with thrombin in the presence of extracellular Ca²⁺, another mechanism also takes place, namely the entry of Ca²⁺ from the extracellular space, in this case through capacitative Ca²⁺ channels, since Ca²⁺ entry induced by thrombin in platelets is mainly capacitative [7–10]. Again, platelets from BDL rats had a greater increase in Ca²⁺ in response to thrombin than controls, although this was significant only for the two lower concentrations of the agonist. In comparison with the previous experiment, platelet stimulation with thrombin in the presence of Ca²⁺ increases [Ca²⁺], not only because of its release from the internal stores, but also by Ca²⁺ that enters the cell induced by depletion of the internal stores, the so-called CCE. We have estimated the entry of Ca²⁺ under our experimental protocol as the difference between
the rise in \([\text{Ca}^{2+}]_i\), induced by thrombin in the absence and presence of extracellular \(\text{Ca}^{2+}\) (Figure 4). As observed, this difference was greater in platelets from BDL rats, which indicates that the thrombin-induced increase in intracellular \(\text{Ca}^{2+}\) is enhanced, at least for the lower thrombin concentrations used. Thus, as these lower thrombin concentrations are close to the physiological levels that platelets may encounter in the vascular system, our results suggest that activation of platelets from BDL rats with thrombin will trigger \(\text{Ca}^{2+}\)-dependent mechanisms more easily, resulting in the complete activation of platelet adhesion and aggregation. At high thrombin doses, however, an increased net entry of \(\text{Ca}^{2+}\) was not observed, and this may be due to the fact that higher doses of thrombin that produce greater increases in \([\text{Ca}^{2+}]_i\), are also expected to induce a greater activation of \(\text{Ca}^{2+}\) extrusion and storage mechanisms [28].

In order to confirm that CCE was altered in platelets from BDL rats, additional and more direct experiments were performed. The first approach used a direct depletion of the intracellular stores using thapsigargin, which inhibits \(\text{Ca}^{2+}\) uptake by SERCA [23,29] and this opens capacitative \(\text{Ca}^{2+}\) channels in the plasma membrane [30–32]. Interestingly, although the effect of thapsigargin in elevating \(\text{Ca}^{2+}\) levels was modest in comparison with that of thrombin, we observed a significant difference between the groups. Thus \([\text{Ca}^{2+}]_i\) was greater in platelets from BDL rats after thapsigargin treatment, and this may be due to the increased \(\text{Ca}^{2+}\) accumulation into the stores, possibly secondary to an enhanced pumping activity of SERCA. In support of this possibility, we found that the enzymatic activity of SERCA was significantly increased in microsomes of platelets from BDL rats. However, in spite of a greater \(\text{Ca}^{2+}\) depletion of the internal stores, CCE induced by treatment with thapsigargin was clearly lower in platelets from BDL rats. In another set of experiments, we analysed the rate of entry of \(\text{Mn}^{2+}\) which, by quenching fura-2 fluorescence, allows for the monitoring of \(\text{Mn}^{2+}\) entry also occurring by the same store-operated channels used by \(\text{Ca}^{2+}\) [16]. These experiments confirmed the results obtained with thapsigargin, indicating that CCE, secondary to inhibition of SERCA, is reduced in platelets from BDL rats. The mechanisms underlying this alteration are not known. It has been suggested that nitric oxide inhibits CCE in human platelets by activating the refilling of the stores [33]. Whether an excess of nitric oxide present in this rat model of liver cirrhosis and cholestasis [11–13] is involved in the alterations shown in the present study is not known at the present time.

Two separate \(\text{Ca}^{2+}\) stores, the dense tubular system and an acidic organelle, with high and low sensitivity to thapsigargin respectively, and high- and low-leakage rates, have been reported in platelets [18,23,34]. Depletion of each store induces CCE by different mechanisms that are differentially regulated by cellular components, such as the actin cytoskeleton [35]. Although thrombin is able to release \(\text{Ca}^{2+}\) from both stores at concentrations as low as 0.1 unit/ml [36], both \(\text{Ca}^{2+}\) compartments show a different sensitivity to thapsigargin, which might explain the differences observed between thapsigargin-or thrombin-induced CCE in platelets from BDL rats compared with controls. Our results indicate that thapsigargin-induced CCE, which is mostly mediated by depletion of the dense tubular system, is reduced in platelets from BDL rats. This effect might be compensated by an enhanced CCE mediated by depletion of the acidic stores after treatment with thrombin or by an increased non-CCE, which has been suggested to occur at high thrombin concentrations [37].

Our results are in keeping with those of Pihusch et al. [5], who described alterations in platelet function in cholestatic patients compatible with platelet activation status that may explain the more favourable outcome of portal hypertensive bleeding found in patients with advanced chronic cholestatic liver disease. Our results offer a possible mechanism that could explain those alterations; however, more experiments will be necessary in order to confirm these alterations in \(\text{Ca}^{2+}\) signalling in cirrhotic patients.

In conclusion, chronic bile-duct ligation alters intracellular \(\text{Ca}^{2+}\) homoeostasis in platelets, such that an enhanced \(\text{Ca}^{2+}\) release is evoked by thrombin. This may be due to an increased amount of \(\text{Ca}^{2+}\) stored in the intracellular organelles and secondary to enhanced activity of SERCA. These alterations are already evident before cirrhosis is completely developed and occurs during the cholestasis phase.

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