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Altered calcium signaling in platelets from bile duct-ligated rats

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Summary

Background/Aims: we have analyzed the mechanisms of calcium entry and release in platelets obtained from bile duct-ligated rats (BDL), two (cholestasis group) and four weeks after surgery. *Methods:* platelets were washed and loaded with fura-2. Cytosolic calcium concentration ($[Ca^{2+}]_i$) was determined in cell suspensions by means of fluorescence spectroscopy. *Results:* Basal $[Ca^{2+}]_i$ was similar in platelets from BDL rats as compared with those from their respective controls, both in the absence and presence of extracellular calcium. Platelet stimulation with thrombin in the absence and presence of extracellular calcium induced a rapid rise in $[Ca^{2+}]_i$ that was of greater magnitude in platelets from BDL rats than in controls. Calcium storage was significantly elevated in platelets from BDL rats, as well as the activity of the sarcoendoplasmic reticulum calcium ATP-ase (SERCA). Capacitative calcium entry, as evaluated by inhibition of SERCA with thapsigargin, is also altered in platelets from BDL rats, showing lower rates of calcium entry. *Conclusions:* chronic BDL alters intracellular calcium homeostasis in platelets, so that an enhanced calcium release is evoked by thrombin, which may be due to an increased amount of calcium stored in the intracellular organelles and secondary to an enhanced activity of SERCA. These alterations are already evident before cirrhosis is completely developed, during the cholestasis phase.

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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 recent study 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 (4). In fact, membrane density of platelet receptor CD 42b was higher in cholestatic than in non-cholestatic patients (5). This antigen is part of the platelet adhesion receptor gplb/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 cytosolic calcium concentration ($[Ca^{2+}]_i$) is a major signal underlying platelet activation (7-10). Physiological agonists such as ADP and thrombin stimulate both the release of calcium from intracellular stores and the entry of calcium across the plasma membrane, which contributes to full platelet activation. When agonist stimulation ceases, a number of mechanisms remove calcium from the cytosol to restore the resting $[Ca^{2+}]_i$. Among these, the most relevant are calcium reuptake into the intracellular stores by the sarcoendoplasmic reticulum Ca^{2+} -ATPase (SERCA) and calcium extrusion carried out by two different transporters, the plasma membrane Ca^{2+} -ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (7-10).

Thus, in order to further characterize the molecular basis of the activation of platelets in biliary cirrhosis, we have studied the mechanisms involved in calcium

signaling in platelets obtained from an experimental model of liver cirrhosis and cholestasis, the bile duct-ligated rat.

Materials and methods.

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 approved by the Ethics Committee of the Universidad de Murcia.

Experimental groups.

Animals weighing around 200 g were subjected to bile duct ligation and excision (BDL) or sham operation as previously described (11-13). Normal rat chow and tap water were offered with no restrictions. Experiments were performed in the fourth week after surgery (BDL group) and on the 11-13th day (cholestasis group).

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

This procedure has been previously published (14). Animals were anesthetized with thiobutabarbital (Inactin, 100 mg/kg, i.p., RBI, Massachusetts, USA) and blood obtained from the abdominal aorta in a plastic tube containing an anticoagulant solution (mM: sodium citrate, 80; citric acid, 52; glucose, 180). After obtaining platelet rich plasma, platelets were washed and incubated with 2.5 μ M fura-2 AM (Molecular Probes, Leiden, The Netherlands) for 45 minutes at room temperature. Then, after washing out fura-2, platelets were stored at room temperature (19°C) and in the dark until calcium measurements. Liver biochemistry was analyzed in plasma samples by an automated method (Hitachi 737, Boehringer Mannheim, Germany).

Platelets were placed in fluorescence-free cuvettes (C-0793, Sigma) in the optical field of a fluorescence spectrometer (Aminco Bowman 2, Microbeam, Barcelona, Spain), and excited alternatively with light of 340 and 380 nm wavelength and the light emitted at 510 nm collected. Changes in $[Ca^{2+}]_i$ were obtained by using the fura-2 340/380 fluorescence ratio and calibrated as described (14-15). Only one concentration of each drug was tested in 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:

1. *The response to thrombin (0.03, 0.1, 0.3, and 1 U/ml)* was studied in the absence of extracellular calcium (no calcium added plus EGTA 0.5 mM) and in its presence (1 mM $CaCl_2$). In separate experiments, 2-aminoethoxydiphenyl borate (2-APB, 100 μ M) was used to block the IP_3 receptor.
2. *Capacitative calcium entry (CCE)*: platelets in Ca^{2+} -free medium were stimulated with thapsigargin (1 μ M). Then, 180 s. later, calcium (1 mM) was added and changes in $[Ca^{2+}]_i$ monitored for another 180 s. In a second set of experiments, CCE was evaluated as the rate of manganese (Mn^{2+}) influx (16). $MnCl_2$ (100 μ M) was added and fura-2 fluorescence, monitored at 360 nm, observed for 180 s.
3. *Ca^{2+} accumulation into intracellular stores*: platelets suspended in a Ca^{2+} -free medium (+ EGTA 100 μ M) were challenged with ionomycin (5 μ M) and thapsigargin (1 μ M). In additional experiments, platelets were incubated with 0.5 mM $CaCl_2$ during 5 min. previous to the experiment, to make sure that platelets were not depleted of calcium, and then stimulated as mentioned above, after chelating the calcium added with EGTA 1 mM.

Determination of calcium ATP-ase 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 inorganic phosphate release was measured, in microsomes, by the colorimetric method of Lanzetta (20). Experiments were also performed in the presence of 1 μ M thapsigargin to inhibit SERCA.

Drugs.

Fura-2 AM was dissolved in DMSO. All the products used were from Sigma Chemical (Madrid, Spain), except where indicated. Drug 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.

Statistical analysis.

Data are expressed as the mean \pm S.E. In order to compare the responses between groups, the area under the curve (AUC) of the individual calcium responses was calculated as the integral of the rise in $[Ca^{2+}]_i$ for 180 s above basal taking a sample every second. The resulting values as well as baseline values were compared by one-way analysis of variance and a Student-Newman-Keuls as post-hoc test. A probability level of $p < 0.05$ was considered to be a significant difference.

Results

All the BDL rats used in the present study showed the typical features of this model: jaundice, enlarged liver and spleen and mesenteric edema. Ascites was not present in any animal. Sham operated animals did not show any of these alterations. Plasma total bilirubin was elevated at day 12 (12.8 ± 0.8 mg/dL vs 0.02 ± 0.01 in

controls) and decreased slightly in the BDL group (9.8 ± 0.4). A similar pattern was observed with gamma-glutamyl transpeptidase (97.4 ± 26.4 U/L, 1.2 ± 0.3 and 55.6 ± 5.6 , respectively), with alkaline phosphatase (154.4 ± 37.5 U/L, 10.5 ± 3.9 and 96.5 ± 14.8 , respectively) and with alanine amino-aminotransferase (174.7 ± 24.7 , 89.5 ± 15.7 and 86.4 ± 8.2 , respectively).

Resting $[Ca^{2+}]_i$ in platelets was similar in all groups, both in the presence of calcium (43.1 ± 3.4 nM in BDL rats, 39.4 ± 4.1 in the cholestasis group and 47.0 ± 3.4 nM in controls) and in a Ca^{2+} -free medium (35.1 ± 3.4 nM, 32.4 ± 2.9 and 32.1 ± 2.0 , respectively).

In a Ca^{2+} -free medium, thrombin induced a transient and concentration-dependent elevation in $[Ca^{2+}]_i$ in both bile duct-ligated groups (figure 1), and the response in platelets from cholestatic and BDL rats was greater than in controls, as confirmed by the AUC values (figure 2, top).

Platelet stimulation with thrombin in the presence of 1 mM extracellular calcium induced greater elevations in $[Ca^{2+}]_i$ and more sustained than in a Ca^{2+} -free medium. These responses were of greater magnitude in platelets from cholestatic and BDL rats than in controls, but only at lower concentrations, as it can be appreciated in figure 2 (middle).

The estimation of thrombin-evoked net calcium entry (figure 2, lower) was significantly greater at the two lower concentrations of thrombin in platelets from BDL rats. However, this result should be taken with caution, since these data do not take into account the contribution of calcium entering the cell but that can be pumped out of the cell and/or stored by calcium ATPases.

2-APB, used at 100 μ M to block IP_3 receptors (figure 3), almost abrogated the response to 0.1 U/ml thrombin in a Ca^{2+} -free medium (90% as compared with

experiments performed in the absence of 2-APB), although a significant elevation in $[Ca^{2+}]_i$ still remained after the stimulation with a greater concentration of thrombin (after treatment with 0.3 U/ml thrombin, a 20-25% of the response was still evident). Again, the response observed in BDL platelets was greater than that in controls.

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

With regard to CCE, thapsigargin induced a slow and sustained increase in $[Ca^{2+}]_i$ that was greater in platelets from cholestatic and BDL rats (figure 5 and 6, left). The addition of calcium produced a rapid increase in $[Ca^{2+}]_i$ indicative of calcium entry (figure 5). However, the amount of calcium that entered the cells after thapsigargin was significantly smaller in platelets from BDL rats (figure 6, right). A tendency to lower calcium 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), there is a small decay in fluorescence, probably due to the presence of some extracellular fura-2, the 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). When the non-capacitative entry (basal) was subtracted from that induced by thapsigargin, net capacitative entry of Mn^{2+} was obtained (figure 7, lower). As observed, there was a lower entry of Mn^{2+} in platelets from BDL rats, also indicative of a lower CCE.

Calcium removal from the cytosol by SERCA and PMCA was investigated by assessing the enzymatic activity of these Ca^{2+} ATPases in platelet microsomes (figure 8). The activity of the Ca^{2+} ATPases was significantly elevated in the BDL rats, as evidenced by the slope of the relationship (11.4 ± 0.4 in BDL vs 8.1 ± 0.3 in control microsomes). Activity of the pumps in the cholestasis group was also similar to that observed in the BDL group (10.7 ± 0.3). After inhibition of SERCA with thapsigargin, the activity of PMCA still remained, and it was significantly greater in platelets from BDL rats (slopes: 2.8 ± 0.2 vs 3.8 ± 0.2). These data 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 calcium homeostasis. Therefore, in the present study we have analyzed calcium signaling in platelets obtained from a rat model, frequently used for the study of cholestatic and cirrhotic diseases, the bile duct-ligated model.

As it is shown by the present data, platelets obtained from BDL rats exhibited a similar $[Ca^{2+}]_i$ in the resting state. Although platelet calcium levels have been widely measured in other experimental rat models (24-25), we believe this is the first time that platelet $[Ca^{2+}]_i$ is determined in an experimental model of liver cirrhosis and cholestasis.

The responses to thrombin were significantly enhanced in platelets from bile duct-ligated animals, both in the absence and presence of extracellular calcium. When platelets were stimulated with thrombin in the absence of external calcium, the elevation in $[Ca^{2+}]_i$ is the result of calcium release from the internal stores, mainly the endoplasmic reticulum (7-10). Our results indicate that thrombin-stimulated release of calcium from the internal stores is clearly elevated in platelets from cholestatic and BDL rats. The mechanisms responsible for this alteration are unknown, but it may be due to enlarged calcium stores or to enhanced IP_3 generation and/or either enhanced affinity of IP_3 receptors for IP_3 or an increased resistance of these receptors to desensitization. Although we have not measured IP_3 production at the current state, we have used 2-APB, a cell-permeant IP_3 receptor blocker (26-27), and found that it reduced thrombin-induced calcium 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 IP_3 -dependent pathway. Another explanation for the larger release of calcium in BDL platelets is that the amount of calcium stored could be greater. Indeed, when we induced extensive depletion of the intracellular stores, with thapsigargin and ionomycin, we found that the amount of calcium accumulated into the intracellular compartments by platelets from BDL rats was significantly greater than that of controls. These data suggest that the increased calcium release induced by thrombin in platelets from BDL rats may be related to an elevated accumulation of calcium in the intracellular stores.

When platelets were stimulated with thrombin in the presence of extracellular calcium, another mechanism also takes place, the entry of calcium from the extracellular space, in this case through capacitative calcium channels, since, in

platelets, calcium entry induced by thrombin is mainly capacitative (7-10). Again, platelets from BDL rats showed a greater calcium elevation in response to thrombin than controls, although this was significant only for the two lower concentrations of the agonist. Different to the previous experiment, platelet stimulation with thrombin in the presence of calcium, increases $[Ca^{2+}]_i$ not only because of its release from the internal stores, but also by calcium that enters the cell induced by depletion of the internal stores, the so-called CCE. We have estimated the entry of calcium under our experimental protocol as the difference between the rise in $[Ca^{2+}]_i$ induced by thrombin in the presence and absence of extracellular calcium (figure 4). As observed, this difference is greater in platelets from BDL rats, which indicates that thrombin-induced increase in intracellular calcium is enhanced in platelets from BDL rats, at least for the lower thrombin concentrations used. Thus, since these lower thrombin concentrations are close to the physiological levels that platelets may encounter in the vascular system, our result suggest that the activation of platelets from bile duct-ligated rats with thrombin will activate more easily the Ca^{2+} -dependent mechanisms driving to the complete activation of platelet adhesion and aggregation. At high thrombin doses, however, an increased net entry of calcium is not observed, and this may be due to the fact that higher doses of thrombin that produce greater increases in $[Ca^{2+}]_i$ are also expected to induce a greater activation of calcium extrusion and storage mechanisms (28).

In order to confirm that CCE is 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 calcium uptake by SERCA (23, 29), and this opens capacitative calcium channels in the plasma membrane (30-32). Interestingly, although the effect of thapsigargin to elevate

calcium levels was modest, in comparison to that of thrombin, we observed a significant difference between groups. Thus, $[Ca^{2+}]_i$ was greater in platelets from BDL rats, after thapsigargin, and this may be due to the increased calcium 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 calcium depletion of the internal stores, the CCE induced by treatment with thapsigargin was clearly lower in platelets from BDL rats. In another set of experiments, we analyzed the rate of entry of Mn^{2+} , which by quenching fura-2 fluorescence, allows for the monitoring of Mn^{2+} entry, which occurs also by the same store-operated channels used by Ca^{2+} (16). These experiments confirmed the results obtained with thapsigargin, indicating that the CCE of calcium, 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 the 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 present time.

Two separate calcium 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). While thrombin is able to release calcium from both stores at concentrations as low as 0.1 U/mL (36) both calcium compartments show a different sensitivity to thapsigargin, which might explain the differences observed between thapsigargin- or thrombin-induced CCE in BDL platelets

compared to controls. Our results indicate that thapsigargin-induced CCE, which is mostly mediated by depletion of the dense tubular system, is reduced in BDL platelets. 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-capacitative entry of calcium which has been suggested to occur at high thrombin concentrations (37).

Our results are in keeping with those of Pihusch et al who described a platelet function alteration in cholestatic patients compatible with a 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 which could explain those alterations. However, more experiments will be necessary in order to confirm these calcium signalling alterations in cirrhotic patients.

In conclusion, chronic bile duct-ligation alters intracellular calcium homeostasis in platelets, so that an enhanced calcium release is evoked by thrombin, which may be due to an increased amount of calcium stored in the intracellular organelles and secondary to an enhanced activity of SERCA. These alterations are already evident before cirrhosis is completely developed, during the cholestasis phase.

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Figure legends.

1. Changes of intracellular calcium levels in response to thrombin in platelets from control, cholestatic and BDL rats, in the absence of extracellular calcium. The data are the mean of 6 experiments.
2. Area under the curve of the integrated calcium responses after thrombin administration in the absence (top panel) and in the presence (middle panel) of extracellular calcium and difference between them two (lower panel).
3. Changes of intracellular calcium levels in response to thrombin in platelets from control and BDL rats, in the absence of extracellular calcium, and after incubation with 100 μ M 2-APB to block the receptor for IP₃. The data are the mean of 4 experiments.
4. Changes of intracellular calcium levels in response to ionomycin and thapsigargin in control and BDL rats, in the absence of extracellular calcium. The data are the mean of 5 experiments.
5. Capacitative calcium entry in response to thapsigargin in platelets from control, cholestatic and BDL rats. The data are the mean of 5 experiments.
6. Area under the curve of the integrated calcium responses after thapsigargin (left panel) and calcium (right panel) administration.
7. Percentage decay of fluorescence, collected at 360 nm, after administration of 100 μ M MnCl₂ in unstimulated platelets (top panel) and after addition of thapsigargin (middle panel). Lower panel represents net Mn influx, obtained after subtracting basal from thapsigargin-induced decay in fluorescence. The results are the mean of 5 experiments per group.
8. Changes in the enzymatic activity of SERCA as a function of time in microsomes obtained from platelets of control and BDL rats (n=6 each).

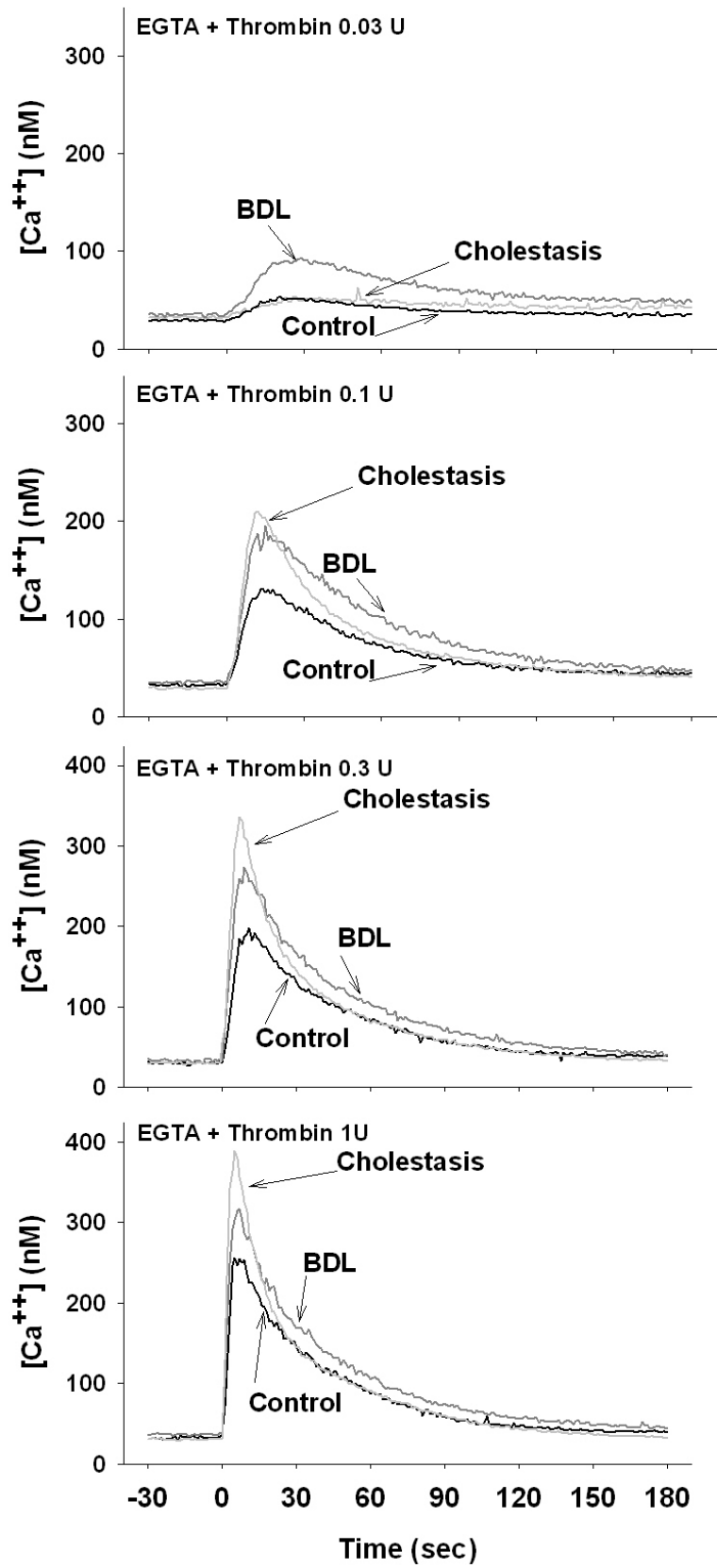


Figure 1

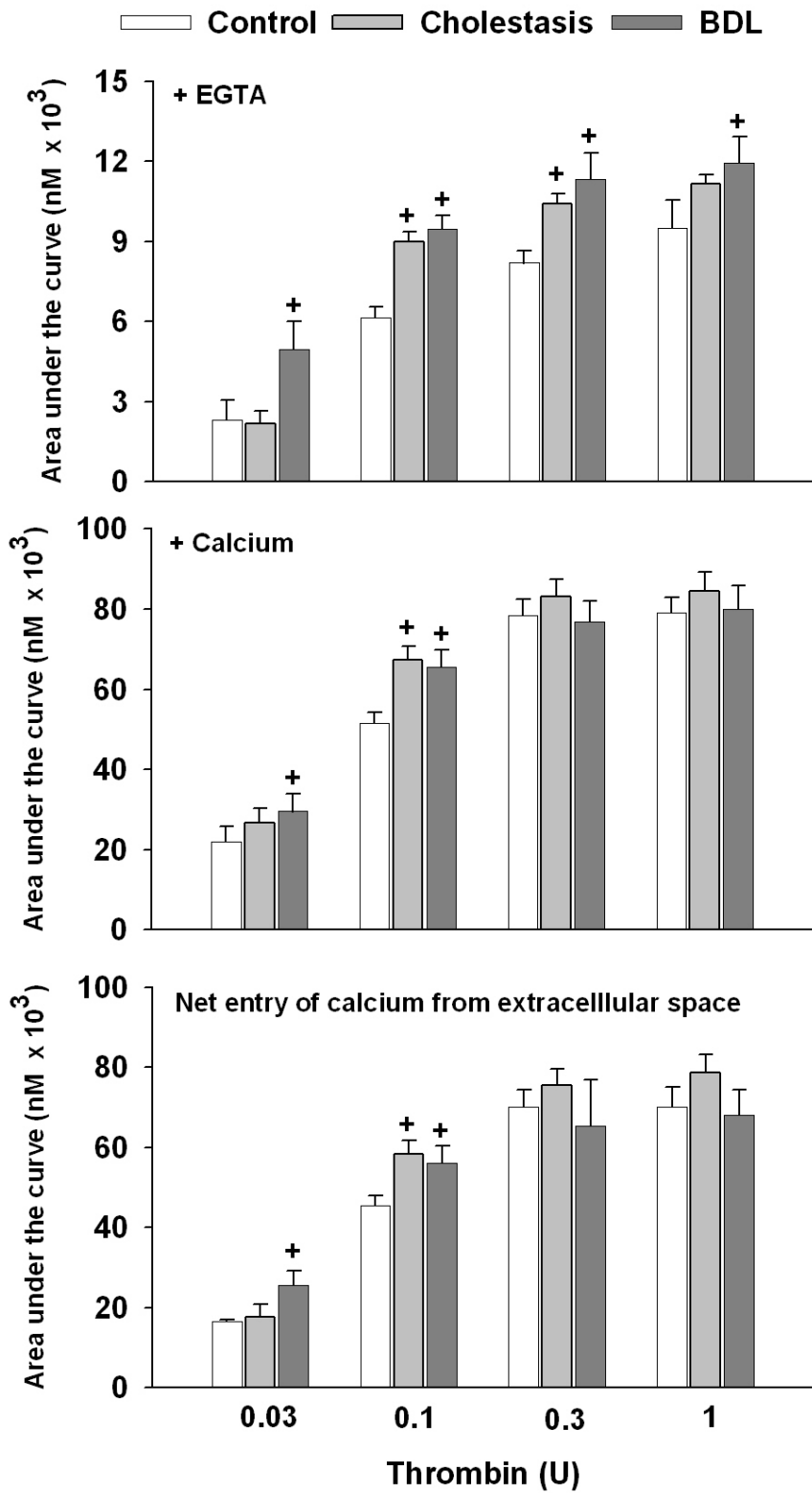


Figure 2

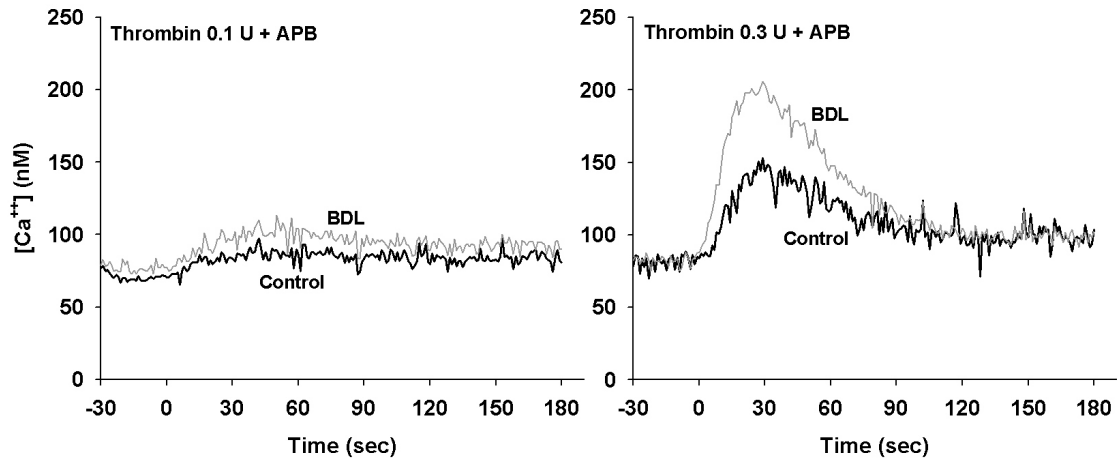


Figure 3

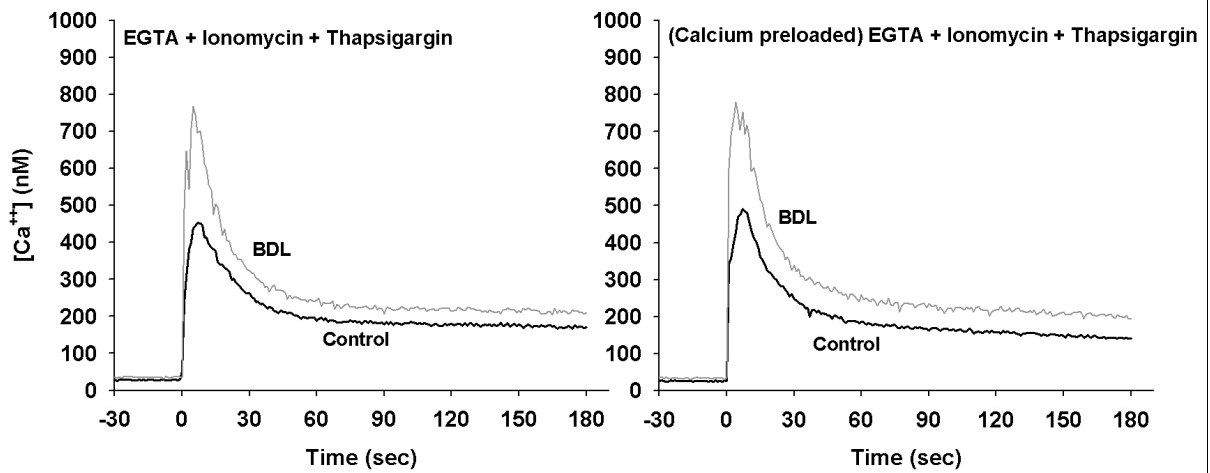
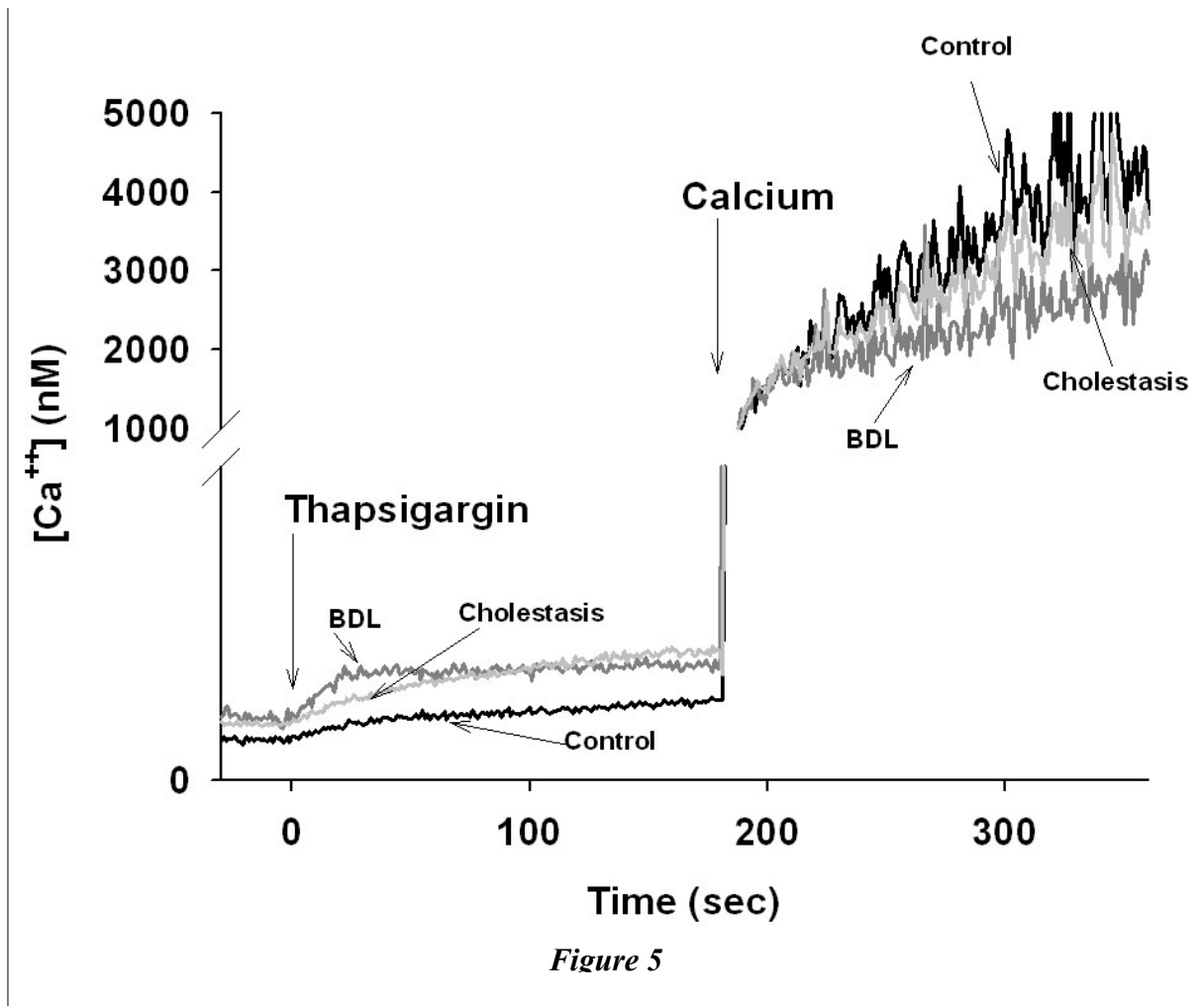


Figure 4



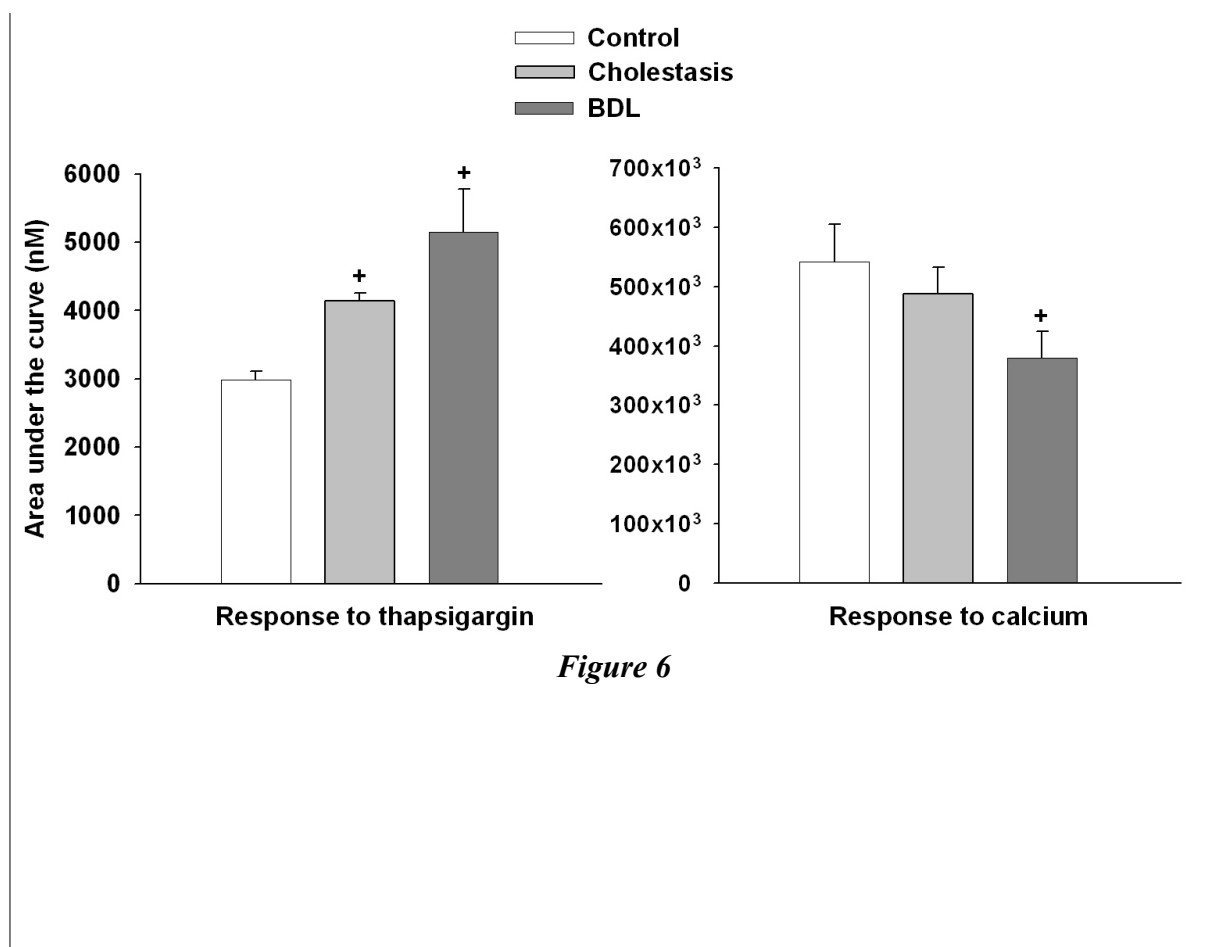


Figure 6

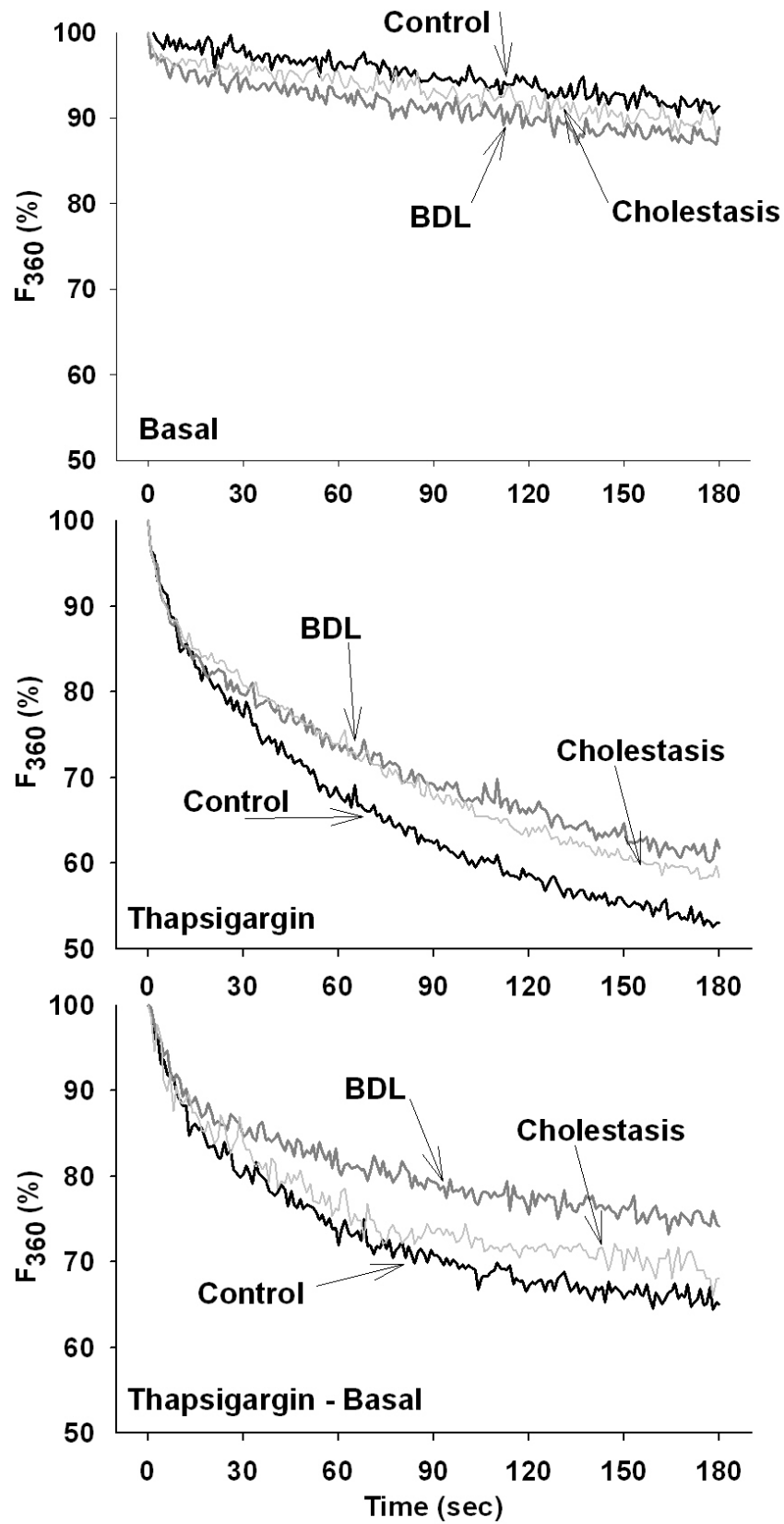


Figure 7

