Cell Communication and Signaling BioMed Central

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Altered calcium signaling in platelets from nitric oxide-deficient hypertensive rats

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Published: 10 May 2004

Cell Communication and Signaling 2004, **2**:1

Received: 12 February 2004
Accepted: 10 May 2004

This article is available from: http://www.biosignaling.com/content/2/1/1

Abstract

Background: In the present study we have analyzed the mechanisms of calcium entry and mobilization in platelets obtained from rats chronically treated with the nitric oxide synthesis inhibitor, N-nitro L-arginine methyl ester [L-NAME, 40 mg/kg/day, 5 days). The platelets were obtained the day of the experiment, washed and loaded with fura-2. The intracellular calcium levels were determined in suspension of cells by means of fluorescence spectroscopy.

Results: Basal calcium levels were always elevated in the platelets of the L-NAME-treated rats, both in the presence and in the absence of extracellular calcium. The administration of thrombin in the absence and in the presence of extracellular calcium induced important elevations in calcium levels that were always of greater magnitude in the platelets of the L-NAME-treated rats than in those of the controls. The addition of calcium to thapsigargin-treated platelets produced a massive elevation in calcium levels in both groups that was significantly greater in the platelets obtained from the hypertensive rats than in those of the controls.

Conclusions: It is concluded that the arterial hypertension induced by the reduction of nitric oxide alters the regulation of platelet calcium levels so that elevated baseline levels and calcium entry and mobilization are enhanced. This could be the result of direct or indirect effects of the lack of nitric oxide synthesis in platelets or in other tissues.

Background

Nitric oxide (NO) is a very important regulator of numerous biological functions, among them the control of vascular tone. The reduction of NO production, either by inhibiting its synthesis or in situations of endothelial dysfunction, leads to arterial hypertension which then affects hemostatic, vascular, renal, and cardiac function, among others [1-3]. Arterial hypertension is also a risk factor for developing thrombotic events, which is related to activation of blood platelets which then can interact easily with a damaged vascular endothelium. It is thought that the mechanism responsible for the activation of platelets in hypertension is related to an increased cytosolic calcium (Ca^{2+}) level and to an enhanced sensitivity to agonists, but the mechanisms responsible for this increase in platelet Ca^{2+} are not known [4,5].

It is well known that NO plays an important role in aggregation of platelets. In fact, platelets were one of the first cell types where the L-arginine-nitric oxide pathway was characterized [6]. Recent studies have also suggested that NO is able to interfere with the regulation of calcium levels in platelets [7] and in other cell types. Thus, a NO-induced reduction of intracellular calcium levels has been demonstrated to occur both at the level of calcium entry to the cell from the extracellular space and at intracellular calcium release sites, such as the endoplasmic reticulum [7]. Thus, we hypothesized that the reduction in NO levels brought about by the chronic inhibition of NO that induces an arterial hypertensive state would induce an elevation in platelet calcium concentration. In order to test this hypothesis, we have performed experiments on platelets obtained from L-NAME-treated hypertensive rats, by using fluorescence spectroscopy.

Results

Mean arterial pressure was increased in the L-NAME-treated animals and averaged 154.3 ± 1.6 mmHg (n = 6). This value was significantly different from that observed in the control animals (105.6 ± 1.1, n = 6). Resting calcium levels were always elevated in the platelets of the L-NAME-treated rats, both in the presence (155 ± 10 nM in these hypertensive rats and 76 ± 8 nM in the controls) and in the absence of extracellular calcium (154 ± 7 nM and 70 ± 4 nM, respectively). The administration of thrombin in the absence of extracellular calcium (figure 1) induced a sharp elevation in calcium levels and a decline back to basal levels, which in the case of the two higher doses, 0.3 and 1 U, reached even values well below the baseline. However, the AUC measured during the first 60 seconds after the administration of the 0.3 U dose was significantly greater in the platelets of the hypertensive rats (866.5 ± 102.4 units) than in those of the controls (513.53 ± 64.9 units). The AUC was not calculated in the experiments with the dose of 1 U because calcium fell below baseline during the first 15 seconds after the drug administration. But, the lower dose of 0.1 U maintained calcium elevated for the entire 180 seconds period and the AUC was again significantly enhanced in the platelets of the hypertensive rats (2727.9 ± 264.2 units) than in those of the controls (1726.6 ± 223.6 units).

The administration of thrombin in the presence of 1 mM extracellular calcium induced important elevations in calcium levels that were maintained during the experiment (figure 2), and that were always of greater magnitude in the platelets of the L-NAME-treated rats than in those of the controls, as it can be concluded from the AUC data presented in figure 3.

Finally, thapsigargin administration to platelets in the absence of extracellular calcium slightly elevated baseline calcium levels, but without significant differences between the groups (figure 4, left). However, the addition of calcium to these thapsigargin-treated platelets produced a massive elevation in calcium levels in both groups, that was significantly greater in the hypertensive rats (area under the curve: 551.9 ± 32 units) than in the controls (429.4 ± 25 units).

Discussion

Several studies indicate that arterial hypertension is associated with alterations in the regulation of intracellular calcium levels in many cell types of human and experimental hypertension [3,4,8,9]. These alterations bring about an elevation in cytosolic calcium levels which seems to be related, among others, with an enhanced vascular tone and with a tendency to platelet activation, which contributes to thrombotic events. Also, platelets are readily available and they share several alterations shown by other cell types such as smooth muscle cells [8]. Because of this, platelets are often utilized for the study of calcium signaling. In the present work, we have used platelets to analyze the regulation of calcium levels in a frequently utilized model of arterial hypertension, the L-NAME-treated rat. This nitric oxide deficient arterial hypertensive rat model is now very much studied because it is very well suited to analyze the pathophysiology of arterial hypertension.

As it is shown by the present data, platelets obtained from the hypertensive rats exhibited greater calcium levels in the resting state. Although platelet calcium levels have been measured frequently in other arterial hypertensive rat models, we believe this is the first demonstration of a similar alteration in the L-NAME-treated hypertensive model. Also, a similar alteration in free calcium concentration has been described in red blood cells of rats chronically treated with L-NAME [10]. This elevated resting level of calcium may be the result of the alterations identified in the present experiments, enhanced calcium entry and mobilization, and likely to be the consequence of the deficit in NO synthesis. These results are in keeping from previous studies from our laboratory [11], showing that the mesenteric vessels of L-NAME hypertensive rats show an enhanced response to vasopressors which is related to calcium entry.

The responses to the agonist thrombin were significantly enhanced in the platelets of the hypertensive animals, both in the absence and in the presence of calcium. When thrombin was administered in the absence of external calcium, the elevation in cytoplasmic calcium levels is the result of calcium release from the internal stores, mainly endoplamic reticulum, because of the production of IP3 and other metabolites produced after the signal transduction pathway has been started [12,13]. Interestingly, in

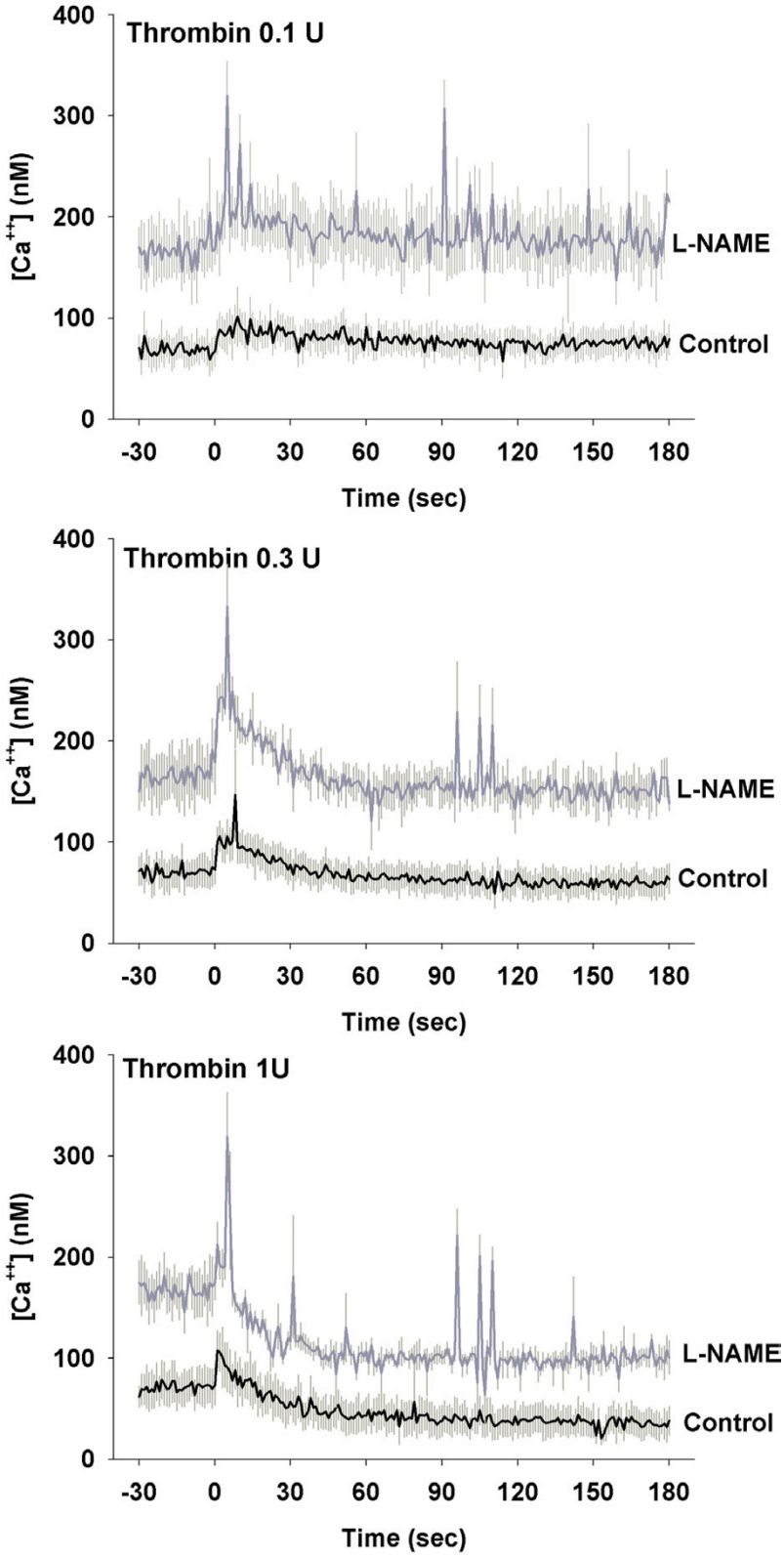

Figure 1
Changes of intracellular calcium levels in response to thrombin in platelets from control and L-NAME hypertensive rats, in the absence of extracellular calcium. The data are the mean and standard error of 6 experiments.

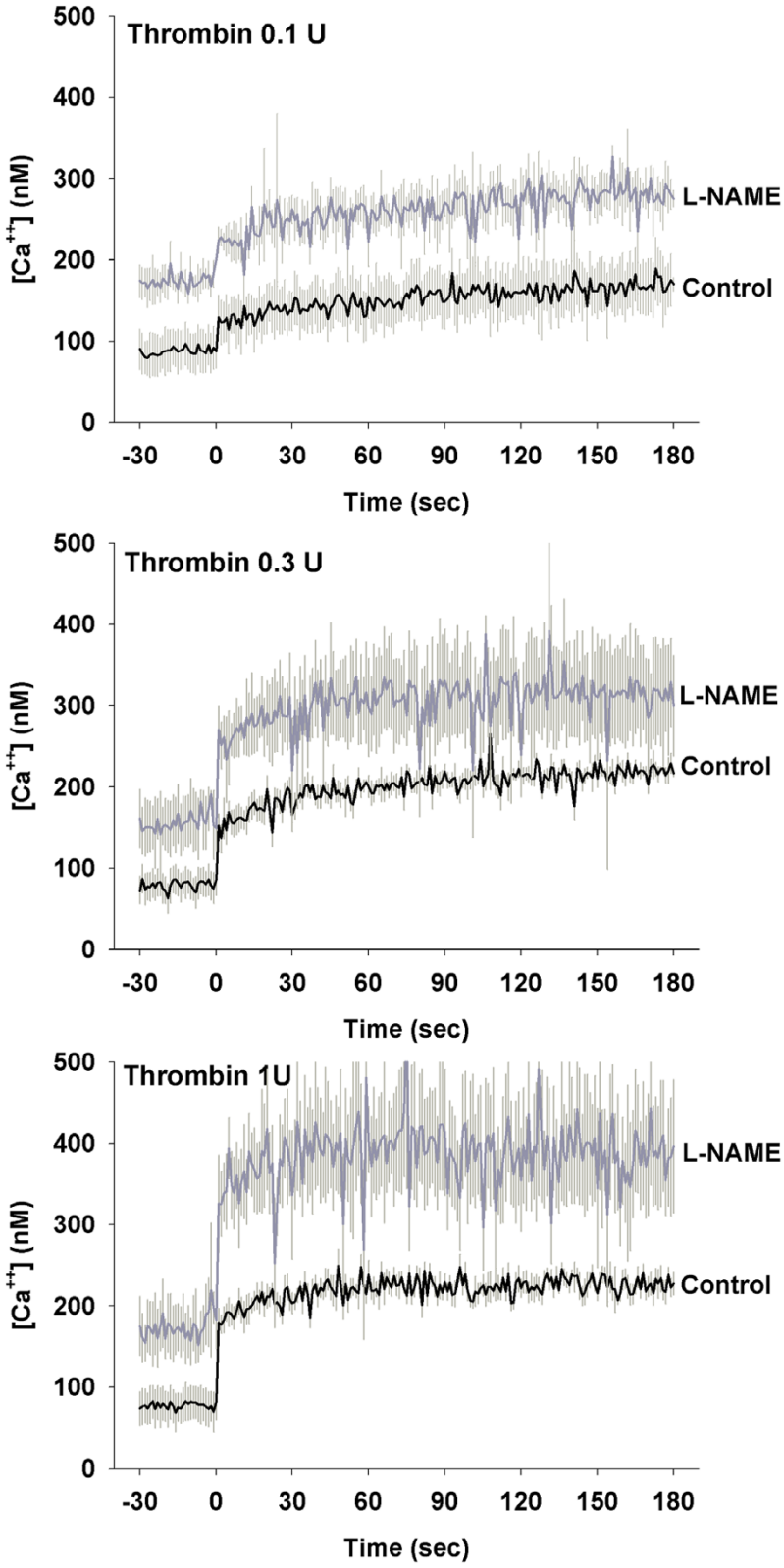

Figure 2
Changes of intracellular calcium levels in response to thrombin in platelets from control and L-NAME hypertensive rats, in the presence of extracellular calcium. The data are the mean and standard error of 6 experiments.

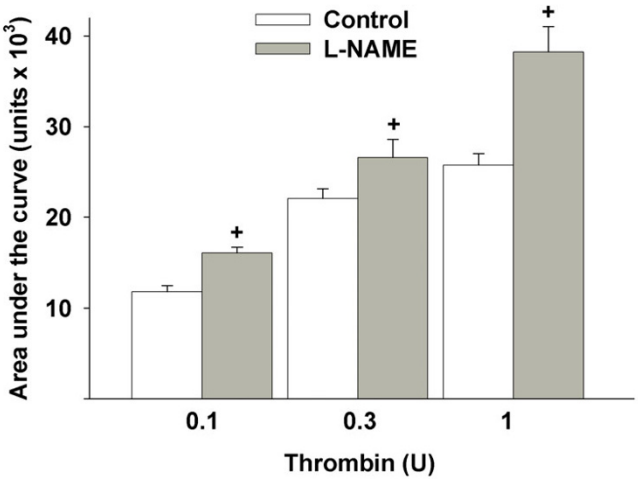

Figure 3
Area under the curve of the integrated calcium responses after thrombin administration in the presence of extracellular calcium.

the experiments perfomed with the two greater doses of thrombin, the calcium levels declined well below the baseline levels and this may be indicative of an activation of cell membrane calcium extrussion mechanisms, such as the plasma membrane calcium ATP-ase [13]. When thrombin is administered in the presence of extracellular calcium, another mechanism of cellular calcium elevation also takes place, the entry from the extracellular space, in this case probably through store operated calcium channels, since calcium entry after thrombin is mainly capacitative [12,13]. Our results clearly indicate that both mechanisms, the entry from the extracellular space and the mobilization from the internal stores, are working in an exaggerated manner in the platelets of the L-NAME-treated hypertensive rats, which results in much higher calcium levels.

Another experiment was used to confirm that capacitative calcium entry is altered in the platelets of the hypertensive rats. Thus, we used thapsigargin to inhibit the uptake of cytoplasmic calcium by the calcium ATP-ase of the endoplasmic reticulum. This effect brings about the opening of the so-called store-operated calcium channels in the plasma membrane, also called capacitative calcium entry. As observed, when calcium was added after the administration of the pump inhibitor, a massive entry of calcium was observed and the effect was again significantly greater in the platelets of the hypertensive rats.

It is very likely that at least part of these effects are due to the absence of nitric oxide, an important regulator of the intracellular calcium levels. Thus, it has been shown that NO inhibits calcium influx indirectly via acceleration of refilling of calcium stores [7]. If less NO is available as a consequence of the inhibition of its synthesis, then calcium influx would be increased, because of the lower refilling of calcium stores. Another possibility is that NO also interferes with the extrusion of calcium from the cell, but this possibility has not been directly tested in the present experiments. Thus, future investigations are needed to clearly define the role of NO in these alterations.

Conclusion

The arterial hypertension induced by the reduction of nitric oxide levels alters the regulation of platelet calcium levels so that elevated resting levels and an enhanced calcium entry and mobilization are very evident. These alterations may be due to direct or indirect effects of nitric oxide interacting with the mechanisms controlling calcium levels in platelets and in other tissues.

Methods

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.

Experimental groups

Animals weighing around 250 g were chronically treated with the NO synthesis inhibitor, L-NAME (40 mg/kg/day in the drinking water) for 5 days. The dose of the inhibitor was adjusted daily according to the water intake and body weight. Control animals were also concurrently maintained with no treatment. Normal rat chow was offered with no restrictions. Blood pressure was determined by the tail cuff method (Cibertec, Madrid, Spain) by using MacLab software (AD Instruments, UK) in a Macintosh LCII computer.

Obtention of platelets and fura-2 loading

Animals were anesthetized with thiobutabarbital (Inactin, 100 mg/kg, i.p., RBI, Massachussets, USA) and blood obtained from the abdominal aorta in a plastic tube containing an anticoagulant solution (composition in mM: sodium citrate, 80; citric acid, 52; glucose, 180). After obtaining platelet rich plasma (700 × g, 5 min), platelets were pelleted (745 × g, 8 min) and resuspended in a low pH-zero calcium buffer (buffer 1, composition in mM: NaCl, 136; KCl, 2.7; $MgCl_2$, 2; HEPES, 5; Glucose, 5.6; NaH_2PO_4, 0.42; EGTA, 0.5, pH 6.6), also containing apyrase (40 μg/ml) and PGE1 (50 μM). After centrifugation (745 × g, 8 min), platelets were resuspended in low-calcium buffer (buffer 2, same composition as buffer 1, except: $CaCl_2$, 0.16 mM and no EGTA was added, pH 7.4).

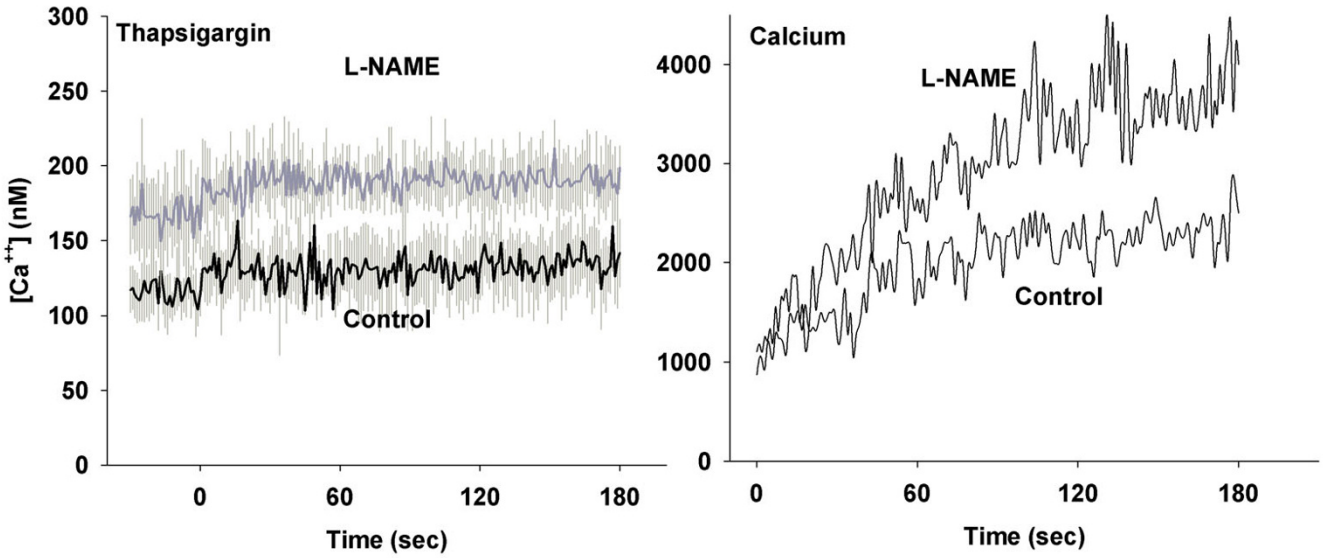

Figure 4
Changes of intracellular calcium levels in response to thapsigargin in platelets from control and L-NAME hypertensive rats, in the absence of extracellular calcium (left) and after addition of 1 mM external calcium (right). The data are the mean and standard error of 6 experiments. Only the mean is provided in the right panel, for the sake of clarity.

Then, the platelets were incubated (37°C) for 45 minutes with 2.5 μM fura-2 AM (Molecular Probes, Leiden, The Netherlands) and same amount of pluronic acid 20% (in DMSO). Fura-2 incubation was terminated by addition of 1 ml of buffer 1 and the tube centrifuged at 745 × g, 8 min. After centrifugation, the supernatant was discarded and the pellet of platelets resuspended in 1 ml of buffer 2 and stored at room temperature and in the dark until calcium measurements.

Calcium measurements

Platelets (100 μl + 1,9 ml of buffer 2) were placed in fluorescence-free cuvettes (C-0793, Sigma) in the optical field of a fluorescence spectrometer (Aminco Bowman 2, Microbeam, Barcelona, Spain), under continuous stirring and at 37°C. The cells were excited alternatively with light of 340 and 380 nm wavelength and the light emitted at 510 nm collected once a second, stored and processed by a PC-compatible computer equipped with a proprietary software. The calibration of $[Ca^{2+}]$ was based on the signal ratio at 340/380 nm and an established protocol as stated below. The $[Ca^{2+}]$ was calculated according to the formula:

$$[Ca^{2+}] = ((R\text{-}R_{min})/(R_{max}\text{-}R)) \cdot (S_f/S_b) \cdot Kd$$

where R is the ratio of the 340/380 nm fluorescence signal. R_{max} is the 340/380 ratio in the presence of saturating calcium (0.5% Triton X-100 containing 1 mM $CaCl_2$), R_{min} is the 340/380 ratio in calcium free buffer (EGTA-Tris 5 mM), Kd is the dissociation constant (225 nM), and S_f/S_b is the ratio of the 380 nm fluorescence measured in calcium free conditions to that in calcium repleted conditions. Background fluorescence was obtained after addition of $MnCl_2$ (1 mM) and subtracted from every value. All drugs and chemicals were added in small volumes (less than 10 μl) to the cuvette, and the platelets were not washed between drug additions. Only one concentration of each drug was tested in every platelets suspension. During the time necessary for each experiment (less than 5 minutes), the baseline did not change in a control experiment without agonists. The calibration procedure was done in every experiment, to take into account differences in the number of platelets between animals.

Experimental protocols

After obtaining baseline values for 30 seconds, the appropriate drug concentration was added and the fluorescence recorded for 180 seconds. Then, the calibration procedure (as stated above) was performed and the experiment was finished. Platelets were studied in the presence (1 mM) and in the absence of extracellular calcium (EGTA 0.5 mM) and were challenged by addition of thrombin (0.1, 0.3, and 1 units/ml). To analyze the capacitative mechanism, platelets with no calcium added were tested with

thapsigargin (1 μM) for 3 min and then calcium (1 mM) for another 3 min.

Drugs

Fura-2 AM was dissolved in DMSO. The rest of products used were from Sigma Chemical (Madrid, Spain), except where indicated. Drug stock solutions were prepared in distilled water and maintained frozen (-20°C). Appropriate dilutions were prepared freshly every day in buffer 2.

Statistical analysis

Data are expressed as the mean ± S.E. In order to compare the calcium responses between control and experimental rats, the area under the curve (AUC) of the individual calcium responses were calculated by summation of all experimental values (180 seconds) minus the averaged baseline (30 seconds), and expressed as arbitrary units. The resulting values as well as baseline values were compared by one-way analysis of variance. A probability level of $p < 0.05$ was considered to be a significant difference.

Competing interests

None declared.

Authors' contributions

DI performed the experiments. NMA and CM-P calculated the calcium concentration, performed the statistical analysis and created the figures. MCO supervised all these tasks. JG-E designed the experiments and wrote the article. All authors read and approved the final manuscript.

Acknowledgements

This work has been performed with grants from Comisión Interministerial de Investigación Científica y Técnica (SAF2000-0157), Fundación Séneca (PB/45/FS/02), and from Instituto de Salud Carlos III (RNIHG, CO3/02).

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