ORIGINAL ARTICLE

Pharmacological characterization of the calcium influx pathways involved in nitric oxide production by endothelial cells

Caracterização farmacológica das vias de influxo de cálcio envolvidas na produção de óxido nítrico por células endoteliais

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❚ **ABSTRACT**

Objective: To characterize the calcium influx pathways implicated in the sustained elevation of endothelial intracellular calcium concentration, required for the synthesis and release of relaxing factors. **Methods:** We evaluated the effect of the newly synthesized pyrazole derivatives, described as selective inhibitors for ORAI (BTP2/Pyr2 and Pyr6) and TRPC3 (Pyr3 and Pyr10) channels, upon endothelium- and extracellular calcium-dependent relaxations stimulated by acetylcholine and thapsigargin, in pre-constricted rat thoracic aortic rings. **Results:** Acetylcholine and thapsigargin responses were completely reverted by Pyr2 and Pyr6 (1 to $3\mu M$). Pyr3 (0.3 to $3\mu M$) caused a rapid reversal of acetylcholine (6.2 ± 0.08mg.s^{-1}) and thapsigargin (3.9 ± 0.25mg.s^{-1}) relaxations, whereas the more selective TRPC3 blocker Pyr10 (1 to $3\mu M$) had no effect. The recently described TRPC4/5 selective blocker, ML204 (1 to $3\mu M$), reverted completely acetylcholine relaxations, but minimally thapsigargin induced ones. Noteworthy, relaxations elicited by GSK1016790A (TRPV4 agonist) were unaffected by pyrazole compounds or ML204. After Pyr2 and Pyr6 pre-incubation, acetylcholine and thapsigargin evoked transient relaxations similar in magnitude and kinetics to those observed in the absence of extracellular calcium. Sodium nitroprusside relaxations as well as phenylephrine-induced contractions (denuded aorta) were not affected by any of pyrazole compounds (1 to $3\mu M$). **Conclusion:** These observations revealed a previously unrecognized complexity in rat aorta endothelial calcium influx pathways, which result in production and release of nitric oxide. Pharmacologically distinguishable pathways mediate acetylcholine (ORAI/TRPC other than TRPC3/TRPC4 calcium-permeable channels) and thapsigargin (TRPC4 not required) induced calcium influx.

Keywords: Endothelial cells; Calcium/metabolism; ORAI1 protein; TRPC cation channels; Aorta; Nitric oxide; Rats; Relaxation

❚ **RESUMO**

Objetivo: Caracterizar as vias do influxo de cálcio envolvidas no aumento sustentado da concentração intracelular de cálcio na célula endotelial, essencial para a síntese e a liberação de fatores relaxantes. **Métodos:** Analisamos o efeito de derivados pirazólicos sintetizados recentemente, descritos como inibidores seletivos para canais ORAI (BTP2/Pyr2 e Pyr6) e TRPC3 (Pyr3 e Pyr10), nos relaxamentos dependentes de endotélio e cálcio extracelular, produzidos por acetilcolina e tapsigargina, em anéis pré-contraídos da aorta torácica de rato. **Resultados:** As respostas de acetilcolina e tapsigargina foram completamente revertidas por Pyr2 e Pyr6 (1 a $3\mu M$). Pyr3 (0,3 a $3\mu M$) produziu reversão rápida dos relaxamentos de acetilcolina ($6,2\pm0,08$mg.s^{-1}) e tapsigargina ($3,9\pm0,25$mg.s^{-1}), enquanto o bloqueador mais seletivo para TRPC3, Pyr10 (1 a $3\mu M$), não apresentou efeito. ML204 (1 a $3\mu M$), bloqueador seletivo de TRPC4,

descrito há pouco tempo, reverteu os relaxamentos induzidos por acetilcolina de forma completa, mas afetou minimamente aqueles produzidos por tapsigargina. Os derivados pirazólicos ou ML204 não afetaram os relaxamentos estimulados com GSK1016790A (TRPV4-agonista). Ainda, após pré-incubação com Pyr2 e Pyr6, acetilcolina e tapsigargina provocaram relaxamentos transitórios semelhantes em magnitude e cinética àqueles observados na ausência de cálcio extracelular. Os relaxamentos do nitroprussiato de sódio e as contrações induzidas pela fenilefrina (aorta sem endotélio) não foram afetados pelos compostos pirazólicos (1 a 3μM). **Conclusão:** Essas observações revelaram uma complexidade desconhecida das vias de influxo de cálcio no endotélio da aorta de rato, que resultam na produção e na liberação de óxido nítrico. Vias distinguíveis farmacologicamente medeiam o influxo estimulado por acetilcolina (ORAI TRPC, diferentes de TRPC3 TRPC4) e tapsigargina (TRPC4 não requerido).

Descritores: Células endoteliais; Cálcio/metabolismo; Proteína ORAI1; Canais de cátion TRPC; Aorta; Óxido nítrico; Ratos; Relaxamento

INTRODUCTION

Accumulated evidence indicates that extracellular calcium is required for the continuous production of nitric oxide (NO) implying that calcium influx is involved.[1-5] For instance, many endogenous and synthetic substances that cause endothelium- and NO-dependent relaxations in isolated arteries, or stimulate NO production in cultured endothelial cells (EC), also induce a rapid rise in intracellular calcium concentration ($[Ca^{2+}]_i$) that is dependent on external Ca^{2+} concentration. Indeed, a correlation between changes in endothelial $[Ca^{2+}]_i$ and NO production has been shown.[4,5] Despite these data indicating that calcium influx in EC is necessary for the synthesis and release of relaxing factors, the identity of the channels involved in this influx, and whether distinct extracellular Ca^{2+}-dependent stimuli of EC involve the same or a different set of channels remain unknown.

Several proteins involved in calcium entry pathways were identified over the past two decades.[6] Of special interest are the recently discovered ORAI proteins, as well as some members of non-selective cation permeable TRP channel family, which can mediate either store-operated calcium entry (SOCE) (capacitative) or non-capacitative Ca^{2+} influx in a variety of cells.[6,7] Since ORAI and TRP channels are found in EC, these molecules have emerged as putative mediators of Ca^{2+} influx required for production of NO by these cells.

Consequently, we took advantage of the recent availability of pyrazole derivatives, which cause selective blockade of ORAI (pyr2; pyr6) and TRPC3 (pyr3; pyr10) channels, as well as of the TRPC4

blocker ML204,[8-11] and investigated their effects upon acetylcholine and thapsigargin induced endothelium dependent relaxations.

OBJECTIVE

To characterize pharmacologically the calcium influx pathways involved in the production of nitric oxide by endothelial cells in response to acetylcholine and thapsigargin.

METHODS

Animals

Male Wistar rats (200 to 220g) bred in the animal facilities at *Universidade de São Paulo* (USP), Ribeirão Preto *campus*, were used in this study. Animals were housed under controlled 12/12 hours light-dark cycle and temperature, with food and water *ab libitum*. Procedures were conducted in accordance with the Ethical Principles in animal research adopted by the *Colégio Brasileiro de Experimentação Animal* (COBEA) and approved by the institutional Animal Investigation Ethical Committee (CETEA, protocol 024/2011).

Rats were deeply anesthetized with pentobarbital sodium (50mg/kg, intraperitoneal) and euthanized by exsanguination. After opening of thoracic cavity, the descending thoracic aorta was carefully excised, and immediately transferred to Petri plates containing a modified Krebs solution (116.0mM of NaCl; 4.5mM of KCl; 1.16mM $MgCl_2$; 2.5mM of $CaCl_2$; 1.14mM of NaH_2PO_4; 25.0mM of $NaHCO_3$; and 11.1mM of glucose). Arteries were cleaned of any adhering perivascular tissue and then cross-sectioned into small cylindrical segments (rings, 3.5 to 4mm in length). Special care was taken to preserve the endothelium, but in some experiments a mechanical removal of the endothelium was made intentionally by gently rubbing the luminal surfaces of aortic rings between the fingers for 40 seconds.

Thoracic aortic rings (TAR) were then suspended in an organ bath (Letica Scientific Instruments, Spain) containing Krebs solution at 37°C, continuously bubbled with 95%O_2/5%CO_2, for recording isometric contractions. Variations in aortic rings force were continuously monitored with a data acquisition system (PowerLab, ADInstruments/LabChart software v.5.0).

Thoracic aortic rings were gradually stretched up to 2g and then allowed to equilibrate for 1 to 2 hours (washout at 20-minute intervals). Resting tension was readjusted to 2g, if alterations occurred during the first

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40 minutes. Contractions were evoked by the addition of phenylephrine (PE, 30-100nM) every 60 minutes. To avoid vasomotor interferences of cyclooxygenase-derived products, diclofenac (10μM) was always present in the Krebs solution. For the experiments, a concentration of PE that causes submaximal contractions (60 to 80% of maximal response) was selected. Substances were tested once the plateau of the contractions was attained (about 7 to 8 minutes).

Viability of endothelium was assessed by determining the relaxation response to ACh (1μM). Thoracic aortic rings were considered endothelium-intact (+E, $\geq$80% relaxation) or endothelium-denuded (-E, no relaxation).

Chemicals

Acetylcholine, PE and GSK1016790A were purchased from Sigma Aldrich; diclofenac, thapsigargin, Pyr2 and sodium nitroprusside (SNP) were purchased from Calbiochem™; HC-067047 and ML204 were from Tocris Bioscience; and L-NNA was purchased from Research Biochemicals International-USA. Pyrazole compounds (Pyr3, Pyr6 and Pyr10) were synthesized as described in Glasnov et al.,[12] and provided by Dr. Klaus Groschner (Institute of Biophysics-MedUni Graz, Austria).

Data representation and analysis

Relaxations or contractions were expressed as changes in tension of PE-induced contraction, calculated according to the following formula: $\Delta T(\%) = [(T_{final} - T_{PE-plateau})/(T_{PE-plateau} - T_{resting})] \times 100$; negative values denote relaxations. Analysis of results (time-course, area under a curve − AUC, duration and kinetics) was performed using GraphPad Prism (v.5.01) and LabChart Pro (v.7.3.8). All data are expressed as mean±standard error of the mean (SEM). Student's t-test was used to determine statistical significance (p>0.05: not significant). Asterisk indicates significant differences: *p<0.05; **p<0.01; ***p<0.001.

▌RESULTS

Initially, the experiments were conceived to confirm the requirement of endothelium, NO synthesis and extracellular calcium concentration ([Ca^{2+}]$_o$) for ACh, Thap and GSK101 (TRPV4-activator) relaxations. The three agents caused sustained relaxations, which were strictly endothelium-dependent and abolished by L-NNA (Figures 1A to 1E); the onset of these responses was rapid for ACh/Thap (<30 seconds) and

slower for GSK101 (>2 minutes) and they reached a steady state in 4-6 minutes, which persisted for at least 10 minutes. L-NNA had no effect on endothelium-independent relaxation caused by SNP. The role of [Ca^{2+}]$_o$ was evaluated by incubating arterial preparations (endothelium-intact) in a nominally Ca^{2+}-free solution for 40 minutes (Figures 1A to 1C, right). In these conditions, the endothelium-dependent relaxations elicited by ACh and Thap were transformed into transient relaxations while those elicited by GSK101 were abolished. Figure 1F shows the time-course of these responses in both conditions. Note their distinct profile in Ca^{2+}-free solution. To quantitate these differences, the AUC (%.s) was determined (Figure 1G). AUC values indicated that NO-dependent relaxations in rat aorta are impaired in the absence of [Ca^{2+}]$_o$; ACh and Thap transient relaxations exhibited similar amplitude, but respective AUC values were different. Interestingly, the initial kinetics of ACh and Thap-elicited relaxations was not affected by the absence of [Ca^{2+}]$_o$. Furthermore, the transient relaxations in Ca^{2+}-free medium showed similar time to peak relaxation and duration of the response (Figure 1H).

Next, the potential pathways involved in calcium influx in response to both agents was examined. Initially, the effect of Pyr2 and Pyr3 was determined on pre-constricted TAR without endothelium to detect potential effects of these compounds directly on the smooth muscle. As shown in figure 2A to 2B, both compounds at 1 to 3μM exhibited negligible effect on PE-contractions, causing a slow decrease in tension at 10μM. Therefore, in the subsequent experiments, Pyr2/Pyr3 were used at concentrations below 10μM. Both Pyr2 and Pyr3 caused a complete and concentration-dependent reversal of ACh- and Thap-elicited relaxations, whereas relaxations of comparable magnitude elicited by GSK101 were unaffected. Furthermore, ACh and Thap effects do not appear to involve calcium influx through the TRPV4 channel since the selective TRPV4 blocker, HC-067047, did not reverse their responses at a concentration that fully reversed the GSK101 relaxations (Figures 2C to 2G).

Since the selectivity of Pyr2 and Pyr3 for ORAI and TRPC3 channels has been questioned,[9] for instance, Pyr3, a previously suggested selective inhibitor of TRPC3, may also inhibit ORAI1- and TRPC3-mediated Ca^{2+} entry, we next tested the effects of two novel pyrazole compounds, Pyr6 and Pyr10, which exhibited improved selectivity. Pyr6 and Pyr10 are able to distinguish between TRPC and ORAI-mediated Ca^{2+} entry and may serve as useful tools for the analysis
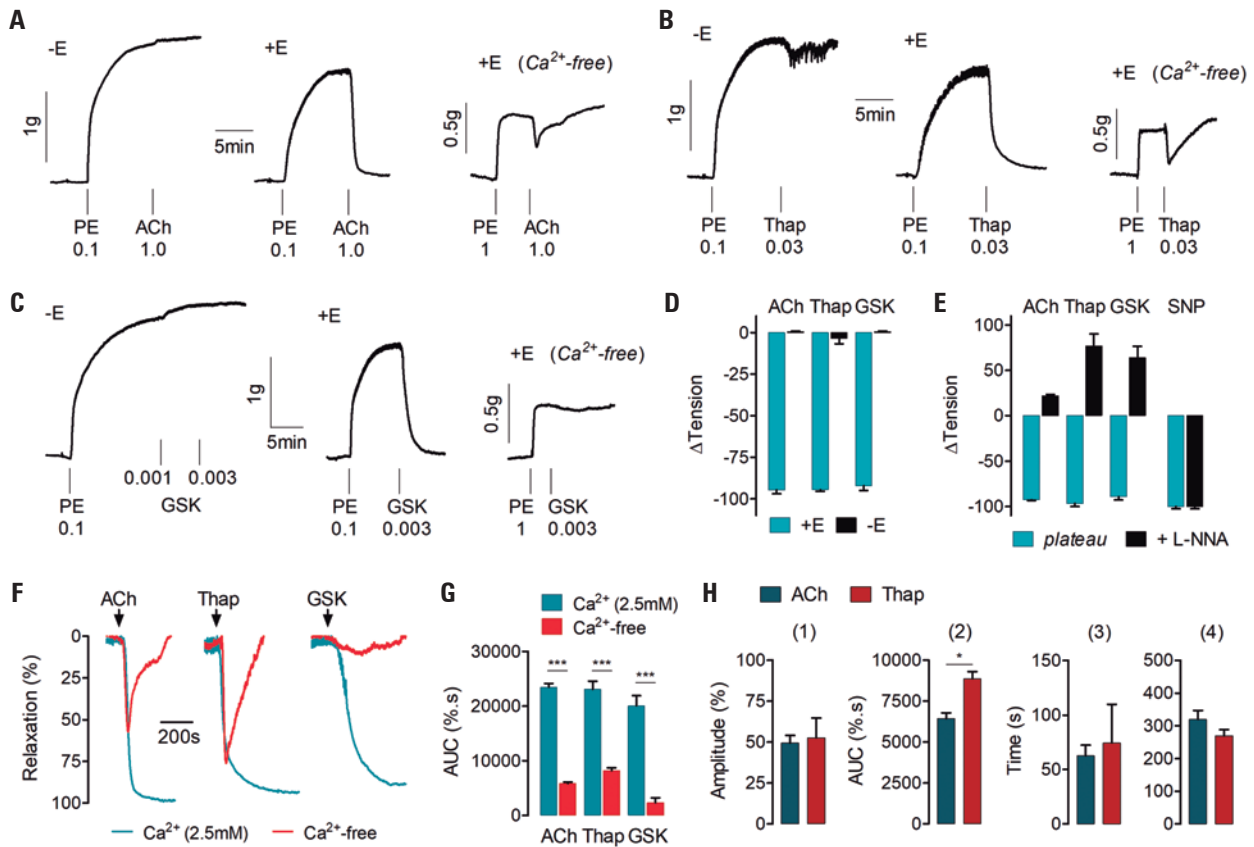
Figure 1. Sustained endothelium-dependent relaxations elicited by ACh and Thap require extracellular calcium. Original recordings showing the effect of ACh (A), Thap (B) and GSK101 (C) in rat thoracic aortic rings pre-constricted with phenylephrine (PE). Effects were analyzed in endothelium-intact (+E), endothelium-denuded (-E) or when rat thoracic aortic rings with endothelium were incubated in nominally calcium free solution (Ca2+-free, prepared omitting CaCl2) during 40 minutes. Numbers below abbreviations indicate drug concentration in μM; scale bars, time in minute, (horizontal); and tension in g (vertical) variations. (D) and (E) Changes in tension (Δ) in percentage of PE-induced contraction produced by ACh, Thap and GSK101 in +E and –E aortic rings. (E) Effect of L-NNA (100μM) added on the plateau of relaxations caused by ACh, Thap and GSK101 or SNP (100nM). (F) Representative time-course of the relaxations produced by ACh (1μM), Thap (30nM) or GSK101 (3nM) in the presence (blue traces) or absence (red traces) of extracellular calcium. Responses were normalized to reduction (%) in the PE-contraction in each condition. (G) Area under a curve (AUC) of the ACh, Thap and GSK101 responses obtained in calcium-containing and calcium-free medium. (H) ACh and Thap transient relaxations parameters: (1) peak, (2) area under curve, (3) time to reach peak and (4) to return to basal (N=3-6 experiments)

of cellular functions of the underlying Ca²⁺ channels.[9] Pyr6 reversed rapidly and completely the relaxations elicited by ACh whereas Pyr10 (1 to 3μM), a more selective TRPC3 blocker, did not affect to any extent such relaxations (Figures 2E to 2F). Interestingly, Pyr6 also reversed Thap responses although with a slower kinetics than that of ACh-induced relaxation.

Considering that Pyr10 was not able to reverse ACh/Thap relaxations and taking in account that TRPC4 had been shown to mediate calcium influx in mouse aortic EC in response to ATP, it become of interest to determine whether the TRPC4-selective antagonist, ML204, was able to affect endothelium-dependent relaxations. ML204 reversed rapidly and completely ACh-induced relaxations, but minimally affect those caused by Thap (Figures 3A to 3B). In +E

rings incubated with ML204, ACh (1 to 3μM) or Thap (30nM) were still able to produce sustained relaxations. The initial rapid phase of their relaxations, as shown in the time-course analysis, was not affected, but the magnitude of ACh effect was significantly reduced (Figures 3C to 3E). Intriguingly, ML204 pre-incubation augmented the magnitude of PE-elicited contractions (Figure 3F).

To further explore the inhibitory effect of pyrazoles and ML204 on endothelium-dependent relaxations, we examined the time-course of their effect after adding them on the plateau of ACh/Thap responses (Figure 4). Interestingly, it was observed that the compounds segregated into two patterns: a rapid one, which included Pyr3/ML204 and a slower one that included Pyr2/Pyr6, as revealed by the latency to
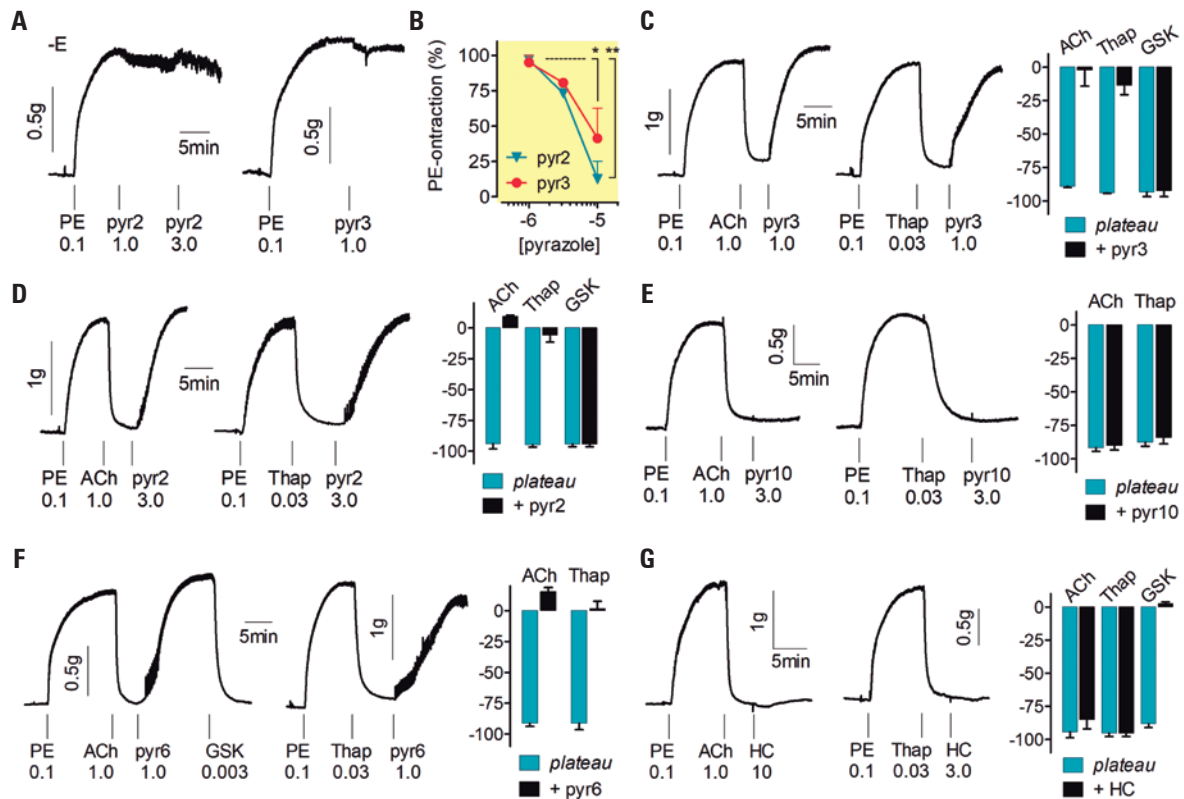
Figure 2. Effect of selective inhibitors for ORAI, TRPCs and TRPV4 channels upon endothelium-dependent relaxations elicited by ACh, Thap and GSK101 in rat thoracic aorta. (A) Original recordings showing the effect of Pyr2 and Pyr3 (1 to 3μM) on the phenylephrine (PE)-induced contraction in endothelium-denuded rings (-E). (B) Concentration-response curve for the inhibitory effect of pyrazole compounds (1 to 10μM) on PE-contraction. (C to G) Original recordings showing the effect of addition of Pyr3 (1μM), Pyr2 (3μM), Pyr10 (3μM), Pyr6 (1μM) or HC-067047 (3 or 10μM) on the plateau of relaxations produced by ACh or Thap. Respective bar graphs summarize the effect of pyrazole compounds and HC-067047 added on the plateau of ACh (1μM), Thap (30nM) or GSK (3nM) relaxations. Results are presented as changes in the tension (Δ) expressed as percentage of PE-evoked contraction in rat aortic rings (N=3-6 experiments)

start the reversal and by the first derivative analysis (dT/dt, mg.s^{-1}). Intriguingly, while Pyr2 and Pyr3 exhibited a faster kinetics against ACh (5.6±0.44mg.s^{-1} and 6.1±0.08mg.s^{-1}, respectively) than against Thap (3.6±0.39mg.s^{-1} and 3.9±0.25mg.s^{-1}), Pyr6 exhibited similar latency and dT/dt values in reversing ACh and Thap.

Finally, we evaluated ACh/Thap responses in TAR pretreated with the different calcium influx blockers. Pyr2 or Pyr6 pre-incubation transformed the sustained endothelium-dependent responses to ACh and Thap into transient relaxations. More interestingly, responses to GSK101 (3 to 10nM) remained unaffected in these experiments (Figures 5A to 5C). Representative ACh or Thap-induced relaxations time course in the presence of pyrazoles, ML204 and HC-067047 is shown in figure 5D to 5E. Pyr3 and Pyr10, as well as HC-067047, had no effect on ACh or Thap relaxations. The amplitude of Thap effect was slightly more affected by Pyr2/Pyr6 than that

of ACh. There were no significant differences between ACh and Thap-induced relaxations in terms of AUC and duration (Figure 5F). Moreover, the transient relaxations obtained with Pyr6/Pyr2 pretreatment were comparable to the relaxations elicited by ACh and Thap in the absence of [Ca^{2+}]$_o$ (compare Figures 5F and 1F).

These results suggest that continuous calcium influx require at least two pharmacologically distinguishable pathways. Therefore, to determine whether Pyr3-sensitive pathway interact with Pyr2 sensitive one, a combination of Pyr2/Pyr3 was tested on the relaxant effect of ACh. Pyr3 pre-incubation facilitated the reversal produced by 0.3μM Pyr2 (Figure 6). These findings indicate that ORAI and TRPCs channels operate in different phases of endothelium-dependent relaxations as summarized schematically in figure 6F. The initial rapid phase would require ORAI channels activation while influx of calcium through TRPCs would be responsible for the sustained phase.
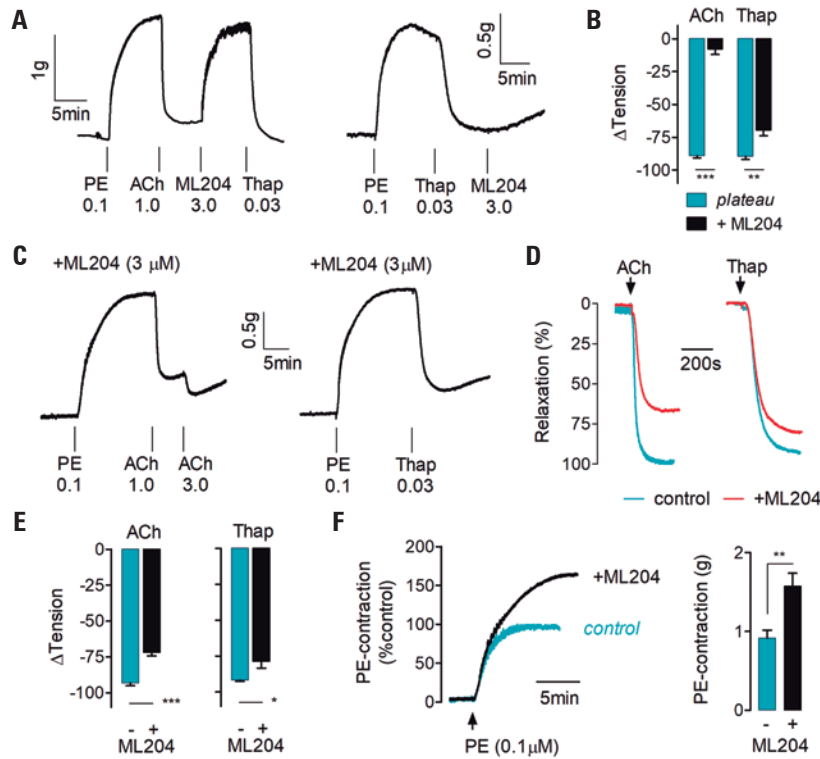
Figure 3. Effects of selective TRPC4 channel inhibitor in rat thoracic aorta. (A) Representative tracings of the effect of ML204 (3μM) added on the plateau of the relaxations produced by ACh (left) or Thap (right) in rat thoracic aortic rings pre-constricted with phenylephrine (PE). (B) ML204 effect presented as tension (%). **p<0.01 and ***p<0.001, Student t-test (paired, two tailed). (C) Original recordings showing the relaxing responses to ACh (1 to 3μM) or Thap (30nM) observed in rat thoracic aortic rings pre-incubated with ML204 (+ML204, 3μM, for 5 minutes). (D) Representative time course of ACh and Thap relaxations before (control, blue traces) and after (red traces) pre-incubation of arterial rings with ML204. (E) Changes in tension (Δ) in percentage of PE-contraction produced by ACh or Thap in the absence (-) and presence (+) of ML204. (F) Left: time-course of PE-contraction in endothelium-intact rings (ACh relaxation >95%) before (control) and after pre-incubation with ML204. Right: PE-contraction in grams (g) for these rings. **p<0.01, Student t-test (paired, two tailed) (N=5-11 experiments)
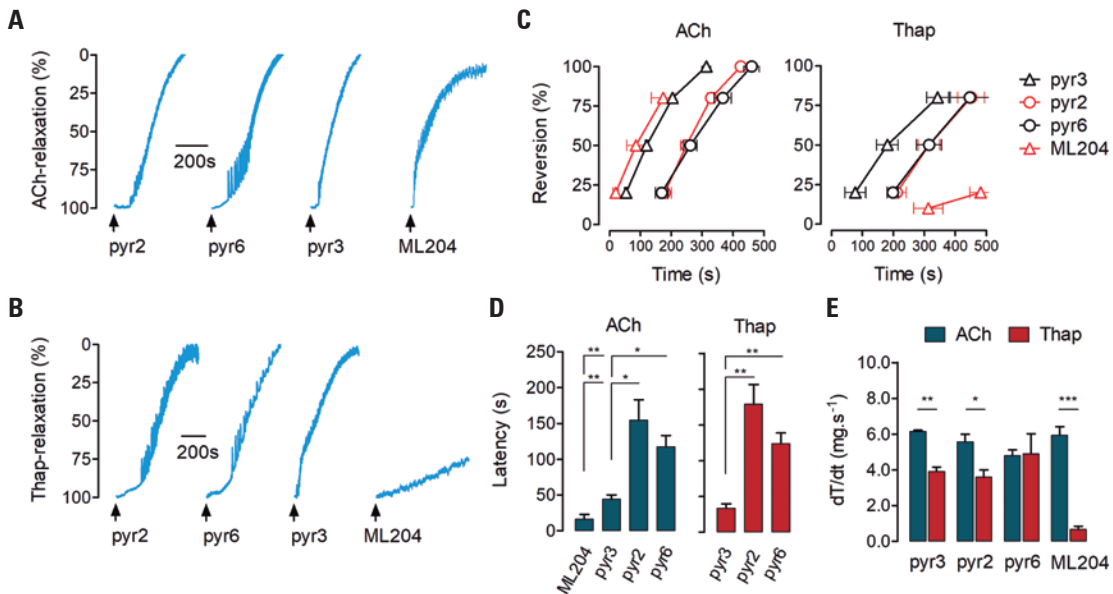

Figure 4. Kinetics of the reversal produced by ORAI and TRPCs channels inhibitors. (A to B) Representative time course for the reversing effect of pyrazole compounds and ML204 on ACh (1μM, A) and Thap (30nM, B) responses. Plateau of the ACh or Thap relaxations was taken as 100%. Concentrations tested: Pyr2 (3μM), Pyr6 (1μM), Pyr3 (1μM) and ML204 (3μM). (C) X-Y plot comparing the time required for ORAI and TRPCs inhibitors to produce a reversal of 20%, 50%, 80% and 100% of ACh or Thap relaxations. (D) Latencys to start the reversal of ACh (1μM) and Thap (30nM) responses. Inhibition was considered initiated when drugs caused a 10% reversal. (E) Velocity (dT/dt, mg.s^{-1}) of the reversal produced by each compound on endothelium-dependent relaxations indicated (ACh, Thap) (N=3-6 experiments)
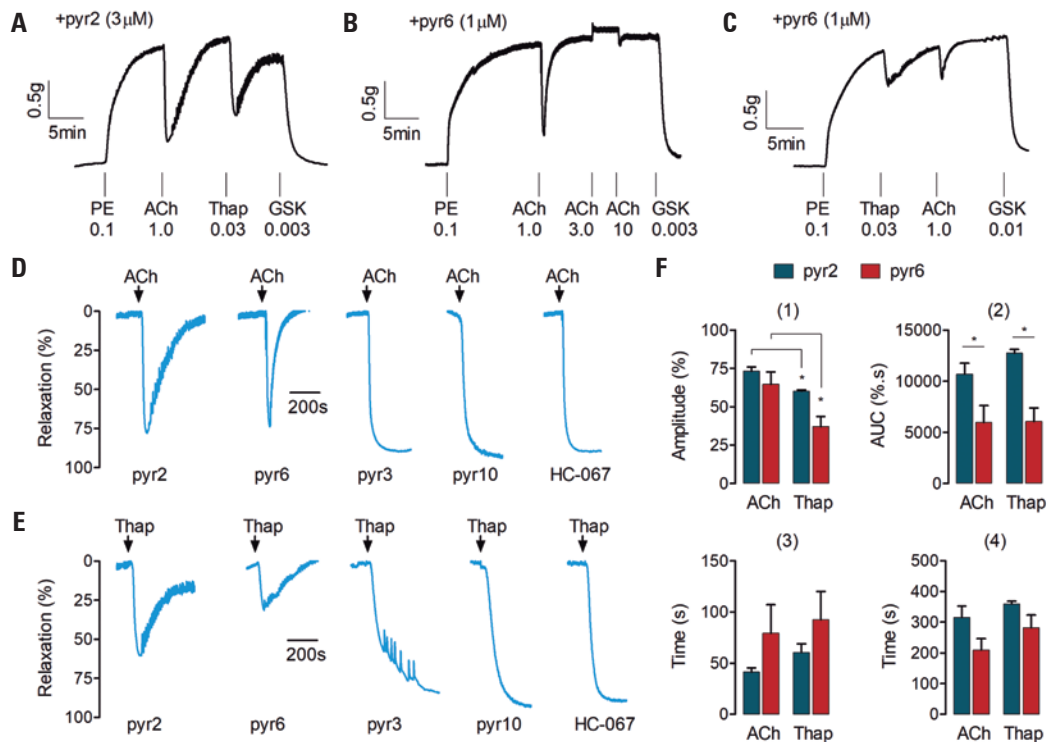
Figure 5. Effects of the pre-incubation with ORAI, TRPCs and TRPV4 channels blockers on endothelium-dependent relaxations. (A to C) Original recordings in endothelium-intact rings of rat thoracic aorta (ACh relaxation >95%) showing the effect of a (5 minutes) pre-incubation with Pyr2 (3μM, A) and Pyr6 (1μM, B and C) on endothelium-dependent relaxations elicited by ACh, Thap or GSK101. (D to E) Representative time course of the relaxations exhibited by ACh (1μM, D) or Thap (30nM, E) in the presence of Pyr2 (3μM), Pyr6 (1μM), Pyr3 (1μM), Pyr10 and HC-067047 (both at 3μM). Arrows indicate the moment of ACh or Thap application. Responses were normalized to reduction (%) in the phenylephrine (PE)-induced contraction in each preparation. (F) Parameters of the transient relaxations produced by ACh and Thap in the presence of Pyr2 or Pyr6: (1) peak, (2) area under curve (AUC), (3) time to reach peak and (4) to return to basal (N=3-5 experiments)
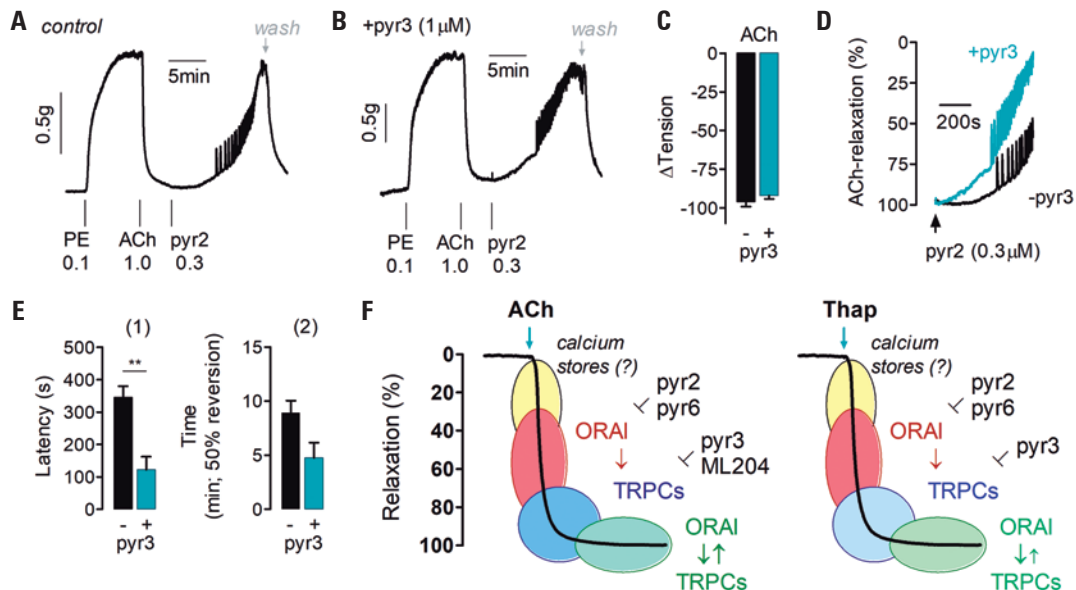

Figure 6. The pre-incubation of rat aortic rings with Pyr3 facilitated the reversal of ACh relaxations by Pyr2. (A to B) Original recordings showing the reversal of ACh-induced relaxations by Pyr2 (0.3μM) in rat aortic rings pre-constricted with phenylephrine (PE). In (B), arterial rings were pre-incubated with Pyr3 (1μM, 5 minutes). (C) Changes in the tension (Δ) of PE-contraction produced by ACh before (-) and after (+) pre-incubation of rat thoracic aortic rings with Pyr3. (D) Representative time course comparing the reversal produced by Pyr2 (0.3μM, arrow) in the absence (-) and presence (+) of Pyr3 (1μM). Plateau of the ACh relaxation was taken as 100%. (E) Parameters of the Pyr2 reversal measured in the absence (-) and presence (+) of Pyr3: (E1) latency in seconds to start (stated as the interval between the addition of Pyr2 and the production of 10% of reversal) (E2) time (minute) needed to produce 50% of reversal of ACh response (n=4 experiments). (F) Schematic representation showing the involvement of ORAI and TRPCs channels in the different phases of endothelium-dependent relaxations produced by ACh or Thap. It was proposed considering kinetics properties of the reversal caused by pyrazole compounds and the effect of their pre-incubation on ACh/Thap relaxant effect

DISCUSSION

The main findings of this study revealed a previously unrecognized complexity in rat aorta endothelial calcium influx pathways activated by ACh and Thap. Furthermore, these findings confirm that in TAR, NO production and release solely accounts for the endothelium-dependent relaxations elicited by ACh/Thap; they also demonstrate in an incontrovertible manner that $[Ca^{2+}]_o$ influx is required for the continuous production of NO in response to these agents. The lack of effect of the selective calcium influx blockers on PE-elicited contractions in endothelium-denuded rings indicate that EC constitute the main site of action of these inhibitors, to reverse or prevent endothelium-dependent relaxations. Noteworthy, the fact that pyrazoles and ML204 did not affect the relaxations elicited by the NO donor, SNP, allows us to interpret these findings in terms of their main pharmacological action, *i.e.*, blockade of calcium influx. In addition, the selectivity of these compounds was confirmed in our experiments by the lack of effect of the pyrazole compounds and ML204 upon responses to GSK101, which promotes calcium influx through the activation of endothelial TRPV4 channels. The simplest interpretation of these findings consists in proposing that (1) pharmacologically distinguishable channels appear to participate in the initiation and in the maintenance of calcium influx; and (2) that pharmacologically distinguishable channels maintain calcium entry during ACh or Thap stimulation (as depicted in Figure 6F).

The observation that Pyr2 or Pyr6 pre-incubation (but not Pyr3/ML204) resulted in a transient relaxation in response to ACh/Thap which is very similar to that produced in the absence of $[Ca^{2+}]_o$, is fully consistent with the interpretation that calcium influx is required for the sustained production and release of NO. The transient relaxations elicited by these agents could be attributed to calcium release from endoplasmic reticulum (ER) calcium stores. ACh has been shown to interact with cell membrane muscarinic receptors, coupled to phospholipase C activation and the subsequent IP_3 generation, which in turn activates IP_3R located in ER leading to calcium release.[6] Thap effect is attributed to an inhibitory action on SERCA, which leads to a passive calcium flow towards the cytosol with the consequent depletion of ER calcium stores; this depletion is sensed by STIM which interacts with and promotes clustering of ORAI channels resulting in calcium influx;[6,13] this sequence of events have been named "store-operated calcium entry".[13] Our findings would indicate that

in rat aortic EC, both ACh and Thap are promoting calcium entry through an initial Pyr2/Pyr6 sensitive pathway, implicating that STIM/ORAI channels are mediating calcium entry after depletion of ER calcium stores. However, it is worth reminding that calcium entry activation by agonists of cell surface receptor does not necessarily requires store depletion.[13] Since Pyr3 was able to reverse the steady state relaxation elicited by ACh/Thap, but no to prevent the relaxing responses when pre-incubated, it is likely that Pyr3 target is recruited to the calcium influx pathway after the activation of this pathway by Pyr2/Pyr6 targets (STIM/ORAI). The fact that Pyr2 and Pyr6 were also able to reverse the relaxations suggests that ORAI channels continue to be required for the sustained calcium influx, implicating a multi-component STIM/ORAI/TRPC complex in the mediation of calcium influx during the sustained phase of NO synthesis and release following ACh and Thap stimuli. Indeed, a TRPC: ORAI complex was proposed to mediate store- and receptor-operated calcium entry in HEK-293 cells heterologously expressing these proteins.[14] Furthermore, it has been shown that STIM can also interact with several TRPC channels promoting their function as SOCE channels.[15]

Although Pyr3 was initially described as TRPC3-selective inhibitor, ACh/Thap-elicited relaxations were not affected by a recent described selective TRPC3 blocker Pyr10,[9] suggesting that TRPC3 might not be the channel involved in the sustained phase of calcium influx activated in rat aortic cells. This interpretation is consistent with previous observations showing that endothelium-dependent relaxations observed in hind limb perfusion experiments or endothelial dependent NO production in TAR were not altered in TRPC3-KO mice.[16]

The fact that Thap responses were completely reversed by Pyr3 but negligibly by ML204 could indicate that the calcium influx pathways involved in the maintenance of calcium influx activated by ACh and Thap are pharmacologically distinguishable. As ML204 did not reversed Thap-elicited relaxation, TRPC4 channels seem to play a minor contribution to the sustained phase of calcium influx triggered by Thap. The observed effect of ML204 upon ACh relaxation is entirely consistent with the report that TAR from TRPC4-KO mice exhibit reduced but not absence of ACh-induced relaxations.[17] Finally, although it has been shown that TRPC4 interacts constitutively with ORAI,[18] the fact that TRPC4 channels contribute to

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calcium influx only in ACh-stimulated cells indicates this channel is not activated by mere ER calcium store depletion. Since TRPC4 channels are insensitive to or inhibited by DAG, a product of PLC-mediated hydrolysis of PIP_2, it is likely that its activation results from the depletion by PIP_2 which has been shown to inhibit TRPC4 channel activity.[19]

Although the current findings indicate a contribution of pyrazole-sensitive channels mediating Ca^{2+} entry in EC (which is required for the production and release of NO), direct measurements of cytosolic Ca^{2+} concentration in EC would be necessary to confirm our interpretations/results obtained *in vitro*. Moreover, considering that we analyzed the relaxant effect of NO released by rat aorta EC (stimulated by ACh/Thap), further experiments measuring simultaneously endothelial Ca^{2+} concentration and smooth muscle relaxation could help us better understand and elucidate the actual mechanisms. However, in assays aimed to determine ($[Ca^{2+}]_i$ and its mobilization using fluorescent calcium indicators and confocal microscopy, the initial findings indicated that calcium mobilization responses to acetylcholine are impaired and/or absent in primary EC cultures prepared from the rat aorta.

CONCLUSION

The STIM/ORAI/TRPC channels are integral components of the calcium influx pathway activated by ACh and Thap, leading to nitric oxide release in rat thoracic aorta. Furthermore, our findings are the first evidence indicating that the composition of the calcium influx pathways is stimulus dependent, *i.e.*, TRPC4 contributes to ACh but not to Thap elicited store-operated calcium entry. These observations might be clinically relevant since identifying the calcium channels involved in the sustained production of nitric oxide by endothelial cells could lead to the development of specific drugs to block these channels in case of excess production of nitric oxide (circulatory shock), or to activate them to promote vasodilation in cases of reduced blood flow (ischemia).

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AUTHORS' INFORMATION

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