

# Human umbilical artery smooth muscle exhibits a 2-APB-sensitive capacitative contractile response evoked by vasoactive substances and expresses mRNAs for STIM, Orai and TRPC channels

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**ABSTRACT:** After depletion of intracellular  $\text{Ca}^{2+}$  stores the capacitative response triggers an extracellular  $\text{Ca}^{2+}$  influx through store-operated channels (SOCs) which refills these stores. Our objective was to explore if human umbilical artery smooth muscle presented this response and if it was involved in the mechanism of serotonin- and histamine-induced contractions. Intracellular  $\text{Ca}^{2+}$  depletion by a  $\text{Ca}^{2+}$ -free extracellular solution followed by  $\text{Ca}^{2+}$  readdition produced a contraction in artery rings which was inhibited by the blocker of Orai and TRPC channels 2-aminoethoxydiphenyl borate (2-APB), suggesting a capacitative response. In presence of 2-APB the magnitude of a second paired contraction by serotonin or histamine was significantly less than a first one, likely because 2-APB inhibited store refilling by capacitative  $\text{Ca}^{2+}$  entry. 2-APB inhibition of sarcoplasmic reticulum  $\text{Ca}^{2+}$  release was excluded because this blocker did not affect serotonin force development in a  $\text{Ca}^{2+}$ -free solution. The PCR technique showed the presence of mRNAs for STIM proteins (1 and 2), for Orai proteins (1, 2 and 3) and for TRPC channels (subtypes 1, 3, 4 and 6) in the smooth muscle of the human umbilical artery. Hence, this artery presents a capacitative contractile response triggered by stimulation with physiological vasoconstrictors and expresses mRNAs for proteins and channels previously identified as SOCs.

## Introduction

The human umbilical artery is a vessel of fundamental importance for fetal-placental blood flow. Since the human umbilical arteries are branches from the iliac

arteries of the fetus, the properties of these blood vessels could offer information of the circulatory system of the newborn. Hence, the knowledge of the mechanisms implicated in the regulation of cytosolic  $\text{Ca}^{2+}$  concentration and therefore, contraction of their vascular smooth muscle cells (VSMC) is very important.

In VSMC  $\text{Ca}^{2+}$  influx through voltage-activated  $\text{Ca}^{2+}$  channels in response to membrane depolarization or to vasoactive agonists represents the most studied pathway. However, an increasing number of investigations in blood vessels have demonstrated that depletion of

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intracellular  $\text{Ca}^{2+}$  stores triggers a  $\text{Ca}^{2+}$  influx from the extracellular space through ionic channels present in the cellular membrane. This phenomenon is known as “capacitative” or “store-operated”  $\text{Ca}^{2+}$  entry and it is likely involved in the refilling of intracellular  $\text{Ca}^{2+}$  stores (Parekh and Penner, 1997; Putney *et al.*, 2001; Bolotina and Csutora, 2005; Leung *et al.*, 2008). This mechanism has not been previously studied in the human umbilical artery nor in other vessels of feto-placental circulation, and hence, its physiological relevance in agonist-induced contractions in this system is unknown.

Channels implied in the capacitative response have been named store-operated channels (SOCs) and it has been shown that they increase their open probability when the sarcoplasmic reticulum (SR) is depleted, producing an inward  $\text{Ca}^{2+}$  current (Albert *et al.*, 2007; Parekh and Putney, 2005). Several reports in the literature propose as possible SOC proteins the transmembrane proteins named Orai (Frischauf *et al.*, 2008; Hewavitharana *et al.*, 2007; Soboloff *et al.*, 2006; Deng *et al.*, 2009; Baryshnikov *et al.*, 2009; Peinelt *et al.*, 2006; Roberts-Thomson *et al.*, 2010) and the Transient Receptor Potential (TRP) channels family, especially those of the Canonical type (TRPC channels) (Leung *et al.*, 2008; Parekh and Putney, 2005; Dietrich *et al.*, 2006; Ng *et al.*, 2010). Finally, the Stromal Interacting Molecules (STIM) types 1 and 2 located in the membrane of SR are  $\text{Ca}^{2+}$  sensors of SR  $\text{Ca}^{2+}$  content (Frischauf *et al.*, 2008; Hewavitharana *et al.*, 2007; Soboloff *et al.*, 2006; Deng *et al.*, 2009; Peinelt *et al.*, 2006). When SR  $\text{Ca}^{2+}$  concentration diminishes, these proteins could activate SOC proteins present in the cellular membrane to allow the capacitative  $\text{Ca}^{2+}$  influx.

Although there is now much information about the structures involved in the capacitative response, further research is necessary to completely understand how these structures interact in order to produce SR refilling and, mainly, what is the physiological importance of this mechanism for each cellular type. Different kinds of experimental protocols have been used for this purpose. For instance, inducing SR depletion with  $\text{Ca}^{2+}$  chelating agents like BAPTA or EGTA in extracellular solutions followed by  $\text{Ca}^{2+}$  re-addition (Putney *et al.*, 2001; Bird *et al.*, 2008), blocking  $\text{Ca}^{2+}$  entry to the SR with sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) inhibitors like thapsigargin or cyclopiazonic acid (CPA) (Putney *et al.*, 2001; Bird *et al.*, 2008), or directly inducing  $\text{Ca}^{2+}$  release from the SR by increasing intracellular inositol 1,4,5-trisphosphate concentration (Fasolato *et al.*, 1993; Fasolato and Nilius, 1998; Parekh and Penner, 1995).

However, in vascular smooth muscle, SR  $\text{Ca}^{2+}$  release and depletion are physiologically produced by mechanisms involving activation of membrane receptors. Hence, in the present work we demonstrate using classical protocols that the human umbilical artery presents a capacitative response, and then that it can be activated by stimulation with serotonin (5-HT) and histamine, both of which produce tonic contractions by coupling to membrane receptors, phospholipase C (PLC) signaling and SR  $\text{Ca}^{2+}$  release and depletion. To strengthen these data, we demonstrate by the polymerase chain reaction (PCR) technique that the mRNAs for STIM, Orai and for the different subtypes of cationic TRPC channels involved in capacitative entry mechanisms are present in the smooth muscle cells of this artery.

## Material and Methods

### Sample collection

Umbilical cords were obtained after vaginal and caesarean deliveries performed in the Instituto Central de Medicina of La Plata, Argentina. They were placed in a transport solution of the following composition (in mM): 130 NaCl, 4.7 KCl, 1.17  $\text{KH}_2\text{PO}_4$ , 1.16  $\text{SO}_4\text{Mg}$ , 24  $\text{CO}_3\text{HNa}$ , 2.5  $\text{Cl}_2\text{Ca}$ , pH 7.4 at 4°C and immediately taken to our laboratory where they were stored at 4°C and used within 24 hrs. All the vascular preparations were classified as surgical discard specimens and thus they were exempted from patient consent requirements.

### Isometric force measurements

The vessels were placed in a Petri dish filled with Krebs Ringer bicarbonate solution (KRB, see composition later), cleaned of adherent connective tissue, and cut into 3-4 mm wide rings. Special care was taken in not damaging the inner endothelial layer or overdistingending the vessel. The ring was gently suspended between two stainless steel wires in a water-jacketed organ bath kept at 37°C and filled with a KRB with the following composition (mM): 130 NaCl, 4.7 KCl, 1.17  $\text{Na}_2\text{HPO}_4$ , 1.16  $\text{MgSO}_4$ , 24.0  $\text{HCO}_3\text{Na}$ , 2.5  $\text{CaCl}_2$ , and 6.0 glucose, constantly bubbled with a mixture of 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ , giving a pH of 7.40. The lower wire was fixed to a vertical plastic rod immersed in the organ bath, while the upper one was rigidly attached to a force transducer (Letica TRI-201). The preparations were then stretched to obtain a passive force of  $\approx 2$  grams. In some experiments we used as bath solutions a  $\text{Ca}^{2+}$ -

free KRB solution (KRB without  $\text{CaCl}_2$ ) or a  $\text{Ca}^{2+}$ -free KRB with the addition of 1 mM of EGTA (see results for details).

*Total RNA isolation and Reverse Transcriptase Polymerase chain reaction (PCR)*

Samples from seven umbilical arteries were used for this analysis. The endothelial layer was carefully eliminated by gently rubbing the inner surface with a cotton swab. Total RNA was obtained from homogenates using Trizol Reagent (Gibco-BRL, Rockville, MD, USA) (Chomczynski and Sacchi, 1987). The integrity of isolated RNA was checked by 1% agarose-formaldehyde gel electrophoresis. Possible contamination with protein or phenol was controlled by measuring the 260/280 nm absorbance ratio, while DNA contamination was avoided using 1 U/ $\mu\text{l}$  DNAase I (Gibco-BRL). RT-reaction was performed using the Super Script III reverse transcriptase (200 U/ $\mu\text{l}$ ; Gibco-BRL) and total RNA (50 ng) as template. For PCR, specific primers were used based on the human cDNA sequence to detect TRPC 1, 3, 4, 5, 6 and 7 (TRPC 2 was not analyzed since it is a

pseudogene (Parekh and Putney, 2005), STIM (1 and 2) and Orai (1, 2 and 3), as well as the von Willebrand factor (endothelial marker used to assure that endothelial cells were not present in our samples) and  $\beta$ -actin (positive control of the PCR). GenBank accession numbers, primer sequences used and expected size of PCR products are shown in Table 1. Possible contamination with genomic DNA was checked by performing PCR with or without the SuperScript III RT.

The cycling profile was the following: 3 min at 95°C followed by 30 sec at 95°C, 30 sec at 58°C, and 1 min at 72°C for 35 cycles and 10 min at 72°C. PCR products were separated by electrophoresis on a 1.5% (w/v) agarose gel and stained with ethidium bromide.

*Statistical analysis*

Results were expressed as means  $\pm$  SEM. Paired or unpaired Student's t-tests were used to evaluate statistically significant differences between two groups. ANOVA followed by the Tukey's test was used for multiple group comparisons. The level of statistical significance was set at  $p < 0.05$ .

**TABLE 1.**

**Data regarding the different primers used for PCR experiments shown in figure 4: GenBank accession numbers, forward and reverse primers sequences, and size (in bps).**

Gene of interest	GeneBank accession number	Sequence of forward primer	Sequence of reverse primer	Product size (bps)
<b>hTRPC1</b>	Z73903	5'-GAACATAAATTGCGTAGATG-3'	5'-CGATGAGCAGCTAAAATGACAG-3'	180
<b>hTRPC3</b>	U47050	5'-GACTTTGGGATGCTGTCCAT-3'	5'-GTACGCAATCCGAGAGAAGC-3'	250
<b>hTRPC4</b>	AF175406	5'-CAGGCTGGAGGAGAAGACAC-3'	5'-GACCTGTCGATGTGCTGAGA-3'	214
<b>hTRPC5</b>	AF054568	5'-CAACTGTCGTGGAATGGATG-3'	5'-AGTGCTTCCGCAATCAGAGT-3'	244
<b>hTRPC6</b>	AF080394	5'-GCCAACAGCAACTTCTCTCC-3'	5'-TCCCAGAAAAATGGTGAAGG-3'	218
<b>hTRPC7</b>	NM_020389.1	5'-GCTGCCTACTTGTCCCTGTC-3'	5'-CACGCCCACTACAAAATCCT-3'	150
<b>h STIM 1</b>	NM_003156.3	5'-CCAGAGCCTCAGCCATAGTC-3'	5'-CATCGTCCATCAGTTTGTGG-3'	174
<b>h STIM 2</b>	NM_001169118.1	5'-TGGATGTGGTTTTGTTTGA-3'	5'-CTTCCTGTGCCTTTTCAAGC-3'	228
<b>h ORAI 1</b>	NM_032790.3	5'-GTCAAGTCTTGCCCTCAA-3'	5'-GGCTAACAGTGAGCGGTAG-3'	182
<b>h ORAI 2</b>	NM_001126340.1	5'-CATAAGGGCATGGATTACCG-3'	5'-ACTCGCTGATGGAGTTCAGG-3'	350
<b>h ORAI 3</b>	NM_152288.2	5'-GAAGCTGTGAGCAACATCCA-3'	5'-AACTTGACCCAACCAACCAG-3'	153
<b>hvW Factor</b>	BC022258	5'-TGACGGTGAATGGGAGACTGG-3'	5'-GCATGAAGTCATTGGCTCCGTTTC-3'	228
<b>hB-actin</b>	NM_001101.3	5'-AAATCTGGCACCACACCTTC-3'	5'-CTCCTTAATGTCACGCACGA-3'	392

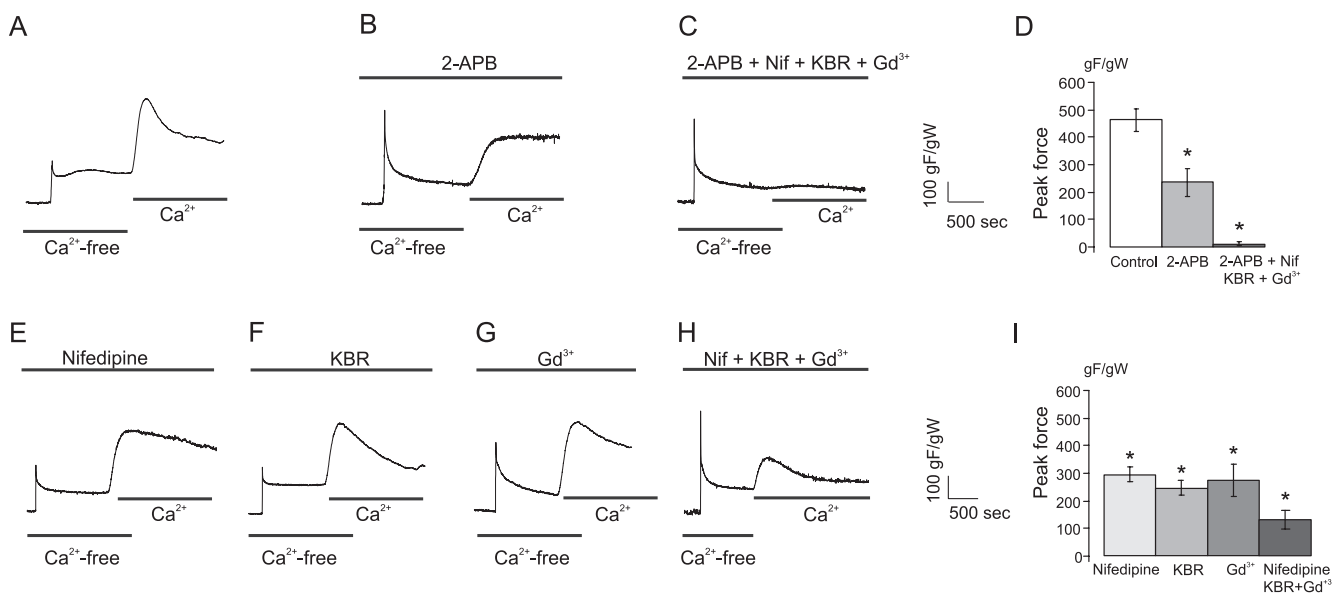
## Results

### Presence of a capacitative response

We first used a classical protocol, such as exposition to a  $\text{Ca}^{2+}$ -free extracellular medium and then  $\text{Ca}^{2+}$  readdition, to identify the presence of a capacitative response in the human umbilical artery, where this mechanism had not been described before. Hence, vessel rings were first incubated during 15 min at room temperature in a  $\text{Ca}^{2+}$ -free Krebs Ringer bicarbonate ( $\text{Ca}^{2+}$ -free KRB) solution containing 5 mM EGTA to eliminate all the extracellular  $\text{Ca}^{2+}$  that could remain attached to connective tissue. Then, they were mounted in the force transducer and exposed during 15 minutes to a KRB solution without  $\text{Ca}^{2+}$  (at  $37^\circ\text{C}$ ) in order to stabilize the temperature. Then, they were stretched to 2 grams and left for another 15 min in this  $\text{Ca}^{2+}$ -free solution. Interestingly, during this time 32% of the vessel rings developed a small spontaneous and transient contraction of  $42 \pm 7$  gF/gW ( $n=13$ ), probably caused by SR release or leak, induced by the extracellular  $\text{Ca}^{2+}$ -free solution. After that,  $\text{Ca}^{2+}$  was restituted to the bath reaching a final concentration of 2.5 mM. Immediately, the rings developed a rapid contraction which reached a maxi-

mal force (peak) and then stabilized at a minor force value (Fig. 1A). To see if all, or part, of this contraction could be inhibited by a blocker of capacitative  $\text{Ca}^{2+}$  entry, the same protocol was repeated in presence of 100  $\mu\text{M}$  2-aminoethoxydiphenyl borate (2-APB), which is widely used to study this mechanism (Bootman *et al.*, 2002) (Fig. 1B). The peak force induced by addition of  $\text{Ca}^{2+}$  now decreased significantly respect to the control contraction, and it did not fall to a minor value. So, these results suggest that the exposition of artery rings to a  $\text{Ca}^{2+}$ -free solution, during almost 45 minutes, is able to produce SR depletion and SOC activation.

Afterwards, we tested if the remaining force could be inhibited by blocking other  $\text{Ca}^{2+}$  entry pathways that we know are present in smooth muscle cells of the human umbilical artery. In fact, if the artery rings were incubated with 100  $\mu\text{M}$  2-APB plus 5  $\mu\text{M}$  nifedipine (a voltage-operated  $\text{Ca}^{2+}$  channel inhibitor), 200  $\mu\text{M}$   $\text{Gd}^{3+}$  (a non selective cationic channels blocker) and 5  $\mu\text{M}$  KB-R7943 (at this concentration, an inhibitor of the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger reverse mode), the contraction induced by  $\text{Ca}^{2+}$  restitution was abolished (Fig. 1C). When these were tested separately, all of them had a significant inhibitory effect but none produced a complete inhibition (Fig. 1E, F and G). Figure 1H shows results obtained



**FIGURE 1.** The human umbilical artery exhibits a capacitative contractile response induced by a period of extracellular  $\text{Ca}^{2+}$  deprivation followed by  $\text{Ca}^{2+}$  readdition. Typical recordings of force development in artery rings subjected to a protocol of 30 min in a Krebs solution without  $\text{Ca}^{2+}$  followed by  $\text{Ca}^{2+}$  readdition in **A**: control conditions; **B**: with 100  $\mu\text{M}$  2-APB; **C**: with 100  $\mu\text{M}$  2-APB, 5  $\mu\text{M}$  nifedipine, 200  $\mu\text{M}$   $\text{Gd}^{3+}$  and 5  $\mu\text{M}$  KB-R7943 (KBR); **E**: with 5  $\mu\text{M}$  nifedipine; **F**: with 5  $\mu\text{M}$  KB-R7943; **G**: with 200  $\mu\text{M}$   $\text{Gd}^{3+}$ ; and **H**: with 5  $\mu\text{M}$  nifedipine, 5  $\mu\text{M}$  KB-R7943 and 200  $\mu\text{M}$   $\text{Gd}^{3+}$ . gF/gW: grams of developed force / weight of wet tissue. **D**: mean  $\pm$  SEM values of experiments shown in A ( $n=40$ ), B ( $n=13$ ) and C ( $n=7$ ). **I**: mean  $\pm$  SEM values of experiments shown in E ( $n=13$ ), F ( $n=14$ ), G ( $n=10$ ) and H ( $n=6$ ). In all cases, \* indicates statistically significant differences from control conditions ( $p < 0.05$ ).

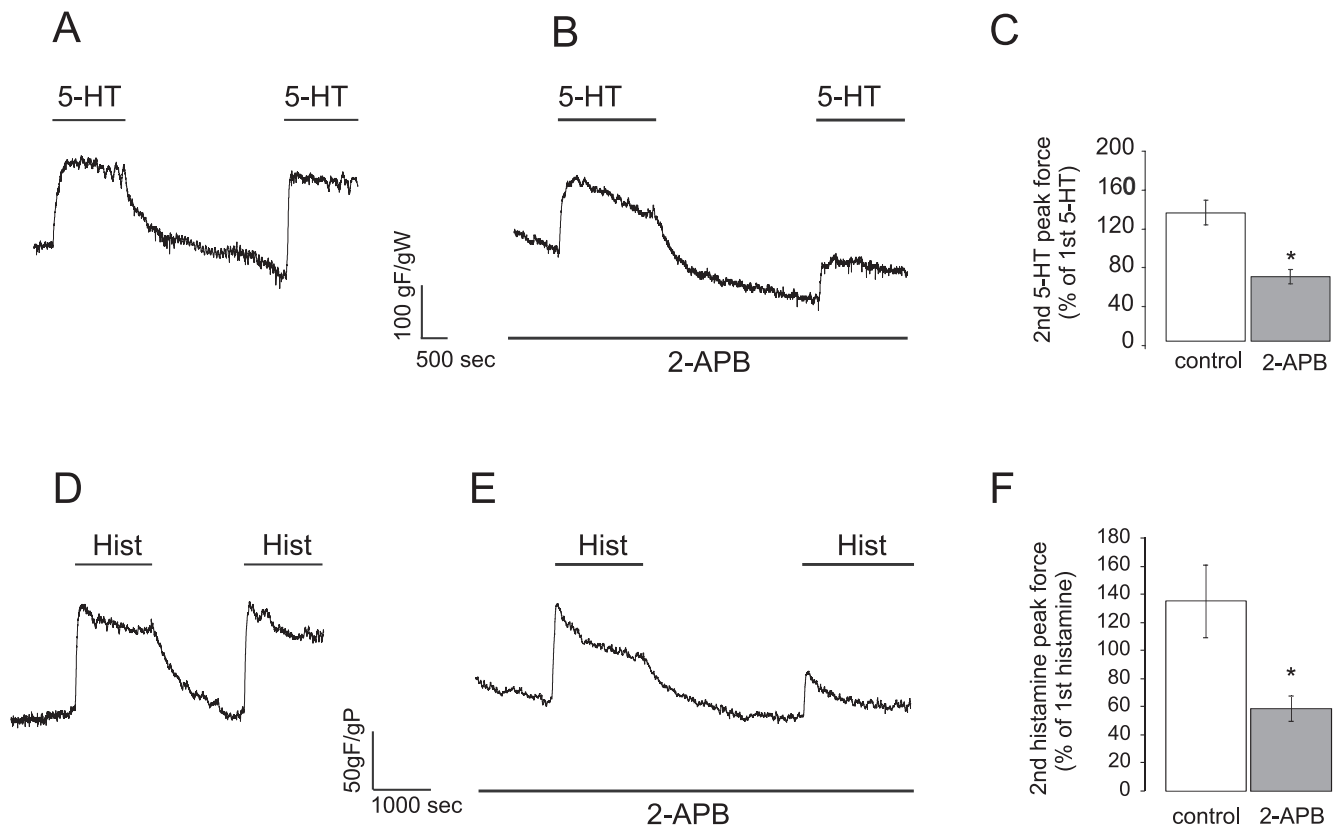
using a combination of nifedipine,  $Gd^{3+}$  and KB-R7943 but not 2-APB, which would leave only the 2-APB-sensitive capacitative response as a  $Ca^{2+}$  entry mechanism to induce the artery ring contraction.

#### *Vascular agonists and capacitative response*

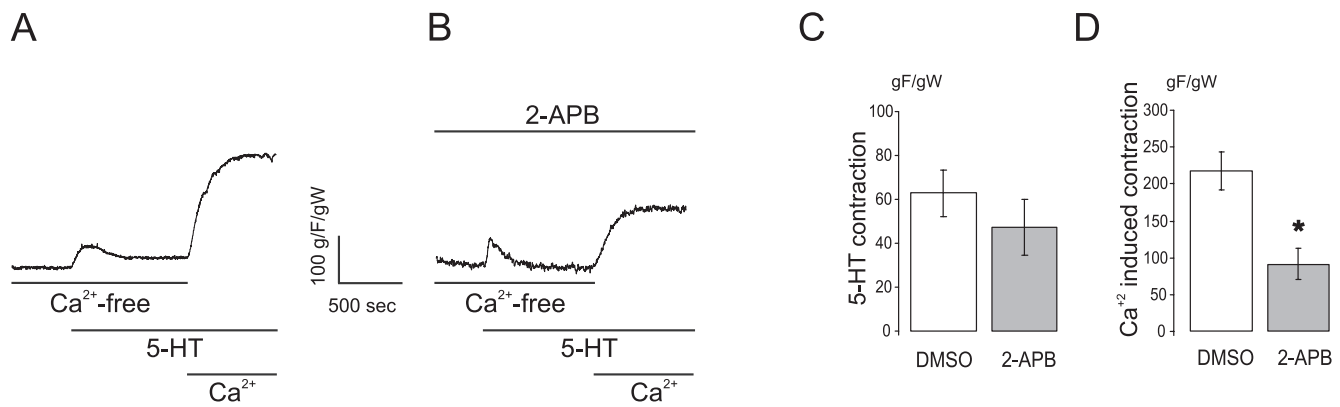
In human umbilical artery both 5-HT and histamine are potent contractile agonists acting through G-coupled  $5-HT_2$  receptors and H1 receptors, respectively (Bertrand and St-Louis, 1999; Lovren *et al.*, 1999). It is known that they produce activation of inositol 1,4,5-trisphosphate sensitive  $Ca^{2+}$  channels ( $InsP_3$  receptors) which allow  $Ca^{2+}$  release from sarcoplasmic reticulum (SR) and its consequent depletion (Tufan *et al.*, 2003). Since this will cause a reduction in SR  $Ca^{2+}$  content, we hypothesized that it could trigger a capacitative  $Ca^{2+}$  entry. Therefore, we applied two consecutive stimuli with  $1 \mu M$  5-HT or  $10 \mu M$  histamine separated by a 25 minutes period of agonist

wash-out. The obtained results presented in Figure 2A and 2D show that for both vasoconstrictor agents, the second contraction had the same magnitude as the first one. However, when the same protocol was repeated in the presence of  $100 \mu M$  2-APB, the second contractions were significantly lower than the first ones (Fig. 2B and 2E). Also, the bars in Figure 2 are showing the mean force relationships between the force values of the first and second contraction for 5-HT (2C) and for histamine (2F) in both conditions.

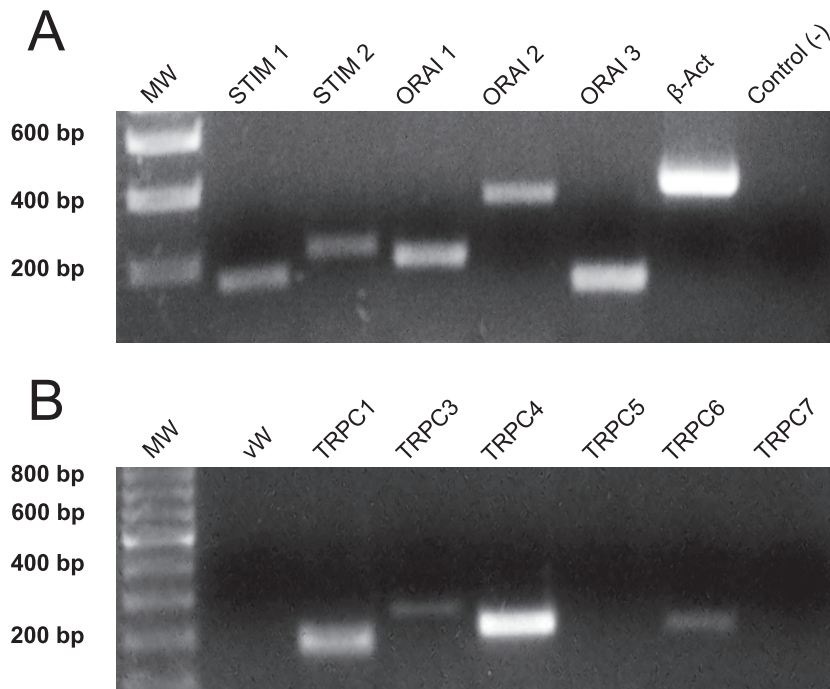
It is known that 2-APB can also inhibit  $InsP_3$  receptors (Bootman *et al.*, 2002; Maruyama *et al.*, 1997), so the results presented in Figure 2B and 2E may be interpreted as a late effect of 2-APB on  $InsP_3$  receptors. In order to test this possibility, we performed a control experiment where 2-APB was tested on a contraction evoked by 5-HT on an arterial ring exposed to a  $Ca^{2+}$ -free extracellular solution (with  $1 mM$  EGTA), so only the contraction due to SR  $Ca^{2+}$  release would be ob-



**FIGURE 2.** The capacitative contractile response participates in the mechanism of 5-HT and histamine force development in smooth muscle of the human umbilical artery. **A:** Typical recording of two  $1 \mu M$  5-HT-induced contractions separated by 25 min without 5-HT (gF/gW: grams of developed force / weight of wet tissue). **B:** Typical recording of an experiment similar to that shown in A but in the presence of  $100 \mu M$  2-APB. **C:** mean  $\pm$  SEM values of experiments shown in A (n=10) and B (n=12). **D:** Typical recording of two  $10 \mu M$  histamine-induced contractions separated by 25 min without histamine (gF/gW: grams of developed force / weight of wet tissue). **E:** Typical recording of an experiment similar to that shown in D but performed in the presence of  $100 \mu M$  2-APB. **F:** mean  $\pm$  SEM values of experiments shown in D (n=10) and E (n=10). In all cases, \* indicates statistically significant differences from controls ( $p < 0.05$ ).



**FIGURE 3.** 2-APB does not interfere with 5-HT-induced Ca<sup>2+</sup> release from the sarcoplasmic reticulum but it inhibits the capacitative contractile response in the human umbilical artery. **A:** Typical recording of 1 μM 5-HT contraction of artery rings in a Ca<sup>2+</sup>-free KRB followed by extracellular Ca<sup>2+</sup> readdition. The chamber had an appropriate amount of the vehicle of 2-APB (DMSO) (gF/gW: grams of developed force / weight of wet tissue). **B:** Typical recording of an experiment similar to that shown in A but performed in the presence of 100 μM 2-APB. **C:** mean ± SEM values of peak 5-HT force of experiments shown in A (n=9) and B (n=10). **D:** mean ± SEM values of stable force after Ca<sup>2+</sup> readdition as shown in A (n=9) and B (n=10).



**FIGURE 4.** The PCR technique shows that smooth muscle cells of the human umbilical artery express mRNA for several structures implicated in the capacitative response. **A:** photograph of a typical PCR experiment showing the presence of mRNAs for STIM 1 and 2, and Orai 1, 2 and 3. Also shown are positive (with primer) and negative (without primer) results for β-actin. **B:** photograph of a typical PCR experiment showing the presence of mRNAs for TRPC 1, 3, 4 and 6, and the absence of TRPC 5 and 7 (TRPC 2 was not tested, see text for details). A negative result for von Willebrand factor (vW) indicates that there is no contamination of the sample with endothelial cells. A positive controls for β-actin was performed but is not shown.

served. In this setting, stimulation with 1  $\mu\text{M}$  5-HT produced a significant transient contraction whose magnitude was not modified by 2-APB. Moreover, when extracellular  $\text{Ca}^{2+}$  was restored, a force development was observed which was now significantly diminished by the presence of 2-APB. Figure 3 shows typical recordings and mean values for these experiments.

#### *Expression of mRNAs for structures implicated in capacitative response*

STIM and Orai proteins and TRPC channels, are proposed in the literature to be involved in the capacitative response. Hence we explored the presence of the mRNA for these proteins in the human umbilical artery by the PCR technique. Although samples from 7 different arteries were processed in the same way (see Methods), the endothelial marker (von Willebrand factor) was not expressed by only two of them, so only the results from these samples were considered for conclusions about TRPC channels, STIM and Orai present in vascular smooth muscle (each of these two samples was analyzed twice). As shown in Figure 4, we found expression of mRNA for STIM 1 and 2, and for Orai 1, 2 and 3. The TRPC1, 3, 4 and 6 were also present in smooth muscle cells from the human umbilical artery, while we were not able to demonstrate the expression of TRPC5 and 7. The  $\beta$ -actin control was positive for all the PCRs performed.

## Discussion

Our work presents results suggesting that the vasoconstrictors 5-HT and histamine produce an activation of the capacitative response in the human umbilical artery, which seems useful to allow a quick restoration of SR  $\text{Ca}^{2+}$  content. This conclusion is based on the results presented in Figure 2, where it can be seen that the vessels stimulated twice with these agonists evoked two similar contractions in control conditions, while in the presence of 2-APB the second contractions were significantly lower than the first ones. Our interpretation of these data is that during the first stimulation with the vasoconstrictor, the SR depletion caused by the agonist activated a capacitative response which would contribute to the SR refilling. When SOCs were inhibited by 2-APB the  $\text{Ca}^{2+}$  entry was diminished or inhibited and the SR did not completely refill after the first agonist addition, producing then a second contraction of smaller magnitude.

An alternative explanation for our results would be that the second 5-HT or histamine contraction was smaller than the first one because 2-APB had a late inhibitory effect on  $\text{InsP}_3$  receptors. However, we can exclude this possibility because when the stimulus on the SR was performed without extracellular  $\text{Ca}^{2+}$ , 2-APB had no effect on the 5-HT induced contraction, but instead significantly diminished the force produced by  $\text{Ca}^{2+}$  re-addition.

Additionally, although we cannot completely exclude the possibility that the endothelium could have modified these responses, we have previously observed (unpublished data) that endothelial NO and protacyclins inhibition does not modify the contractile force induced by 5-HT. This suggests that the observed reduction of contractile force produced by 2-APB may be mostly due to inhibition of the smooth muscle capacitative response.

Our results are in accordance with the data presented by Gregory *et al.* (2001) in freshly isolated hepatocytes, where 2-APB (up to 100  $\mu\text{M}$ ) did not block  $\text{InsP}_3$  receptors but was able to inhibit the  $\text{Ca}^{2+}$  inflow when  $\text{Ca}^{2+}$  was restituted after vasopressin-induced SR depletion in a free- $\text{Ca}^{2+}$  extracellular medium. Moreover, in cells incubated with normal extracellular  $\text{Ca}^{2+}$ , they showed that 2-APB decreased  $\text{Ca}^{2+}$  entry once the SOC response was activated with vasopressin.

The degree of involvement of capacitative  $\text{Ca}^{2+}$  entry in the response to vasoconstrictors seems to be variable in different vessels and species. For instance, Moneer *et al.* (2005) propose that in A7r5 cells 5-HT produces extracellular  $\text{Ca}^{2+}$  entry only through a capacitative response. On the other hand, Guibert *et al.* (2004) showed the presence of a capacitative  $\text{Ca}^{2+}$  entry in small intrapulmonary arteries of the rat but provide evidence that it is independent of 5-HT-induced changes in cytosolic  $\text{Ca}^{2+}$  concentration and vessel diameter. However, the same researchers, showed that in human pulmonary artery a part of 5-HT-induced contractions was due to voltage-independent  $\text{Ca}^{2+}$  entry which could be mediated by SOCs (Rodat-Despoix *et al.*, 2009). These results in human pulmonary artery are very similar to our findings in the umbilical artery, and it is interesting to note that both are human vessels in which 5-HT plays an important physiological role.

In order to further characterize the capacitative response in the human umbilical artery we also used a classical protocol of SR depletion induced by extracellular  $\text{Ca}^{2+}$  deprivation, and subsequent development of a contraction by  $\text{Ca}^{2+}$  readdition. This force development was partly inhibited by 2-APB, hence suggesting that part of this response was produced via SOCs due

to the SR depletion. Similar results were shown by Potocnick *et al.* (2001) in skeletal muscle arterioles, where a capacitative  $\text{Ca}^{2+}$  influx produced by re-addition of extracellular  $\text{Ca}^{2+}$  following pre-treatment with an extracellular  $\text{Ca}^{2+}$ -free solution (plus nifedipine) could be inhibited by 2-APB. They also demonstrated that 2-APB diminished the contractile response and  $(\text{Ca}^{2+})_i$  transient evoked by phenylephrine but not by KCl. In vascular smooth muscle,  $\text{Ca}^{2+}$ -permeable non-selective cationic channels able to be activated by  $\text{Ca}^{2+}$  store-depletion were also described in rabbit portal vein (Albert and Large, 2002), in human pulmonary artery (Golovina *et al.*, 2001), in rabbit pial arterioles (Xu *et al.*, 2006), in mouse and rabbit aorta (Trepakova *et al.*, 2001) and in mouse pulmonary artery (Ng *et al.*, 2009), among others.

It is interesting to note that in the human umbilical artery, 2-APB did not block all the force development induced by  $\text{Ca}^{2+}$  readdition. Instead, this was only eliminated by a combination of 2-APB and other antagonists of  $\text{Ca}^{2+}$  entry pathways (nifedipine,  $\text{Gd}^{3+}$  and KB-R7943). All of these blockers partially inhibited force development when added independently, meaning that there are multiple pathways which act in a concerted way to let  $\text{Ca}^{2+}$  into the cell after a period of extracellular  $\text{Ca}^{2+}$  deprivation. Some of these routes are related to the capacitative response, as evidenced by 2-APB effects (assuming that 2-APB is more selective for SOCs).

Our results suggest that smooth muscle cells in this artery express all the structures which many bibliographical data point as necessary for the production of a capacitative response. Namely, we present evidences that smooth muscle cells of the human umbilical artery express the mRNAs for STIM 1 and 2, for Orai 1, 2 and 3 and also for some of the members of the TRPC channels family, namely the 1, 3, 4 and 6 subtypes. The mRNAs for TRPC5 and 7 were found not to be present in these cells. According to the literature, the TRPC channel most likely to be a SOC in cells presenting capacitative response is the TRPC1 subtype, and to a lesser extent TRPC3, TRPC4 and TRPC6 (Leung *et al.*, 2008; Parekh and Putney, 2005; Dietrich *et al.*, 2006; Ng *et al.*, 2010). Meng *et al.* (2007) report expression of mRNAs for TRPC 1, 3, 4 and 6 in cultured smooth muscle cells derived from the human umbilical artery. Our results may be considered a step further of those presented by these authors, since we used native cells, while they worked with cultured cells, where the pressure induced by growth factors in the culture media may have altered channel expression (Sandow and Hilton Grayson, 2009).

STIM 1 and 2, as well as Orai 1 and 3, seem to be broadly expressed in different tissues (Frischauf *et al.*, 2008). However, Orai 2 is more expressed in the brain and less in other tissues (Frischauf *et al.*, 2008). Both STIM 1 and 2, are able to activate the plasma membrane Orai proteins (Frischauf *et al.*, 2008) and there are some recent reports that STIM1 could also interact with TRPC channels (Soboloff *et al.*, 2006). Moreover, Liao *et al.* (2008) have reported that a STIM 1/Orai 1/TRPC 1 interaction is necessary to produce a capacitative response.

All these structures are expressed in the human umbilical artery smooth muscle, but it is beyond the scope of this work to establish if in these cells TRPCs, Orai, or a combination of both, are the target of STIM proteins. However, this subject merits further research in order to clarify the physiological roles of these structures in the human umbilical artery and other human vessels. This may provide more data for the understanding of vascular pathological processes affecting fetoplacental blood flow.

In this work we have shown for the first time that smooth muscle of the human umbilical artery is able to evoke a capacitative contractile response inhibited by 2-APB, that stimulation with physiological vasoactive agonists which release  $\text{Ca}^{2+}$  from the SR is enough to induce this response, and that this artery expresses mRNA for STIM 1-2, Orai 1-3 and TRPC 1, 3, 4 and 6 channels which in the literature have been demonstrated to be involved in this mechanism.

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