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Effects of 1-(2-trifluoromethylphenyl)-imidazole (TRIM) on receptor-independent and -dependent contractile responses in rat aorta

Çiğdem SELLİ[1], Yasemin ERAÇ[1], Metiner TOSUN[2,*]
[1]Department of Pharmacology, Faculty of Pharmacy, Ege University, İzmir, Turkey
[2]İzmir University of Economics, Faculty of Medicine, 35330 İzmir, Turkey

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Background/aim: This study investigates whether 1-(2-trifluoromethylphenyl)-imidazole (TRIM), originally proposed as a nitric oxide synthase inhibitor and also suggested to be an inhibitor of store-operated calcium entry in mouse anococcygeal muscle, inhibits receptor-independent and -dependent responses in rat thoracic aorta.

Materials and methods: Cyclopiazonic acid- and serotonin-induced vascular responses were investigated in aortic segments isolated from male Sprague Dawley rats using isolated tissue experiments. Changes in intracellular calcium levels were also monitored via front surface fluorescence measurements in fura-2-loaded embryonic rat vascular smooth muscle cell line A7r5.

Results: TRIM inhibited serotonin-mediated vascular contractions without affecting cyclopiazonic acid-induced responses. In addition, TRIM caused a nonlinear rightward shift in the serotonin concentration-response curve, possibly via serotonin receptor modulation.

Conclusion: TRIM may have an impact on investigation of tissue-specific receptor-independent and -dependent vascular responses. It may also be used as a lead compound in the development of selective serotonin receptor modulators.

Key words: Concentration-response curve, cyclopiazonic acid, serotonin, store-operated calcium, TRIM

1. Introduction

1-(2-Trifluoromethylphenyl)-imidazole (TRIM), originally proposed as a nitric oxide synthase (NOS) inhibitor (1), also inhibits thapsigargin-induced contractions and store-operated Ca^{2+} entry (SOCE) in mouse anococcygeal muscle (2). TRIM has been also shown to have antidepressant-like effects in rat forced-swimming models, possibly acting on 5-HT receptors (3). Its SOC channel blocking effect seems to be plausible as TRIM shares a common N1-substituted imidazole structure with other SOCE inhibitors, SKF96365 and miconazole (4). Depletion of intracellular Ca^{2+} stores activates a sarcolemmal Ca^{2+} influx, a process called SOCE (5). Although not clear, the mechanism that couples store depletion to SOCE appears to be tissue-dependent. Aside from the activation mechanism, the main effect of SOCE is to keep intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) elevated during long-term agonist stimulation and refill the sarcoplasmic reticulum (SR) even after agonist removal (5–7).

Stimulation of many nonexcitable cells by growth factors and hormones triggers sequential events such as phospholipase C (PLC) activation, inositol 1,4,5-trisphosphate (IP_3) production, and Ca^{2+} release from internal IP_3-sensitive stores. Transient Ca^{2+} elevation is followed by sustained Ca^{2+} influx through SOC channels (7,8). Serotonin (5-hydroxytryptamine, 5-HT) is a potent vasoconstrictor (9) agent that mediates arterial responses through activating its G protein-coupled receptors (GPCR), $5\text{-}HT_{2A}$ and $5\text{-}HT_{1B}$ (10). 5-HT-induced intracellular Ca^{2+} changes in primary cultured smooth muscle cells have been characterized by two steps, a transient phase due to IP_3-induced Ca^{2+} release from intracellular stores and a plateau phase that depends on extracellular Ca^{2+} influx (11). Previously, it was shown that 5-HT leads to a selective Ca^{2+} release from cyclopiazonic acid (CPA)-sensitive stores (12,13). In another study, a noncapacitative, arachidonic acid-sensitive, receptor-operated Ca^{2+} channel was also suggested to contribute to 5-HT-induced sustained Ca^{2+} elevations (14).

In addition to IP_3-releasing GPCR agonists, sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitors (CPA; thapsigargin, Tg; etc.) activate SOCE and yield contractions in various smooth muscles (15). Although SOC channels can be activated indirectly by SERCA inhibitors, specific SOC channel activators remain unidentified. SOC channels have been thought to consist of transient receptor potential (TRP) family proteins (16), which were initially identified as an essential component of phototransduction in *Drosophila melanogaster* (17). Among the TRP canonical

* Correspondence: metiner.tosun@ege.edu.tr

subfamily, TRPC1 was suggested to mediate SOCE in vascular smooth muscle cells (18) through heteromultimeric combination with TRPC4 and TRPC5 (19). Selective blockers are needed to delineate the SOCE activation mechanism and the tissue-specific SOC channel composition.

Briefly, this study investigates the effects of TRIM on CPA-induced $[Ca^{2+}]_i$ elevations and force development as well as 5-HT-mediated responses, which are partly associated with depletion of CPA-sensitive Ca^{2+} stores.

2. Materials and methods

2.1. Animals

All animal experiments were performed in accordance with EU guidelines and were approved by the institution's Committee on Animal Use in Research and Education, Ege University (Ethical Committee Approval #: B30.2.EGE.0.03.00.01/106). Rats (Sprague Dawley, male, n = 5) were maintained in appropriate conditions (temperature, humidity, and a 12-h light-dark cycle) with ad libitum access to food and water.

2.2. Chemicals

All chemicals were from Sigma and were dissolved in appropriate solvents as follows: TRIM (PubChem CID: 1359) (10^{-2} M), 0.9% NaCl; 5-HT (10^{-2} M) in distilled water (DW); phenylephrine (PE, PubChem CID: 5284443) (10^{-1} M) in DW; CPA (PubChem CID: 54695722) (10^{-1} M) in dimethyl sulfoxide (DMSO); indomethacin (10^{-2} M) in EtOH; verapamil HCl (10^{-2} M) in DW; 2-aminoethoxydiphenyl borate (2-APB, PubChem CID: 1598) (10^{-1} M) in DMSO; HA-1077 (Fasudil, PubChem CID: 16219471) (10^{-1} M) in DW.

2.3. Isolated tissue experiments

A detailed experimental protocol was used as described earlier (20). Briefly, the thoracic aorta was removed from adult rats (300–350 g), asphyxiated with CO_2, cleaned of extraneous fatty tissue, and cut into 3-mm rings. The endothelium was removed by gently rubbing the intimal surface of the vessel with a cotton swab. Endothelium removal was functionally confirmed by the lack of acetylcholine (ACh)-induced relaxations. Tissues were mounted on a holder attached to an isometric force transducer in organ baths containing Krebs-Ringer bicarbonate solution (in mM: NaCl 118.00, KCl 4.73, $MgSO_4$ 1.20, $CaNa_2EDTA$ 0.03, NaH_2PO4 1.20, $CaCl_2$ 2.50, $NaHCO_3$ 25.00, glucose 11.00, 37 °C, 95% O_2-5% CO_2) (21). Next, 10 μM indomethacin was added into the solution to eliminate the release of endogenous prostanoids. Rings were gradually stretched to the previously established optimal point of the resting tension (20 mN) for rat aorta. Changes in isometric force were recorded with a data acquisition system (BioPac Systems, MP100A-CE). Force was normalized to cross-sectional area [force (F) (in mN)/ cross-sectional area (CSA) (in mm^2) = F/CSA= (change in force × circumference)/2 × wet weight].

2.4. Cell culture and measurement of $[Ca^{2+}]_i$

Vascular smooth muscle cells derived from embryonic rat thoracic aorta (A7r5, ECACC) were cultured in DMEM/ Ham's F12 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and penicillin-streptomycin.

A detailed protocol for monitoring $[Ca^{2+}]_i$ in A7r5 cells was previously described (22). Furthermore, the methodology of the Ca^{2+} monitoring system used in the study was recently published (23). Briefly, A7r5 cells grown on round glass coverslips were incubated with 5 μM fura-2/ AM containing HEPES-buffered saline (HBS, in mM: NaCl 135, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 11.6, $NaHCO_3$ 5, glucose 11.5, pH 7.3). The coverslip was mounted vertically in a polymethacrylate spectrophotometer cuvette, placed into the spectrofluorometer, and continuously perfused with HBS (5 mL/min) at room temperature. Cells were excited at 340 and 380 nm wavelengths and the emission intensities at 510 nm were monitored using a dual wavelength spectrofluorometer with a sampling rate of 1 Hz (PTI QM8/2005, Photon Technology International). Data were expressed as the ratio of fluorescence intensities [ratio (340/380)].

2.5. Data analysis

The results are given as mean ± standard error of the mean. "n" represents the number of rats used. The significance of differences was evaluated by Student's t-test and Newman–Keuls test where appropriate. $P < 0.05$ was considered significant. Force was normalized to cross-sectional area (F/CSA) in all isolated tissue experiments. Each data point in the concentration-response curve was normalized to its own observed maximal effect (Emax). An iterative nonlinear least-squares method was used for curve fitting and 50% effective concentrations (EC_{50}) were derived for each cumulative concentration-response curve (GraphPad Prism5, La Jolla, CA, USA).

3. Results

3.1. Inhibition of CPA-induced contractions and $[Ca^{2+}]_i$ elevations

The inhibitory effects of TRIM on receptor-independent responses were initially tested using isolated rat thoracic aorta in organ bath studies. CPA at 10 μM, a concentration that reportedly depletes SR Ca^{2+} (24), was used to induce contractions in endothelium-denuded rat thoracic aorta. CPA-induced vascular contractions were abolished by 2-APB (100 μM) and Rho kinase inhibitor HA-1077 (50 μM), whereas they were not affected by 200 μM TRIM (Figure 1).

The effects of TRIM on CPA-induced $[Ca^{2+}]_i$ elevations were investigated further by monitoring $[Ca^{2+}]_i$ changes in A7r5 vascular smooth muscle cell culture. Similar to the results of our isolated tissue experiments, in fura-2-loaded A7r5 cells, CPA-induced $[Ca^{2+}]_i$ elevations were not apparently affected by TRIM, whereas they were readily

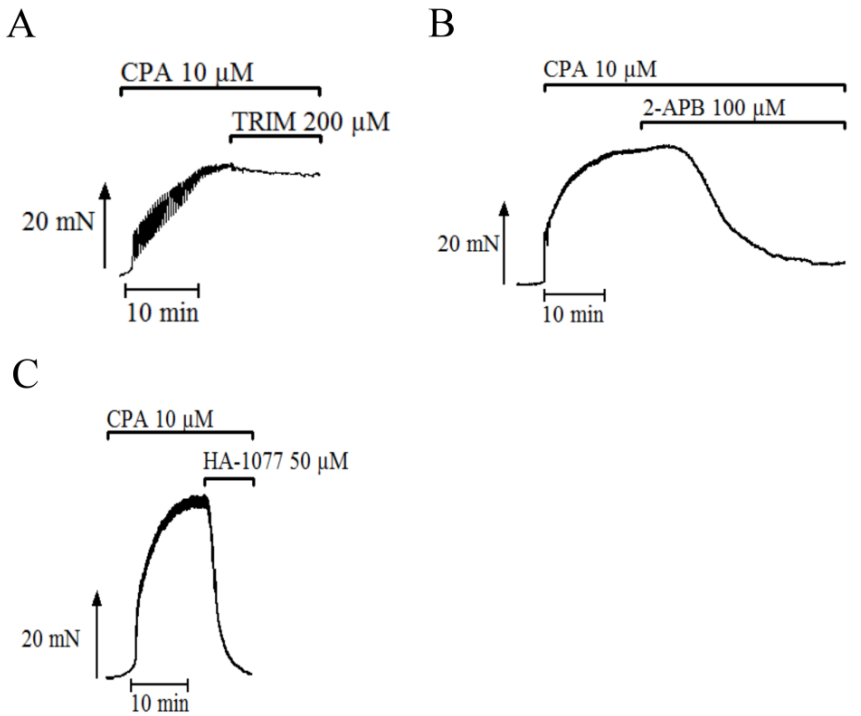

Figure 1. Effects of TRIM, 2-APB, and HA-1077 on CPA-induced contractions. Purported SOCE blockers TRIM (200 μM, A) and 2-APB (100 μM, B) and Rho kinase inhibitor HA-1077 (50 μM, C) were added to 10 μM CPA-induced plateau responses in rat thoracic aorta (n = 1).

abolished by 2-APB, another purported blocker of SOCE (Figure 2).

3.2. Inhibition of 5-HT-mediated contractions by TRIM

The effects of TRIM on receptor-dependent (5-HT-induced) contractions were also investigated using endothelium-denuded vessels in isolated tissue experiments. TRIM significantly inhibited contractions induced by submaximal concentrations of 5-HT in endothelium-denuded rat thoracic aorta ($P < 0.01$, n = 3, Figure 3). Verapamil was also used to test the contribution of voltage-operated calcium channels in TRIM-insensitive serotonin-induced responses. Verapamil significantly inhibited the remaining 5-HT contractions ($P < 0.01$, n = 3, Figure 3).

To investigate TRIM's antagonistic nature, cumulative 5-HT concentration-response experiments were performed in the absence and presence of increasing concentrations of TRIM (1, 10, 100 μM) in isolated organ chambers. The concentration-response curves (CRCs) constructed by cumulative 5-HT responses were shifted to the right in the presence of TRIM (Figure 4). Although there was a gradual decrease in Emax values calculated from CRCs, a similar depression was also observed in a control tissue segment from which sequential CRCs were obtained in the absence of TRIM (data not shown). EC_{50} values (in μM) were 0.63 ± 0.18 in control and 0.81 ± 0.23, 1.15 ± 0.30, and 2.51 ± 0.72 in TRIM (1, 10, and 100 μM, respectively)-pretreated tissues (not significant, Newman–Keuls test).

4. Discussion

4.1. Inhibition of CPA-induced vascular contractions and $[Ca^{2+}]_i$ elevations

TRIM (1–333 μM) was shown to selectively inhibit Tg-induced contractions of endothelium-denuded mouse anococcygeal muscle in a concentration-dependent manner (2). This effect was reportedly independent of nitric oxide synthase inhibition (1). TRIM also inhibited Tg-induced SOCE in mouse anococcygeal muscle cells expressing TRPC1, -2, -5, and -6 (2). In our study, TRIM (200 μM) had no inhibitory effect on CPA-induced contractions of endothelium-denuded rat thoracic aorta and CPA-induced $[Ca^{2+}]_i$ elevations in A7r5 cells. The main limitation of our study is that some experiments (Figures 1A–1C and 2) were not repeated more than once to keep the animal number at minimum as no or complete response was observed. In addition, inadequate dose ratio (<2) calculated by the data obtained from 3 samples (Figure 4) precluded a more appropriate analysis of competitive antagonism via Schild plot. Limited sample size (n = 3) might have led to statistically insignificant EC_{50} values despite the apparent dextral shift in 5-HT CRCs. TRPC1 has been suggested to be a component of functional SOC channels in several cell types (25). In our previous study, two TRPC subtypes, TRPC1 and -C6, were detected in A7r5 cells (22), consistent with a previous report (26). Furthermore, SOCE was upregulated along with TRPC6 expression in TRPC1-silenced A7r5 cells, suggesting

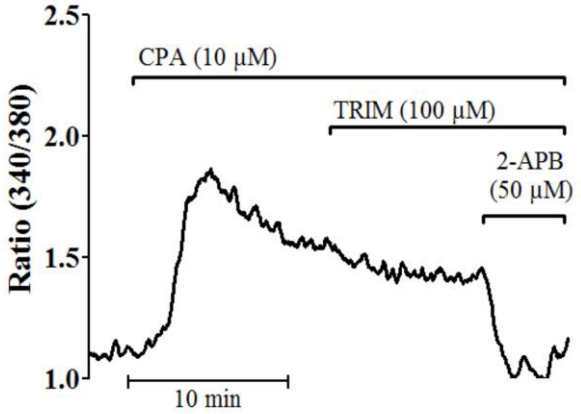

Figure 2. Effects of TRIM and 2-APB on CPA-induced $[Ca^{2+}]_i$ elevations. TRIM (100 μM) and 2-APB (50 μM) were added to 10 μM CPA-induced $[Ca^{2+}]_i$ elevations in A7r5 cells (n = 1).

a SOC regulatory role for TRPC1 in vascular smooth muscle cells (22). Thapsigargin-induced I_{SOC} was reduced in TRPC1-silenced rat mesenteric artery smooth muscle cells expressing TRPC1, -C4, and -C6 (27). In the present study, ineffectiveness of TRIM on CPA-induced contractions in rat thoracic aorta might have resulted from the tissue-specific SOC channel subunit composition, as suggested earlier (2). This may possibly be determined by different expression profiles of proteins participating in SOC channels such as TRPs and Orai1.

Rho-kinase inhibition does not have any effect on Tg-induced SOCE (28), whereas HA-1077 completely inhibited CPA-induced contraction in a rapid manner, supporting the involvement of Rho-kinase in Ca^{2+} sensitization in rat thoracic aorta (present study).

Although initially defined as a membrane-permeable IP_3 receptor antagonist (29), 2-APB was also shown to inhibit SOCE in an IP_3 receptor knockout mouse smooth muscle cell line (30). In our study, 2-APB abolished both CPA-induced contractions and $[Ca^{2+}]_i$ elevations in rat aorta, possibly independent of IP_3 receptor inhibition (31). We also previously showed that a stable TxA2 analogue, U46619, as well as norepinephrine at maximal concentrations did not elevate $[Ca^{2+}]_i$ in 10 μM CPA-treated rat thoracic aorta (12). This observation suggested that 10 μM CPA completely depletes IP_3-sensitive Ca^{2+} stores. Since the IP_3 receptor was shown to be essential for coupling of store-depletion to SOCE (32), the SOCE inhibitory effect of 2-APB may depend on a tissue-specific SOCE activation mechanism. Furthermore, Rho kinase inhibitor HA-1077 inhibited CPA-induced contractions, supporting Rho kinase's role in SOCE via mediating coupling of the IP_3 receptor with TRPC1 (33).

4.2. Inhibition of 5-HT-induced contractions by TRIM

TRIM significantly inhibited the 5-HT-induced contractions in rat thoracic aorta. The effectiveness of TRIM on functionally deendothelialized rat aorta suggests that its inhibitory effect may be independent of NOS inhibition, as reported previously (34). In addition, no effect was observed on endothelin-1-induced contractions in rat thoracic aorta (20). The ineffectiveness of TRIM on contractions induced by ET-1 supports its specific inhibitory effect on 5-HT responses. The rightward shift in the 5-HT concentration-response curves in the presence of increasing TRIM concentrations suggests an antagonistic effect of TRIM on 5-HT responses. Despite the logarithmic increases in TRIM concentrations, the dextral shift in 5-HT concentration-response curve was not proportional as expected for a competitive receptor antagonism. It has been shown that 5-HT concentration-response curve was shifted to the left in a parallel manner in rat thoracic aorta in the presence of 5-HT transporter inhibitors, chlorpheniramine and citalopram (35). The uptake of 5-HT by its transporters may cause the unproportional rightward shift observed in our study.

Since the first phase of the 5-HT CRC in rabbit femoral artery was predominantly mediated by $5\text{-}HT_2$ receptors and the second phase was mediated by α_1-adrenoceptors (36), it can

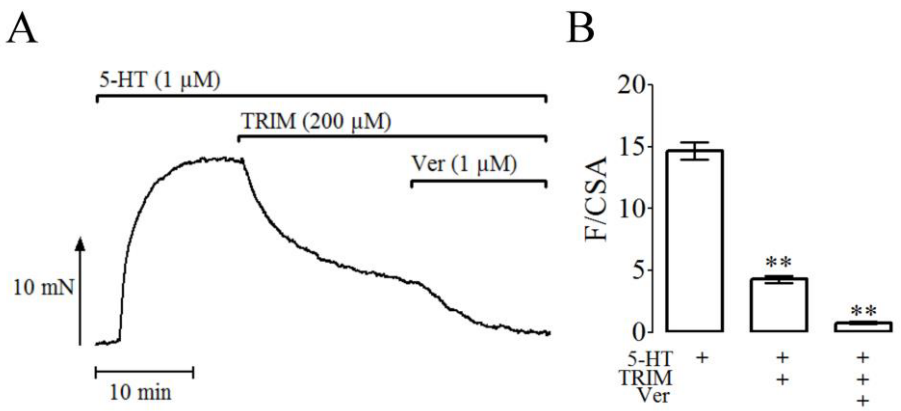

Figure 3. Effects of TRIM and verapamil on 5-HT-induced contractions. TRIM (200 μM) and verapamil (1 μM) were added respectively during 5-HT (1 μM) plateau contractions in endothelium-denuded vessels (**P < 0.01, Newman–Keuls multiple comparison test, n = 3).

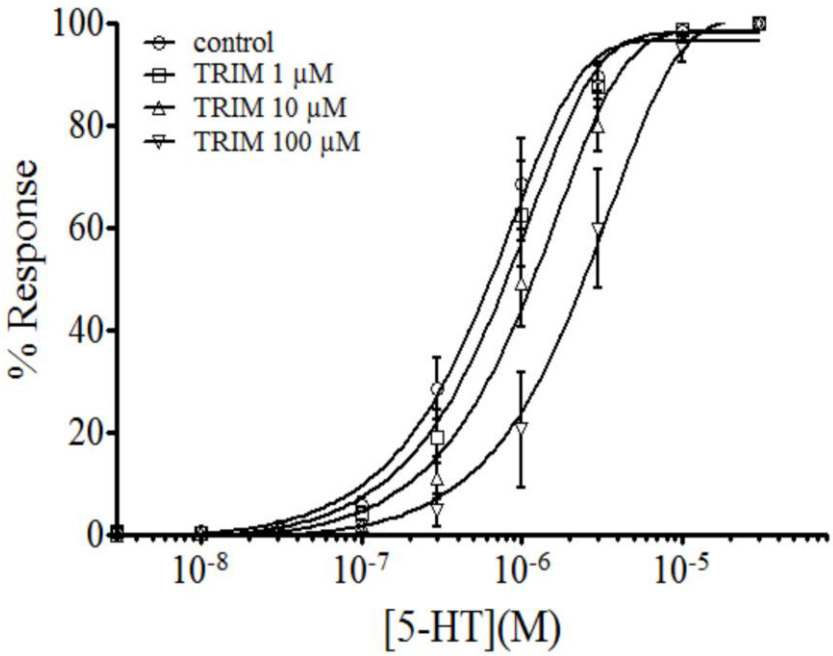

Figure 4. Effects of TRIM on 5-HT concentration-response curves. Concentration-response curve of 5-HT (1 nM to 30 μM) was constructed for control and TRIM-pretreated tissues (n = 3).

be suggested that a possible contribution of α_1-adrenoceptors to 5-HT contractions results in a disproportionate dextral shift. However, in the presence of 100 nM prazosin, which fully inhibits the α_1-adrenergic receptor agonist PE-induced aortic contractions, the 5-HT concentration-response curve was neither depressed nor shifted (37).

5-HT_{2A} and 5-HT_{1B} subtypes have been reported to mediate 5-HT contractions in rabbit epicardial coronary arteries (10). In the presence of the 5-HT_{2A} selective inhibitor ketanserin, the slope of the Schild plot was less than one (0.57) (10). TRIM may have different affinities for 5-HT receptor subtypes, 5-HT_{2A} or 5-HT_{1B}, that mediate vascular smooth muscle contraction. Furthermore, a disproportionate dextral shift suggests to us that TRIM might be an allosteric modulator of 5-HT receptors.

In conclusion, TRIM is an experimental agent for investigation of SOCE; however, its effectiveness may depend on the tissue-specific SOC channel subunit composition. As GPCR agonists activate SOCE indirectly via IP_3-induced store-depletion, TRIM would be valuable in isolating SOCE's contribution in GPCR-mediated responses. The disproportionate rightward shift in the 5-HT concentration-response curves may be caused either by SOCE inhibition and/or allotopic antagonism by TRIM. Modulation of 5-HT receptors by TRIM may be delineated by receptor binding studies.

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