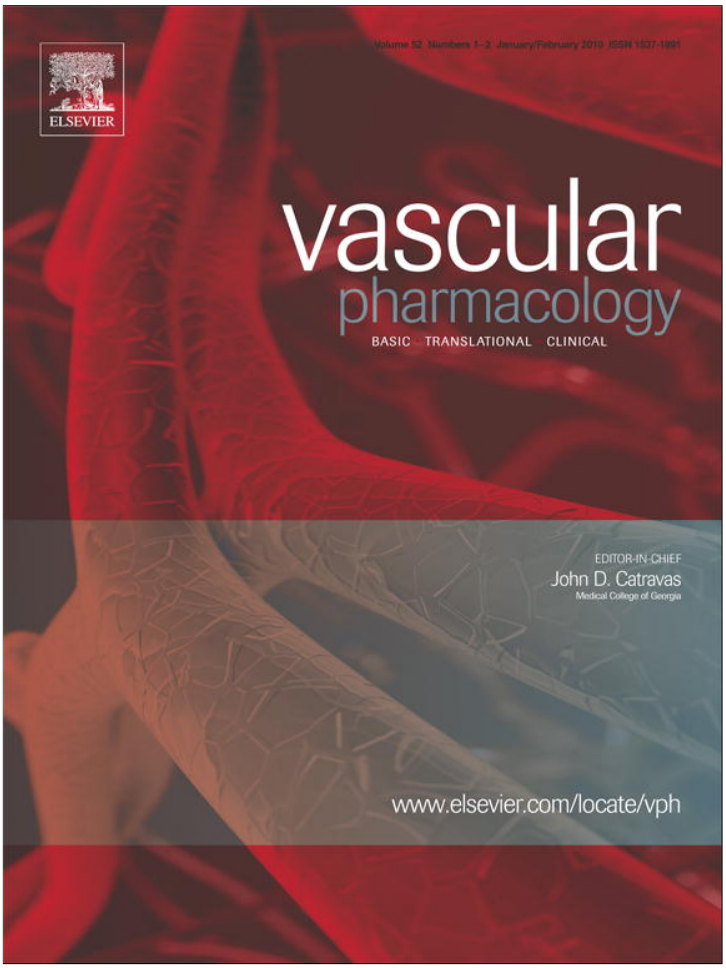

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Vascular activity of a natural diterpene isolated from *Croton zambesicus* and of a structurally similar synthetic trachylobane

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ABSTRACT

The aim of this study was to determine the vasorelaxant activity of a natural diterpene extracted from *Croton zambesicus*, *ent*-18-hydroxy-trachyloban-3-one (DT6), and a synthetic diterpene of similar structure, *ent*-trachyloban-14,15-dione (DT10) in rat aorta. DT6 and DT10 inhibited aorta contraction in a concentration-dependent manner. Both were more potent inhibitors of KCl-evoked contraction than noradrenaline-evoked contraction. Nitric oxide (NO) synthase inhibition did not significantly affect DT6 effect whereas it significantly decreased DT10 inhibitory potency. In fura-2 loaded aorta rings, DT10 simultaneously inhibited KCl-evoked contraction and cytosolic calcium increase in a concentration-dependent manner. Furthermore, DT10 significantly inhibited calcium channel current recorded by the patch-clamp technique in human neuroblastoma cells SH-SY5Y. However, despite potentiation of 8-bromo-cGMP-response, DT6 and DT10 as verapamil depressed acetylcholine-evoked relaxation, DT6 being the most potent, while only DT6 and DT10 depressed SNAP-evoked relaxation. In conclusion, these data suggest that vasorelaxant activity of diterpenes (DT) is associated with the blockade of L-type voltage-operated calcium channels. Inhibition of NO-dependent relaxation by DT could be related to a decrease in NO availability.

1. Introduction

Croton zambesicus Muell. Arg. (*Euphorbiaceae*) is widely used in traditional medicine in Benin, mostly as leaves and roots decoction, to treat hypertension (Adjanohoun et al., 1989; Block et al., 2004). The diterpenoid content is well known in the genus *Croton* (Block et al., 2004). Some trachylobanes and pimaranes have been isolated from the dichloromethane extract of *Croton zambesicus* and have shown vasorelaxant effect in normotensive Wistar rats (Baccelli et al., 2007). Previous observations showed that natural and synthetic diterpenes, the molecular structure of which is relatively similar, depressed rat aorta contractions evoked by KCl-depolarization more potently than contractions evoked by noradrenaline (Baccelli et al., 2007). However, their pharmacological properties have not been studied so far.

The objective of the present study was to investigate the mechanism of the vasorelaxant activity of a natural DT from *Croton zambesicus*, *ent*-18-hydroxy-trachyloban-3-one (DT6) and a synthetic DT, *ent*-trachyloban-14,15-dione (DT10). Their activity on vascular tone, cytosolic calcium concentration, calcium channel current and endothelium function was investigated, and compared to the well-known calcium channel blocker verapamil.

2. Materials and methods

2.1. Diterpenes

ent-18-Hydroxy-trachyloban-3-one or DT6 (Fig. 1) was isolated from the dichloromethane extract of the leaves of *Croton zambesicus* collected in Benin in December 2003 (Baccelli et al., 2007). The synthetic trachylobane, *ent*-trachyloban-14,15-dione or DT10 (Fig. 1) was synthetised as described (Baccelli et al., 2007). The molecular weights of natural and synthetic DT are very close (302.45 for DT6 and 300.44 for DT10).

2.2. Measurement of contractile response of rat aorta

In order to test the vasorelaxant activity of diterpenes (DT), we measured the contractions of isolated thoracic aorta of male Wistar rats (200–350 g) mounted in organ baths. Aortic rings of 2 mm wide were suspended between two hooks under a resting tension of 20 mN in 12.5 ml organ baths filled with physiological solution (composition: see Section 2.5), oxygenated with a gas mixture of 95% O_2 and 5%

Abbreviations: DT, diterpenes; DT6, *ent*-18-hydroxy-trachyloban-3-one; DT10, *ent*-trachyloban-14,15-dione; NNA, N^{ω}-nitro-L-arginine; SNAP, S-nitroso-N-acetyl-D,L-penicillamine; NOS, nitric oxide synthase; VOC, voltage-operated calcium channels.

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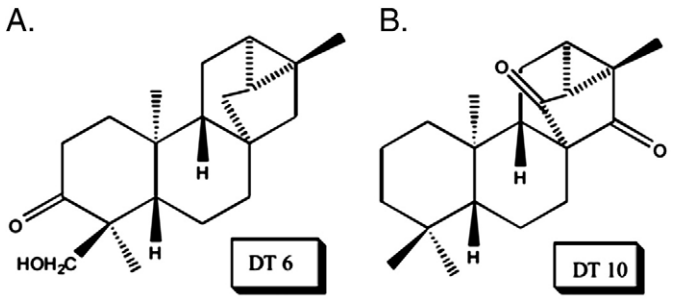

Fig. 1. Molecular structures of the trachylobane diterpenes used in this study. (A) *ent*-18-Hydroxy-trachyloban-3-one (DT6) and (B) *ent*-trachyloban-14,15-dione (DT10).

CO_2 and maintained at 37 °C. Muscle tone was measured with an isometric transducer. After one-hour resting period, physiological solution was changed to a depolarizing 100 mM KCl solution (composition: see Section 2.5) to produce a first contraction. Acetylcholine (1 μM) was added into the bath solution during the plateau phase of the contraction to test the endothelium integrity. After a 60 min resting period in physiological solution, aortic rings were contracted either by changing the physiological solution to a depolarizing KCl (100 mM) solution or by adding noradrenaline (0.5 μM) into the bath solution. The effect of DT on the contraction was tested either by adding cumulative concentrations of DT in the bath during the plateau phase of the contraction, or by testing the contractile response after incubating the aorta in the presence of various concentrations of DT. In the latter protocol, a first contraction was evoked in the absence of drug; DT were thereafter added in the physiological solution for 30 min before a second contraction was evoked in the continuous presence of the drug. To investigate the involvement of nitric oxide (NO) in the effect of the drugs, some experiments were performed in the presence of the NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (NNA, 100 μM).

The relaxation evoked by NO or 8-bromo-cGMP was measured in noradrenaline-contracted artery. At the plateau of the contraction, relaxation was evoked by cumulative addition of increasing concentrations of acetylcholine (10 nM to 10 μM), S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 10 nM to 10 μM), or 8-bromo-cGMP (10 to 100 μM) (Sekhar et al., 1992). The relaxation evoked by SNAP and 8-bromo-cGMP was measured in the presence of NNA, to avoid any influence of endothelium-derived NO.

2.3. Measurement of contractile tension and cytosolic calcium concentration in rat aorta

Cytosolic calcium was measured in endothelium-denuded aortic rings, loaded with 5 μM fura-2 acetoxymethyl ester (fura-2 AM) dissolved in physiological solution containing 0.05% cremophore EL, for 3 h at room temperature. After fura-2 loading period, the aortic ring was suspended in a 3 ml cuvette, which is part of a fluorimeter (CAF110, JASCO, Tokyo), perfused with physiological solution (composition: see Section 2.5) containing 100 μM NNA (Salomone et al., 1995) and gassed with 95% O_2 and 5% CO_2 at 37 ° C for 20 min under a tension of 20 mN. Fluorescence signals at 340 and 380 nm, F_{340}, F_{380}, and ratio F_{340}/F_{380} were recorded simultaneously with contractile tension, measured by an isometric force transducer, by using a data acquisition hardware (MacLab) and data recording software (Chart v3.3, AD Instruments Pty Ltd., Castle Hill, Australia). After washing, the aortic ring was stimulated with 100 mM KCl solution (composition: see Section 2.5). DT were injected into the cuvette at the plateau of KCl contraction. Calcium signal was calibrated at the end of each experiment. Maximal ratio (R_{max}) was obtained by adding ionomycin (10 μM) in KCl solution, while minimal ratio (R_{min}) was measured in the presence of EGTA (2.6 mM). After washing, the autofluorescence of the artery was measured at 340 nm and 380 nm by quenching the fura-2 fluorescence with $MnCl_2$ (6.6 mM). Autofluorescence values were subtracted from experimental values measured at 340 nm and 380 nm in order to calculate cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) by the application of the Grynkiewicz equation, as previously described (Ghisdal et al., 2003; Grynkiewicz et al., 1985).

2.4. Measurement of calcium current in differentiated human neuroblastoma cells SH-SY5Y

Voltage-operated calcium channel (VOC) current was recorded from differentiated human neuroblastoma cells SH-SY5Y, using the patch-clamp technique in the whole-cell configuration. Cells were cultured in Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM:F12) medium supplemented with L-glutamine (2 mM), 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in 5% CO_2 atmosphere. Cells were differentiated in the presence of retinoïc acid (10 μM) during 15 to 19 days, as described (Reuveny and Narahashi, 1993).

Pipettes (resistance: 1–4 MΩ) were pulled and polished using a DMZ-Universal puller (Zeitz Instrument Vertriebs GmbH, Müchen, Germany) and connected to the head stage of a patch-clamp amplifier (Axopatch 200B). Programmed voltage-clamp sequences and data acquisition were performed by specific software (pClamp 9.2) through an A/D–D/A conversion board (Digidata 1322A).

The pipette was filled with low EGTA and high caesium solution, to prevent potassium currents from voltage-gated potassium channels (composition: see Section 2.5) (Cecchi et al., 1987) and cells were continuously perfused with a physiological salt solution (composition: see Section 2.5) containing 1 μM tetrodotoxin in order to block sodium currents (Narahashi et al., 1964).

Cationic current was evoked by 100 ms depolarizing pulses from a holding potential of −100 mV to potentials of −60 mV to 50 mV by steps of 10 mV. After control protocols, DT10 was added to the perfusion solution and the same protocol was applied in the presence of DT10.

2.5. Solutions and drugs

Composition of the physiological solution was (in mM): NaCl, 122; KCl, 5.9; $NaHCO_3$, 15; $MgCl_2$, 1.2; $CaCl_2$, 1.25 and glucose, 11. The KCl solution was composed of (in mM): NaCl, 27; KCl, 100; $NaHCO_3$, 15; $MgCl_2$, 1.2; $CaCl_2$, 1.25 and glucose, 11.

Patch intra-pipette solution contained (in mM): CsCl, 140; $MgCl_2$, 6; Na_2ATP, 5; EGTA, 0.1; HEPES, 10; pH, 7.2 with CsOH. Bath solution composition in mM: NaCl, 130; CsCl, 6; $BaCl_2$, 10; $MgCl_2$, 1.2; glucose, 10; HEPES, 10; pH 7.4 with NaOH; tetrodotoxin 1 μM.

DT were dissolved in dimethyl sulfoxide (DMSO). DMSO, acetylcholine, noradrenaline, NNA, verapamil, EGTA, and cremophore EL were from Sigma-Aldrich (Bornem, Belgium). Mibefradil was from Roche (Switzerland). Fura-2 AM and ionomycin came from Calbiochem (Bierges, Belgium). DMEM-F12 (21331-020), FBS, penicillin, streptomycin, L-glutamine, trypsin-EDTA were from Invitrogen (Merelbeke, Belgium). Tetrodotoxin was from Latoxan (France).

2.6. Analysis

Inhibition of calcium and contractile responses by DT and other drugs was calculated as a percentage of the response measured before addition of inhibitors and was corrected for time-matched controls, which received the same volume of solvent. Results were expressed as mean ± SEM. The drug concentration inhibiting the contractile response by 50% (IC_{50}) was determined by non-linear regression with variable slope factor based on averaged values. Curves were compared using a two-way ANOVA with a Bonferroni post-test. *p*-values lower than 5% indicated significant differences. Patch-clamp results

were analysed by the software Clampfit 9.2. Statistical analysis and graphs were performed with the software GraphPad from Prism.

3. Results

3.1. Diterpenes inhibited rat aorta contraction by blocking voltage-operated calcium channels

Baccelli et al. (2007) showed that maximal tested concentration of DT6 and DT10 relaxed the contraction evoked by KCl (100 mM) depolarization but did not significantly affect noradrenaline contraction of rat aorta. To further characterize the vasorelaxant effect of those DT, concentration–response curves were determined in aorta contracted with KCl or noradrenaline. Synthetic and natural DT (DT10 and DT6 respectively) depressed the KCl-induced contraction in a concentration-dependent manner. DT10 seemed to be more potent than his natural homologous DT6 in the absence of NNA (Fig. 2). NOS inhibition did not significantly affect the effect of DT6 on KCl-evoked contraction whereas NNA significantly decreased the inhibitory potency of DT10 ($-\log IC_{50}$ (M) values were 6.3 ± 0.003 and 5.7 ± 0.01 in the absence of NNA, $p<0.001$, and 5.9 ± 0.006 and 5.7 ± 0.02, in the presence of NNA for DT10 and DT6, respectively).

DT6 and DT10 were more potent inhibitors of KCl-evoked contraction compared to noradrenaline-induced contraction. The relaxation obtained at the highest DT concentration that could be obtained in physiological solution (3 μM), was $29 \pm 2\%$ ($n=8$) and $30\% \pm 2\%$ ($n=3$) for DT10 and DT6, respectively.

The small relaxation observed on noradrenaline-induced contraction and the effective relaxation of KCl-induced contraction suggested that DT could act as calcium channel blockers. Indeed, similar behaviour has been described for verapamil or dihydropyridine derivatives (Morel and Godfraind, 1991). This hypothesis was confirmed by the observation that DT10 was ineffective in aorta contracted by noradrenaline in the presence of 1 μM of verapamil (data not shown).

3.2. DT10 decreased cytosolic calcium level in fura-2 loaded aorta

KCl contraction is associated with an increase in cytosolic calcium caused by the opening of voltage-operated calcium channels in the plasma membrane (Karaki, 2004). In order to determine whether the relaxation of KCl contraction by DT was associated with an inhibition of calcium signal, cytosolic calcium was measured in fura-2 loaded aorta rings. Depolarization of aortic cell membrane by high KCl solution increased the cytosolic calcium concentration from 137 ± 19 to 371 ± 58 nM ($n=19$) and the contractile tension by 17.9 ± 2.5 mN ($n=19$). Addition of DT10 into the bathing solution during the plateau phase of the KCl-response decreased cytosolic calcium and contraction (Fig. 3).

Concentration–response curve of calcium signal inhibition by DT10 (Fig. 3) and contraction were nearly superimposed ($-\log IC_{50}$ for calcium signal inhibition was 6.4 ± 0.03) suggesting that DT10 could act as a calcium channel blocker.

3.3. DT10 inhibited cationic currents in SH-SY5Y

To test the hypothesis that DT could interact with voltage-operated calcium channels, we investigated the effect of DT10 on calcium channel current in human neuroblastoma cells SH-SY5Y by the path-clamp technique in the whole-cell configuration.

In response to depolarizing pulses, differentiated SH-SY5Y cells produced negative inward currents that, in the presence of a sodium channel inhibitor, tetrodotoxin, can be associated with calcium channel current.

Two components could be distinguished in the current (Fig. 4): the first one activated rapidly and was transient, its threshold was around −40 mV and its amplitude was variable; the other component activated and inactivated more slowly, its threshold was about −20 mV and its maximum at 0 mV. *I–V* relationships were determined by current measurement either at 1 or at 10 ms of stimulation (Fig. 4). *I–V* relation measured at 1 ms showed a small shoulder, reflecting the activation of the high threshold, transient component of the current, that disappeared at 10 ms (Fig. 4). This transient component of the current was inhibited by mibefradil whereas the slow component was blocked by verapamil (data not shown). Averaged cell membrane capacitance was 13.4 ± 2.9 pF ($n=11$ cells).

DT10 (3 μM) significantly inhibited the inward current. At 0 mV, the slow component of the current was decreased by $86\% \pm 20\%$ ($n=3$ cells; $p=0.014$). However, the effect of DT10 on the high threshold transient current recorded in response to depolarizing pulses to −30–−40 mV was not significant.

3.4. Diterpenes depressed NO-dependent relaxation

Since the effect of DT10 but not DT6 was sensitive to NNA, interaction of DT with the endothelium function was investigated.

Endothelium-dependent relaxation was evoked by the application of acetylcholine on noradrenaline-contracted artery after 30 min of preincubation in the presence of the tested compound or its solvent. Acetylcholine relaxed noradrenaline contraction in a concentration-dependent manner, with a maximum relaxation reaching 80% of noradrenaline contraction obtained with acetylcholine 10 μM.

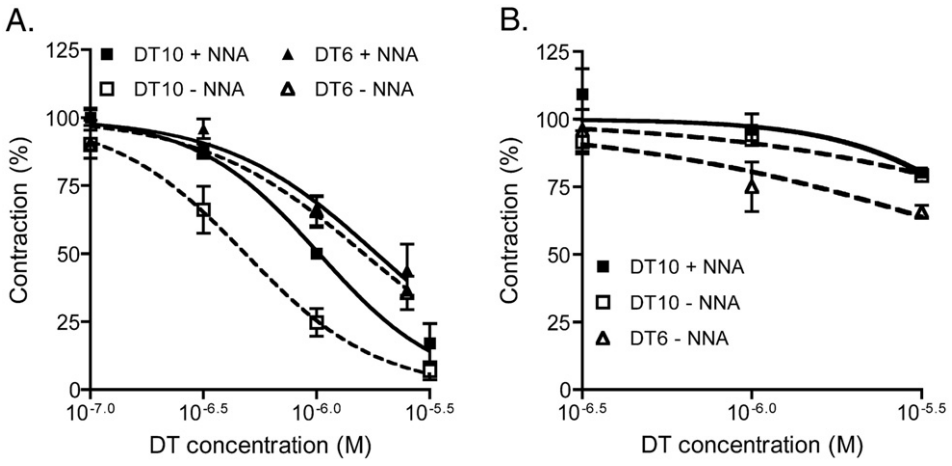

Fig. 2. Effect of natural (DT6) and synthetic (DT10) diterpenes on rat aorta contraction. Concentration–effect curves of DT on KCl-induced (100 mM) contraction (A) or noradrenaline-induced (0.5 μM) contraction (B) with or without NNA (100 μM). Vertical bars indicate the SEM values ($n=2$ to 9 aorta rings).

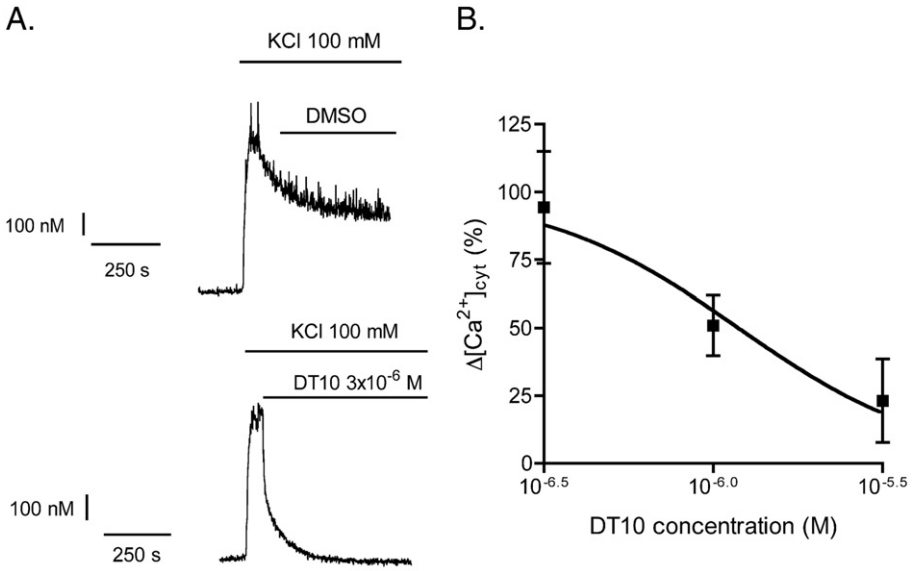

Fig. 3. Effect of DT10 on the increase in cytosolic calcium evoked by 100 mM KCl solution in rat aorta. (A) Typical recordings of the cytosolic Ca^{2+} concentration measured in aortic rings stimulated by KCl. The upper trace shows the effect of the solvent (DMSO), the lower trace shows the effect of DT10 (3 μM) added into the high KCl solution as indicated. (B) Concentration–response curves of DT10 on cytosolic calcium measured 10 min after the addition of DT10 and expressed as % of the calcium concentration measured immediately before the addition of DT10. Values were corrected for time-matched controls. Vertical bars indicate the SEM values ($n = 3$ aorta rings).

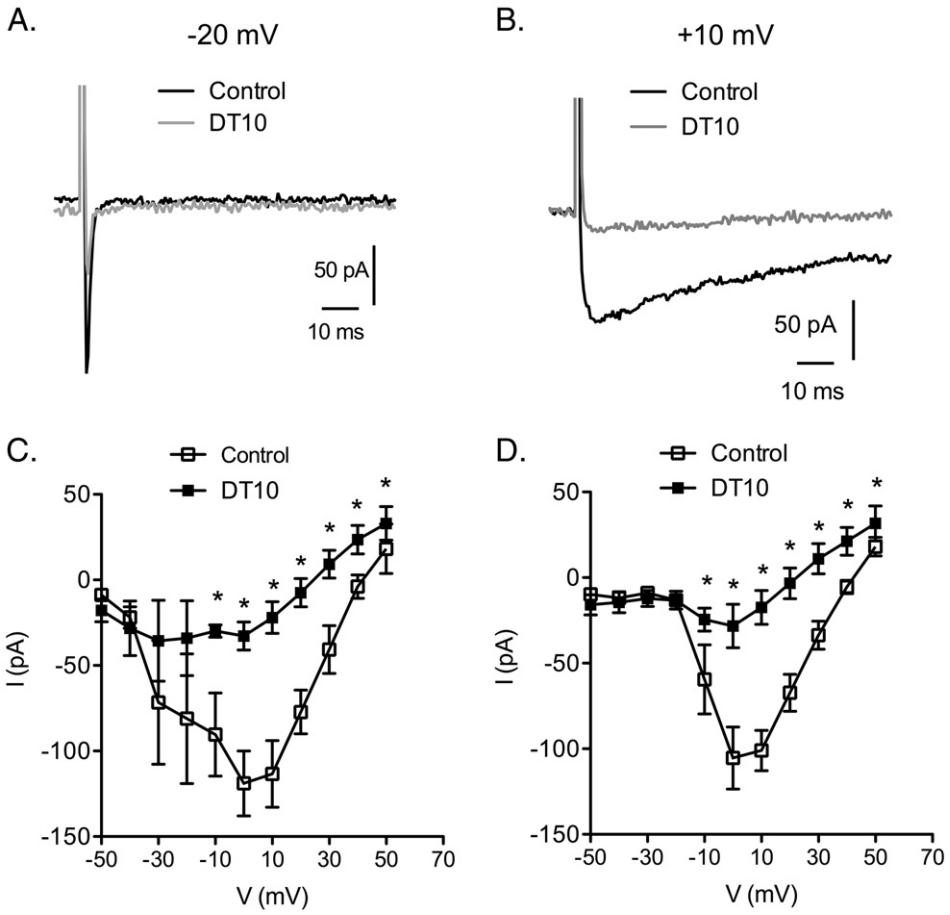

Fig. 4. Effect of DT10 on calcium channel current in SH-SY5Y cells. (A–B): Typical records of the current evoked by pulses to −20 mV (A) and 10 mV (B) from a holding potential of −100 mV in the presence of tetrodotoxin before (control, black line) and after perfusion with DT10 (3 μM, grey line). (C): Current–voltage relationship of the transient current measured at 1 ms in the absence and in the presence of DT10 (3 μM). (D): Current–voltage relationship of the sustained current measured at 10 ms in the absence and in the presence of DT10 (3 μM). For (C) and (D), the holding potential was −100 mV. *indicates a significant difference compared to control value ($p<0.05$). Vertical bars indicate the SEM values ($n = 3$ cells).

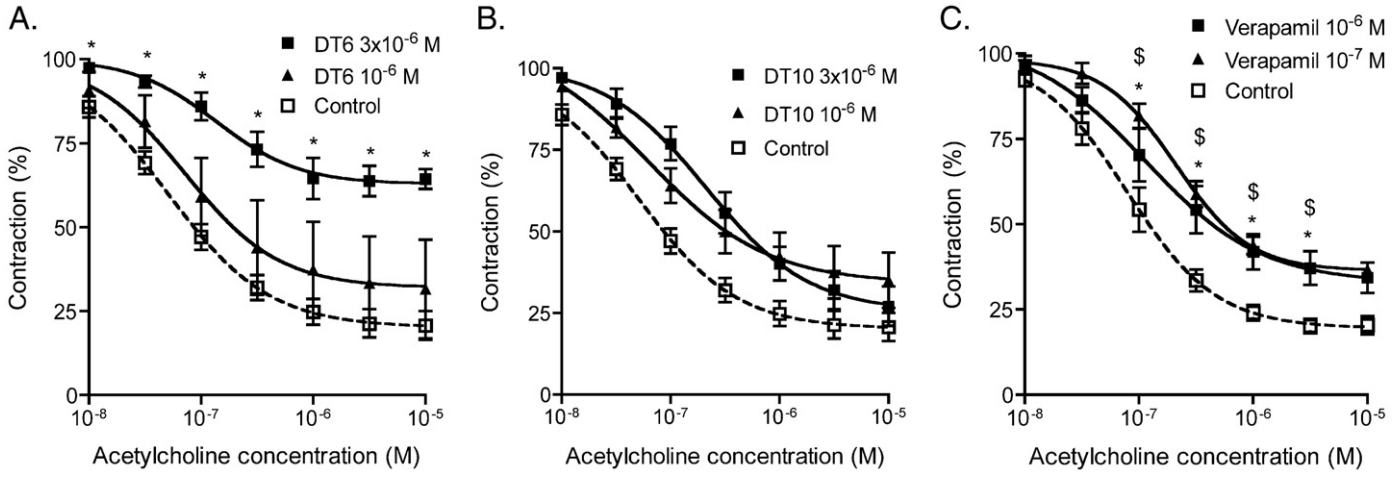

Fig. 5. Acetylcholine-evoked relaxation of noradrenaline contraction. Relaxation was evoked by cumulative addition of acetylcholine on noradrenaline-induced (0.5 μM) contraction of rat aorta in the presence of different concentrations of (A) DT6 ($n=3$), (B) DT10 ($n=5$ to 7) and (C) verapamil ($n=4$). Control relaxations were measured in the presence of the same volume of solvent (dashed line and open symbols). Vertical bars indicate the SEM values. * or $ indicates a significant difference compared to the control value ($p<0.05$).

Acetylcholine-evoked relaxation was completely abolished in the presence of NNA (not shown). In arteries preincubated with DT6 or DT10 (1–3 μM), acetylcholine concentration–response curve was shifted to the right and maximum relaxation was depressed. Relaxation induced by acetylcholine was more affected by DT6 than by DT10 (Fig. 5A and B). Moreover, DT10 effect was not different at 1 and 3 μM, whereas DT6 effect appeared to be dependent on its concentration. To determine whether this effect was a property of DT, or was shared by other calcium channels blockers, we tested the effect of verapamil on relaxation evoked by acetylcholine. Arteries were incubated with concentrations of verapamil, which inhibit KCl-contraction by about 90% (Salomone et al., 1996). Results showed that verapamil also shifted the acetylcholine-evoked relaxation curve to the right and maximum relaxation was depressed (Fig. 5C). As observed in the presence of DT10, verapamil effect was not dependent on its concentration.

In order to further investigate the interaction of DT with NO, we tested the relaxation of noradrenaline-contraction evoked by the NO donor SNAP in the presence of NNA. In the presence of DT6 or DT10, concentration–effect curves of SNAP were displaced to the right compared to the control curve (Fig. 6A and B). It is important to note that the effect of DT on SNAP-evoked relaxation was conversely related to their concentration: the lower concentration produced a greater inhibition of the relaxation. As shown on Fig. 6C, verapamil did not significantly affect the relaxation to SNAP. This effect also was not concentration-dependent, same effect being observed with verapamil 0.1 and 1 μM (Fig. 6C).

3.5. Diterpenes potentiated cGMP-evoked relaxation

NO-evoked relaxation is known to be mediated by the activation of guanylate cyclase and by an increased production of cGMP. To investigate whether DT and verapamil could affect the relaxation evoked by cGMP, we tested the effect of the lipophilic derivative of cGMP, 8-bromo-cGMP in the presence of NNA. Fig. 7 shows that in arteries preincubated in the presence of DT, 8-bromo-cGMP-evoked relaxation was markedly potentiated. A similar effect was observed in the presence of verapamil (Fig. 7C).

4. Discussion

The results reported in the present study show that (1) vasorelaxation evoked by DT is associated with inhibition of voltage-operated calcium channels, (2) DT as verapamil potentiate the relaxation evoked by 8-bromo-cGMP and (3) DT6 and DT10 decrease NO availability.

Experimental evidence suggests that DT interact with voltage-operated calcium channels: (1) they inhibited KCl-evoked contraction

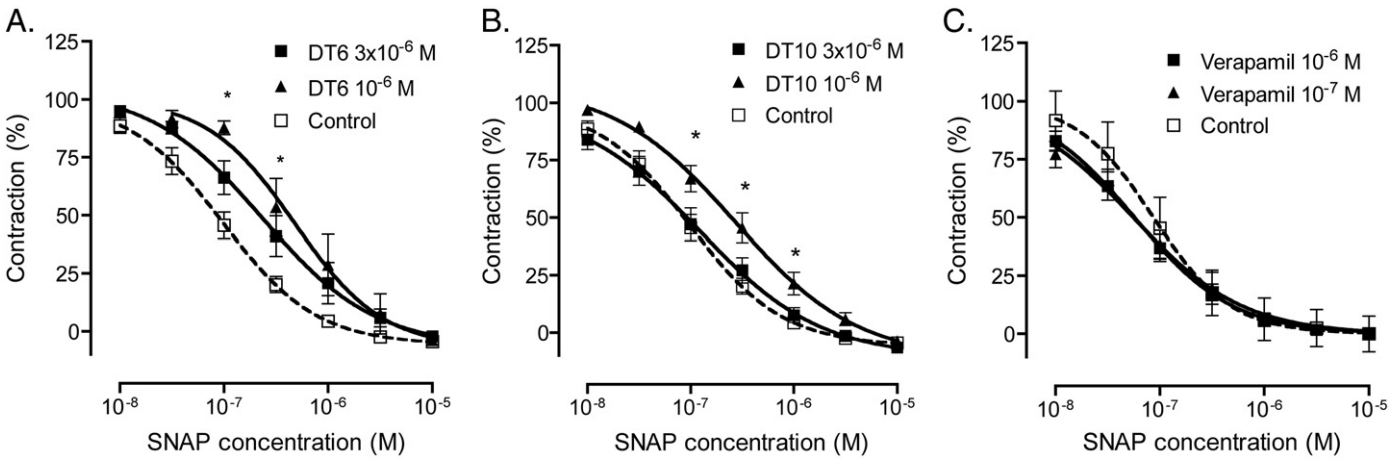

Fig. 6. SNAP-evoked relaxation of noradrenaline contraction. SNAP relaxation was induced by cumulative addition of SNAP on noradrenaline-evoked (0.5 μM) contraction of rat aorta in the presence of NNA and (A) DT6 ($n=3$) (B) DT10 ($n=7$ to 8) and (C) verapamil ($n=8$). Control relaxation (dashed line and open symbols) was measured in the presence of the same volume of solvent. Vertical bars indicate the SEM values. *indicates a significant difference compared to the control value ($p<0.05$).

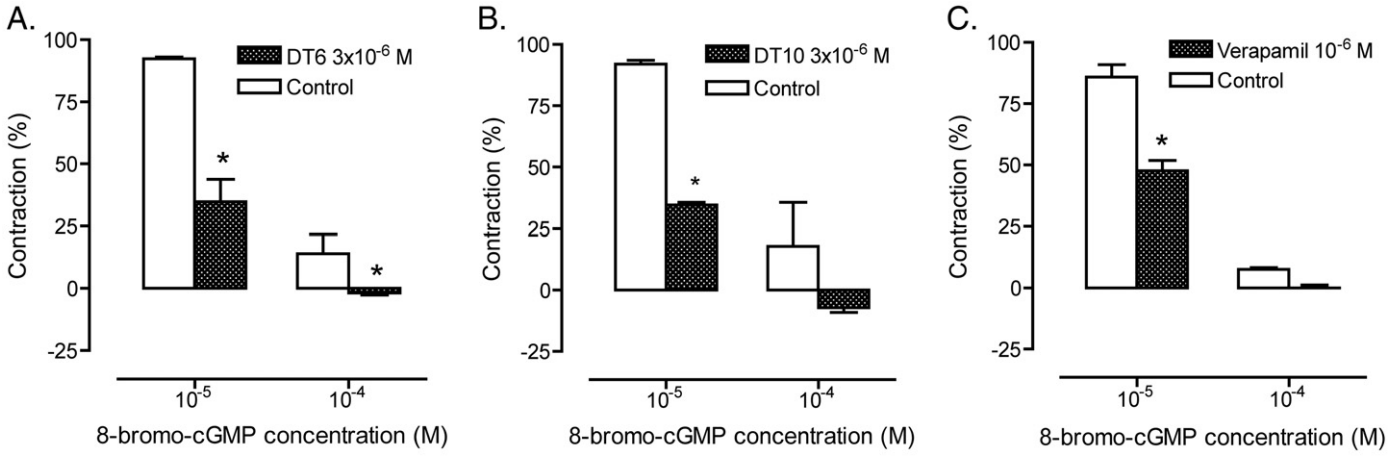

Fig. 7. 8-Bromo-cGMP-evoked relaxation of noradrenaline contraction. Relaxation was evoked by cumulative addition of 8-bromo-cGMP on noradrenaline-induced (0.5 μM) contraction of rat aorta in the presence of NNA and (A) DT6 ($n=3$), (B) DT10 ($n=3$) and (C) verapamil ($n=3$). Control relaxations were measured in the presence of the same volume of solvent. Vertical bars indicate the SEM values. *indicates a significant difference compared to the control value ($p<0.0001$).

more potently than noradrenaline-evoked contraction, (2) this inhibition was associated with a decrease in cytosolic calcium concentration and (3) DT10 inhibited L-type voltage-operated calcium channel current in neuroblastoma cells.

It is well known that an increase in potassium concentration in the extracellular medium of smooth muscle cells induces a sustained contraction as a result of the activation of L-type voltage-operated calcium channels in the plasma membrane following cell membrane depolarization (Karaki, 2004). The opening of calcium channels increases the intracellular calcium leading to smooth muscle cells contraction (Karaki, 2004). Agonists such as noradrenaline, induce the contraction of smooth muscle cells by binding to a specific receptor coupled to a G protein in the plasma membrane, which activates an intracellular signalling pathway leading to contraction (Somlyo and Somlyo, 2000; Webb, 2003). The increase in cytosolic calcium then results from the release of calcium from intracellular stores and from calcium entry through voltage-dependent and independent calcium channels (Webb, 2003). In rat aorta, blockade of VOC with dihydropyridine derivatives or verapamil inhibits the contraction evoked by noradrenaline by 30–40% (Ghisdal et al., 2003; Morel and Godfraind, 1991). Thus, the full inhibition of the contraction and the increase in cytosolic calcium evoked by KCl solution, and the partial inhibition of the noradrenaline-evoked contraction observed in rat aorta in the presence of DT is in agreement with the blockade of VOC by these compounds. This was confirmed by patch-clamp data showing that 1 μM DT10 depressed the calcium current by 86% in neuroblastoma cells SH-SY5Y. Differentiated SH-SY5Y cells express different types of calcium channels, mainly L and N subtypes (Reuveny and Narahashi, 1993). We identified two components in the inward current activated by depolarizing pulses in SH-SY5Y: the fast transient component had a high voltage of activation and was blocked by mibefradil, suggesting that it could be attributed to T-type calcium channels. The sustained component of the current, which activated at more positive voltage and was sensitive to verapamil, is associated with L-type calcium channels. The latter was blocked by DT10.

The inhibitory potency of DT6 on KCl-evoked contraction was unaffected by NO blockade, on the opposite to the decreased potency of DT10 observed in the presence of NNA. The potentiation of the vasorelaxant effect of calcium channels blockers by NO has been reported (Salomone et al., 1996). It is ascribed to the synergy of the effects of increased release of cGMP and calcium channels blockade on the contraction (Salomone et al., 1995). However, despite the potentiation of cGMP-evoked relaxation by DT and verapamil, all three compounds depressed the vasorelaxation evoked by endothelium-derived NO, although to various extent. Such an effect of verapamil has never been described. The inhibition of endothelial NO-dependent relaxation by either DT compounds or verapamil cannot be ascribed to the blockade of L-type calcium channels, as this class of channels are not found on endothelial cells (Adams et al., 1989). Direct interaction of DT with the muscarinic receptor was probably not involved in this effect, since DT similarly depressed the relaxation evoked by the calcium ionophore A23187 (data not shown), or by the NO donor SNAP.

Decreased relaxation to acetylcholine could be part of a negative feedback mechanism by which cGMP attenuates the production of NO by endothelial cells by depressing the increase in endothelial cytosolic Ca^{2+} (Yao and Huang, 2003). Such a mechanism could be amplified in the presence of verapamil and DT. Alternatively, interaction of verapamil and DT with endothelial ion channels cannot be excluded: verapamil has been reported to block Kv channels, in particular HERG channels (Zhang et al., 1999) and Kv1.3 channels (DeCoursey, 1995; Robe and Grissmer, 2000), which are expressed in rat brain endothelial cells (Millar et al., 2008). A role of Kv channels in the regulation of NO production by endothelial cells is suggested by the inhibition of the Ca^{2+} signal activated by acetylcholine in endothelial cells in the presence of the Kv blocker 4-aminopyridine (Ghisdal and Morel, 2001). Intracellular calcium channels might constitute another target of ion channel blockers as inhibition of NO production by endothelial cells through the blockade of calcium release-activated calcium channels has been reported for tetrandrine, a plant alkaloid with calcium channel blocking properties (Kwan et al., 2001). Further investigation will be required to determine the precise mechanism of this effect.

The alteration in the acetylcholine-evoked NO production and release by endothelial cells might not be the only cause for the conflicting effects of DT on the relaxation evoked by endothelial NO and 8-bromo-cGMP. This contradictory observation might also be related to the depression of the effect of exogenous NO measured in the presence of DT, which suggests that these compounds could decrease the availability or the potency of NO at the level of its muscular target and simultaneously potentiate the effect of cGMP. These two opposite actions explain the puzzling observation that the inhibitory effect of DT on SNAP-evoked relaxation was not directly concentration-dependent: the inactivation of NO could be observed at lower concentrations of the antagonist, and thereafter be overwhelmed by the potentiation of the vasorelaxant effect of cGMP.

The vascular activity of DT6 and DT10 was quite similar except the more pronounced inhibition of acetylcholine-evoked relaxation by DT6 than DT10. The latter effect could be related to the larger decrease

in NO-evoked relaxation in the presence of DT6, which might be caused by a higher potency of NO inactivation. Marked inactivation of NO-evoked relaxation in the presence of DT6 could explain the lack of effect of NNA on the vasorelaxant activity of DT6.

In conclusion, the present data indicate that the vasorelaxant effect of DT10 and DT6 is associated with the inhibition of L-type voltage-operated calcium channels and potentiation of the relaxant effect of cGMP, properties that they share with verapamil. However, the decrease in NO-evoked relaxation could reduce the vasorelaxant potentiality of these compounds.

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References

Adams, D.J., Barakeh, J., Laskey, R., Van Breemen, C., 1989. Ion channels and regulation of intracellular calcium in vascular endothelial cells. FASEB J. 3, 2389–2400.

Adjanohoun, E.J., Adjakidje, V., de Souza, S., 1989. Contribution aux études ethnobotaniques et floristiques en République Populaire du Bénin. Agence de Coopération Culturelle et Technique, Paris, France.

Baccelli, C., Navarro, I., Block, S., Abad, A., Morel, N., Quetin-Leclercq, J., 2007. Vasorelaxant activity of diterpenes from Croton zambesicus and synthetic trachylobanes and their structure–activity relationships. J. Nat. Prod. 70, 910–917.

Block, S., Baccelli, C., Tinant, B., Van Meervelt, L., Rozenberg, R., Habib Jiwan, J.L., Llabres, G., De Pauw-Gillet, M.C., Quetin-Leclercq, J., 2004. Diterpenes from the leaves of *Croton zambesicus*. Phytochemistry 65, 1165–1171.

Cecchi, X., Wolff, D., Alvarez, O., Latorre, R., 1987. Mechanisms of Cs+ blockade in a Ca^{2+}-activated K^+ channel from smooth muscle. Biophys. J. 52, 707–716.

DeCoursey, T.E., 1995. Mechanism of K^+ channel block by verapamil and related compounds in rat alveolar epithelial cells. J. Gen. Physiol. 106, 745–779.

Ghisdal, P., Morel, N., 2001. Cellular target of voltage and calcium-dependent K(+) channel blockers involved in EDHF-mediated responses in rat superior mesenteric artery. Br. J. Pharmacol. 134, 1021–1028.

Ghisdal, P., Vandenberg, G., Morel, N., 2003. Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries. J. Physiol. 551, 855–867.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Karaki, H., 2004. Historical techniques: cytosolic Ca^{2+} and contraction in smooth muscle. Trends Pharmacol. Sci. 25, 388–393.

Kwan, C.Y., Leung, Y.M., Kwan, T.K., Daniel, E.E., 2001. Tetrandrine inhibits Ca^{2+} release-activated Ca^{2+} channels in vascular endothelial cells. Life Sci. 68, 841–847.

Millar, I.D., Wang, S., Brown, P.D., Barrand, M.A., Hladky, S.B., 2008. Kv1 and Kir2 potassium channels are expressed in rat brain endothelial cells. Pflugers Arch. 456, 379–391.

Morel, N., Godfraind, T., 1991. Characterization in rat aorta of the binding sites responsible for blockade of noradrenaline-evoked calcium entry by nisoldipine. Br. J. Pharmacol. 102, 467–477.

Narahashi, T., Moore, J.W., Scott, W.R., 1964. Tetrodotoxin blockage of sodium conductance increase in Lobster giant axons. J. Gen. Physiol. 47, 965–974.

Reuveny, E., Narahashi, T., 1993. Two types of high voltage-activated calcium channels in SH-SY5Y human neuroblastoma cells. Brain Res. 603, 64–73.

Robe, R.J., Grissmer, S., 2000. Block of the lymphocyte K(+) channel mKv1.3 by the phenylalkylamine verapamil: kinetic aspects of block and disruption of accumulation of block by a single point mutation. Br. J. Pharmacol. 131, 1275–1284.

Salomone, S., Morel, N., Godfraind, T., 1995. Effects of 8-bromo cyclic GMP and verapamil on depolarization-evoked Ca^{2+} signal and contraction in rat aorta. Br. J. Pharmacol. 114, 1731–1737.

Salomone, S., Silva, C.L., Morel, N., Godfraind, T., 1996. Facilitation of the vasorelaxant action of calcium antagonists by basal nitric oxide in depolarized artery. Naunyn-Schmiedebergs Arch. Pharmacol. 354, 505–512.

Sekhar, K.R., Hatchett, R.J., Shabb, J.B., Wolfe, L., Francis, S.H., Wells, J.N., Jastorff, B., Butt, E., Chakinala, M.M., Corbin, J.D., 1992. Relaxation of pig coronary arteries by new and potent cGMP analogs that selectively activate type I alpha, compared with type I beta, cGMP-dependent protein kinase. Mol. Pharmacol. 42, 103–108.

Somlyo, A.P., Somlyo, A.V., 2000. Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. J. Physiol. 522 (Pt 2), 177–185.

Webb, R.C., 2003. Smooth muscle contraction and relaxation. Adv. Physiol. Educ. 27, 201–206.

Yao, X., Huang, Y., 2003. From nitric oxide to endothelial cytosolic Ca^{2+}: a negative feedback control. Trends Pharmacol. Sci. 24, 263–266.

Zhang, S., Zhou, Z., Gong, Q., Makielski, J.C., January, C.T., 1999. Mechanism of block and identification of the verapamil binding domain to HERG potassium channels. Circ. Res. 84, 989–998.