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Analysis of Rabbit Vascular Responses to DBI, an Ingol Derivative Isolated from *Euphorbia canariensis*

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Abstract

We have analysed the effects of 7,12-*O*-diacetyl-8-*O*-benzoil-2,3-di*epi*ingol (DBI), an ingol derivative isolated from *E. canariensis*, on isometric tension developed by isolated rabbit basilar and carotid arteries.

Concentration–response curves to DBI ($10^{-8}-3 \times 10^{-5}$ M) were obtained cumulatively in both arteries at resting tension and active tone (KCl, 50 mM). At resting tension, DBI induced a concentration-dependent contraction, which was not inhibited in Ca^{2+}-free medium. H7 (1-(5-isoquinoline sulphonyl)-2-methylpiperazine dichloride) (10^{-4} M) inhibited the DBI-induced contraction both in basilar and in carotid arteries. Calmidazolium (10^{-4} M) inhibited the maximum contraction of the carotid artery to DBI, and completely abolished the response in the basilar artery. In pre-contracted basilar arteries DBI induced a concentration-dependent relaxation that was not modified by incubation with N^G-nitro-L-arginine (L-NOARG; 10^{-5} M) or indomethacin (10^{-5} M). In the carotid artery with active tone DBI induced further contractions, which were not significantly modified by L-NOARG (10^{-5} M) and were potentiated by indomethacin (10^{-5} M).

These results suggest that DBI contracts rabbit basilar and carotid arteries by a mechanism that is independent of extracellular Ca^{2+} and involves the participation both of protein kinase C and of calmodulin. DBI relaxes basilar but not carotid arteries by a mechanism independent of the liberation of nitric oxide and prostacyclin. In the carotid artery prostacyclin but not nitric oxide partially counteracts the contractile action of DBI.

The variety of biological actions of natural diterpenes is a matter of increasing interest. Diterpenes isolated from Verbenaceae have cytotoxic activity against carcinoma cells in man and rodents (Habtemariam 1995). Therapeutic properties in the treatment of patients with gastric ulcers, because of the capacity of the compounds to promote epithelial regeneration of gastric mucosa, have been also described (Shirakabe et al 1995). Some diterpenoids have immunosuppressive and anti-rheumatic activity (Gu et al 1995) or are effective in protecting biological systems against oxidative stress (Haraguchi et al 1995). Diterpenes derived from Euphorbiaceae have, on the other hand, been reported to have vascular effects. Phorbol esters can produce slow-developing sustained contraction of cat (Salaíces et al 1990) and canine (Sugawa et al 1991) cerebral arteries by a mechanism that implies the activation of protein kinase C. Jatrophone, a diterpene isolated from some *Jatropha* spp. (Euphorbiaceae), can induce relaxation of the rat aorta (Duarte et al 1992) and portal vein (Silva et al 1995).

The aim of this investigation was to study the contractile or relaxant response of isolated rabbit basilar and common carotid arteries to 7,12-*O*-diacetyl-8-*O*-benzoil-2,3-diepiingol (DBI, Fig. 1), a diterpene isolated from the latex of *Euphorbia canariensis*. We also studied the mechanism of action of this compound by investigating the participation of extracellular calcium, protein kinase C, calmodulin, nitric oxide (NO) and prostacyclin in those responses.

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Materials and Methods

Drugs and solutions

DBI was isolated from the latex of *Euphorbia canariensis* L. in the Department of Organic Chemistry of the University of Valencia (Professor J. A. Marco). Briefly, the latex (ca 90 g), collected in Malpais de Güimar, Tenerife, Canary Islands, in May 1993, was dissolved in hot methanol (700 mL). Cooling to room temperature resulted in a voluminous, white precipitate of common triterpenes which was eliminated by filtration. Evaporation of the solvent in vacuo gave a whitish, oily material. This oil was dissolved in the minimum amount of methanol and reversed-phase silica gel RP-2 was added (3 g of silica gel g^{-1} extracted). The solvent was then totally eliminated in vacuo. The powdery material obtained was placed on the top of an open chromatographic column packed with silica gel RP-2 and eluted under a slight argon pressure (1·5–2 atm) first with water, then with methanol–water 70 : 30

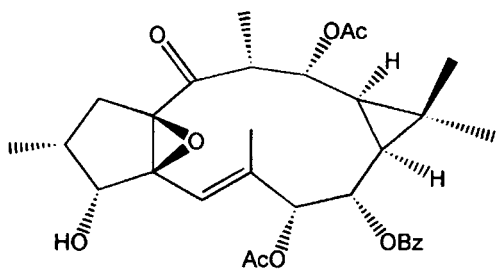

FIG. 1. The chemical structure of DBI (7,12-*O*-diacetyl-8-*O*-benzoil-2,3-di*epi*ingol).

and finally with methanol. Column chromatography of the 70:30 methanol-water fraction (elution with a gradient of hexane–Et_2O 10:1 to Et_2O), followed when necessary by preparative TLC or HPLC, or both, of the intermediate fractions, enabled isolation of DBI. H7 (dihydrochloride), calmidazolium (compound R24571) and indomethacin were obtained from Sigma; L-NOARG was from Peptide Institute Inc.

DBI and indomethacin were dissolved in ethanol and the solution was diluted with twice-distilled water. Calmidazolium was dissolved in DMSO. H7 and L-NOARG were dissolved and diluted in twice-distilled water.

The composition of the modified Ringer-Locke solution was (mM): NaCl, 120; KCl, 5·0; $CaCl_2 \cdot 2H_2O$, 2·2; $MgCl_2 \cdot 7H_2O$, 1·0; $NaHCO_3$, 24; and glucose, 5·6. In KCl-depolarizing solution NaCl was replaced by an equimolar amount of KCl. The Ca^{2+}-free solution was prepared by omitting $CaCl_2$.

Isometric tension recording

New Zealand white rabbits, 2·5–3·5 kg, previously anaesthetized with sodium thiopental (sodium pentothal, Abbott; 2% i.v.), were killed by injection of potassium chloride (10 mEq, i.v.). The whole brain was rapidly removed. The basilar and one common carotid artery were dissected free and cut into cylindrical segments measuring 3 and 4 mm in length, respectively. Each segment was prepared for isometric tension recording in a 5-mL organ bath. Briefly, two L-shaped stainless steel pins, 89 mm and 125 mm in diameter for basilar and carotid arteries respectively, were introduced through the arterial lumen. One pin was fixed to the organ-bath wall and the other was connected to a strain gauge for isometric-tension recording. The vascular preparations were maintained at 37°C in modified Ringer-Locke solution oxygenated with 95% O_2 and 5% CO_2 to give a pH of 7·3–7·4. A resting tension of 0·5 g (basilar) and 2 g (carotid) was applied to the arterial segments and they were allowed to equilibrate for 60–90 min. Tension was readjusted when necessary and the bath fluid was changed every 15 min. After this period of equilibration the reactivity of the arterial segments was checked by depolarization with KCl 50 mM; arteries with a contraction of less than 500 mg (basilar) or 1500 *g* (carotid) were discarded.

Concentration–response curves to DBI ($10^{-8} - 3 \times 10^{-5}$ M) were obtained cumulatively in basilar and common carotid arteries at both resting tension and active tone (KCl, 50 mM).

Experiments in arterial segments at resting tension

The involvement of extracellular Ca^{2+} in the response to stimulation with DBI was studied by obtaining responses to the compound in resting-tension arteries after incubation (20 min) in Ca^{2+}-free medium. The involvement of protein kinase C was studied by obtaining concentration–response curves to DBI after incubation (20 min) with H7 (10^{-4} M), a relatively specific inhibitor of protein kinase C. The involvement of calmodulin was explored by obtaining concentration-response curves to DBI after incubation (20 min) with calmidazolium (10^{-4} M), an inhibitor of calmodulin.

Experiments in arterial segments at active tone

The involvement of NO in the arterial response to DBI was analysed by obtaining concentration–response curves to this diterpene after incubation (20 min) with N^G-nitro-L-arginine (L-NOARG 10^{-5} M), an inhibitor of NO synthesis. The involvement of prostacyclin was assessed by measuring arterial response to DBI after incubation (20 min) with indomethacin (10^{-5} M), an inhibitor of cyclooxygenase.

Statistical analysis

Contractile responses to DBI obtained with arteries at resting tension were expressed as a percentage of the previous contraction induced with 50 mM KCl. In pre-contracted arteries, contractile and relaxant values were expressed as a percentage of the active tone. Only one concentration–response curve was obtained for each arterial segment. For each concentration–response curve the maximum effect (E_{max}) and the concentration of the drug which produced half of E_{max} (ED50) were calculated. Mean ED50 and its confidence limits (95% interval) were calculated by obtaining the mean and the confidence limits of $-\log$ ED50 values because they conformed to a normal distribution.

The mean, standard deviation and standard error of the mean (s.e.m.) were calculated from all the contraction values obtained in each experiment. Statistical analysis of the different concentration–response curves was achieved by applying the Student-Newman-Keuls test, both to $-\log$ ED50 and to E_{max} values. A probability value of less than 5% was considered significant.

Results

Arteries at resting tension

Cumulative addition of DBI ($10^{-8} - 3 \times 10^{-5}$ M) induced a slowly developing, sustained, concentration-related contraction of basilar and carotid arteries at resting tone (Fig. 2). It took about 3–4 h to complete the concentration–response curves. The E_{max} values of the concentration–response curve to DBI for the carotid artery were higher than those obtained for the basilar artery, without significant changes in the ED50 values. Incubation of arterial segments in Ca^{2+}-free medium did not significantly modify the contractile response to DBI in basilar arteries, but significantly potentiated it in carotid arteries, with an E_{max} significantly higher and an ED50 not significantly different from values obtained with control conditions (Fig. 2).

H7 (10^{-4} M) significantly inhibited the contractile response of basilar and carotid arteries to DBI, affecting both ED50 and E_{max} in basilar arteries and the ED50 value only in carotid arteries (Fig. 2). Pre-treatment with calmidazolium (10^{-4} M) completely abolished the DBI-induced contraction of the basilar artery and inhibited the E_{max} of the concentration–response curve to DBI for the carotid artery (Fig. 2).

Arteries with active tone

DBI ($10^{-8} - 3 \times 10^{-5}$ M) induced a slowly developing, sustained, concentration-dependent relaxation of pre-contracted basilar arteries. As for resting tension, the time necessary to complete the concentration–response curves was 3–4 h. This effect was not significantly modified by L-NOARG (10^{-5} M) nor indomethacin (10^{-5} M) (Fig. 3a).

In pre-contracted carotid artery (Fig. 3b), DBI ($10^{-8} - 3 \times 10^{-5}$ M) induced further contraction, which reached a maximum when the concentration of DBI reached 3×10^{-6} M; at higher concentrations a discrete loss of tension was recorded. Indomethacin (10^{-5} M) enhanced the E_{max} of

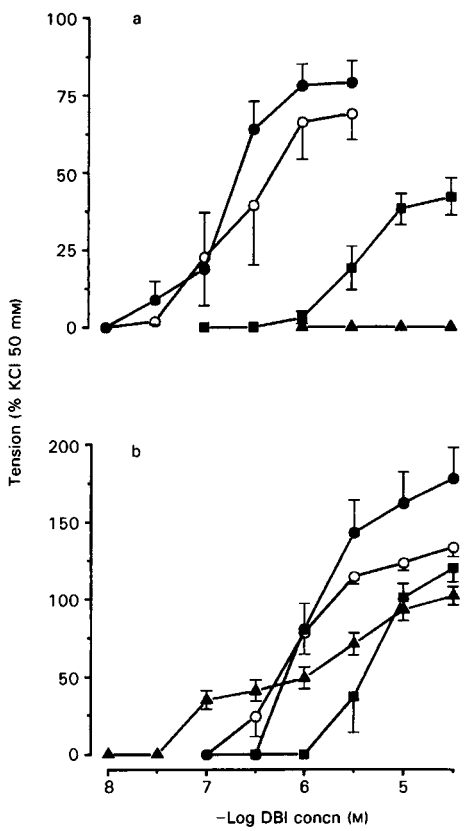

FIG. 2. Concentration–response curves for the action of DBI on isolated rabbit basilar (a) and common carotid (b) arteries under control conditions (○) and after incubation in Ca^{2+}-free medium (●), with H7 (10^{-4} M, ■) or with calmidazolium (10^{-4} M, ▲). Tension values are expressed as percentage of the maximum effect reached by previous depolarization with KCl (50 mM).

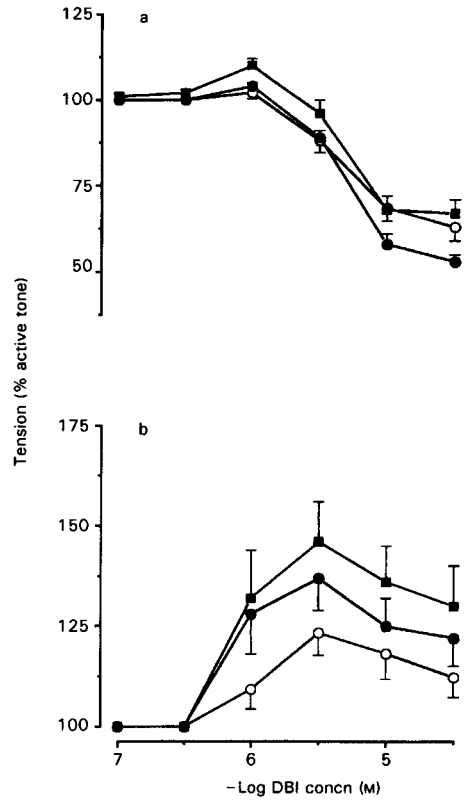

FIG. 3. Concentration–response curves for the action of DBI on pre-contracted (KCl 50 mM) isolated rabbit basilar (a) and common carotid (b) arteries under control conditions (○) and after incubation with N^G-nitro-L-arginine (L-NOARG, 10^{-5} M, ●) or indomethacin (10^{-5} M, ■). Tension values (mean ± s.e.m.) are expressed as percentage of the active tone (= 100).

the concentration–response curve of carotid arteries to DBI, without changes in the ED50 values. This DBI-induced contraction was not significantly affected by L-NOARG (10^{-5} M).

Neither L-NOARG nor indomethacin induced any significant change in the active tone of basilar and carotid arterial segments. Table 1 summarizes the E_{max} and ED50 values of the concentration–response curves to DBI obtained under the different experimental conditions described above.

Discussion

Several studies have demonstrated the vascular effects of some compounds isolated from Euphorbiaceae (Salaíces et al 1990; Sugawa et al 1991). The current results show that DBI strongly contracted rabbit basilar and carotid arteries. In the basilar artery the potency of DBI was similar to that in the carotid artery, but the efficacy was lower. In both arteries, the contraction was developed slowly and was sustained, in agreement

Table 1. Values of the maximum effect (E_{max}) and the concentration of the drug producing half the maximum effect (ED50) for concentration-response curves to DBI in isolated rabbit basilar and common carotid arteries either at resting tension or with 50 mM KCl-induced active tone, under different experimental conditions.

	Basilar artery			Carotid artery		
Resting tension	ED50 (M)	E_{max} (%)	n	ED50 (M)	E_{max} (%)	n
Control	$2{\cdot}7$ ($1{\cdot}8$–$4{\cdot}0$) × 10^{-7}	69 ± 8	7	$6{\cdot}6$ ($5{\cdot}1$–$8{\cdot}5$) × 10^{-7}	134 ± 6‡	12
Ca^{2+}-free medium	$1{\cdot}5$ ($1{\cdot}2$–$2{\cdot}0$) × 10^{-7}	79 ± 7	6	$1{\cdot}1$ ($0{\cdot}9$–$1{\cdot}3$) × 10^{-6}	178 ± 20†	6
H7 (10^{-4} M)	$2{\cdot}9$ ($2{\cdot}4$–$3{\cdot}6$) × 10^{-6}†	42 ± 6*	6	$4{\cdot}5$ ($3{\cdot}6$–$5{\cdot}5$) × 10^{-6}†	120 ± 9	6
Calmidazolium (10^{-4} M)	–	0†	4	$8{\cdot}8$ ($5{\cdot}5$–$1{\cdot}4$) × 10^{-7}	102 ± 6†	5
Active Tone	EC_{50} (× 10^{-6} M)	E_{max} (%)	n	EC_{50} (M)	E_{max} (%)	n
Control	$4{\cdot}5$ ($4{\cdot}0$–$5{\cdot}1$)	− 37 ± 4	8	$1{\cdot}3$ ($1{\cdot}2$–$1{\cdot}5$) × 10^{-6}‡	23 ± 5‡	12
L-NOARG (10^{-5} M)	$5{\cdot}0$ ($4{\cdot}9$–$5{\cdot}1$)	− 47 ± 2	8	$8{\cdot}2$ ($7{\cdot}0$–$9{\cdot}7$) × 10^{-7}	37 ± 8	8
Indomethacin (10^{-5} M)	$5{\cdot}1$ ($4{\cdot}8$–$5{\cdot}5$)	− 33 ± 4	8	$9{\cdot}8$ ($8{\cdot}1$–$11{\cdot}7$) × 10^{-7}	46 ± 10*	8

Results are expressed as a percentage of a previous depolarization with 50 mM KCl (resting tension) or as a percentage of active tone. ED50 values are means and confidence limits; E_{max} values are means ± s.e.m. *$P < 0{\cdot}05$, † $P < 0{\cdot}01$, significantly different from corresponding control values; ‡$P < 0{\cdot}01$, significantly different from corresponding values in basilar artery.

with that induced by phorbol esters (Salaíces et al 1990; Sugawa et al 1991). Contractions induced by phorbol esters are achieved, at least in part, by activation of protein kinase C and they did depend (cat) or did not (dog) on the presence of extracellular calcium (Salaíces et al 1990; Sugawa et al 1991). The similarities between DBI and phorbol esters in respect of origin, chemical structure and contractile activity prompted us to study the degree of involvement of extracellular Ca^{2+} and the possibility that the DBI-induced contraction could be partially mediated, as for phorbol esters, by activation of protein kinase C. The elimination of Ca^{2+} from the extracellular medium did not significantly inhibit the contractile effect of DBI, suggesting that these contractions do not imply entrance of extracellular Ca^{2+} into the sarcoplasm. The higher maximum contraction induced by DBI in carotid arteries in a Ca^{2+}-free medium could be explained by the involvement of this ion in the synthesis and release of several substances (i.e. NO, arachidonic acid metabolites, etc.) that could mediate the vascular response (Abdel-Latif 1986; Moncada et al 1991). H7, an inhibitor of protein kinase C (Hidaka et al 1984), significantly reduced the contractile response of these arteries to DBI, suggesting the involvement of protein kinase C in the response.

In this work we also studied the possibility that calmodulin was involved in the contractile response of rabbit basilar and carotid arteries to DBI. The lack of involvement of Ca^{2+}–calmodulin complex in the contraction induced by phorbol 12,13-diacetate in the canine basilar artery has been reported by Sugawa et al (1991). The calmodulin antagonist calmidazolium (Van Belle 1981) inhibited the maximum contraction induced by DBI in carotid arteries and completely abolished the DBI-induced response in the basilar artery. These results indicate that DBI contracts basilar and carotid arteries by a mechanism that implies the activation of both protein kinase C and Ca^{2+}-calmodulin system.

The possibility that arterial segments had a relaxant response to DBI, as has been reported for jatrophone with rat aorta (Duarte et al 1992) or portal vein (Silva et al 1995), has been also investigated. Concentration–response curves to DBI were obtained for basilar and carotid arteries pre-contracted with KCl. The response of the two arteries tested was completely different. Whereas concentration-related relaxations to DBI were observed for the basilar artery, further contractions were obtained for the carotid artery. We have also examined the possibility that NO or prostacyclin could mediate the relaxant action of DBI in basilar arteries, or could even modulate its contractile action in carotid arteries, as has been described for other vascular beds with several vasoactive substances (Miranda et al 1993; Toda et al 1993). Neither the inhibitor of NO-synthesis L-NOARG (Moore et al 1990) nor the inhibitor of prostacyclin–synthesis indomethacin (Moncada & Vane 1979) modified the relaxant effect of DBI on rabbit basilar artery, indicating that this relaxant action is not mediated by NO or prostacyclin. L-NOARG did not significantly modify the DBI-induced contractile response of the carotid artery, but in the presence of indomethacin this contraction was higher. Because the active tone of arterial segments was not modified by indomethacin, it is reasonable to believe that the release of prostacyclin in pre-contracted carotid arteries is stimulated by DBI, partially counteracting its contractile action.

In summary, DBI has potent action on rabbit basilar and carotid arteries, with important differences in its action and in the mechanism through which it acts. At resting tension, DBI contracts rabbit basilar and common carotid arteries by a mechanism independent of extracellular Ca^{2+} that involves both protein kinase C and calmodulin. DBI relaxes pre-contracted basilar arteries, but not carotid arteries, by a mechanism independent of the liberation of NO and prostacyclin. In carotid arteries, prostacyclin, but not NO, partially counteracts the contractile action of DBI.

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