J. Pharm. Pharmacol. 2000, 52: 1431–1434
Received June 9, 2000
Accepted August 2, 2000

Endothelium-independent Vasorelaxant Effect of Dioclein, a New Flavonoid Isolated from *Dioclea grandiflora*, in the Rat Aorta

F. TRIGUEIRO*, S. F. CÔRTES†, R. N. ALMEIDA* AND V. S. LEMOS

*Laboratório de Hipertensão, Departamento de Fisiologia e Biofísica, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, *Laboratório de Tecnologia Farmacéutica, Universidade Federal de Paraíba, Caixa Postal 5009, CEP 58051-970, João Pessoa, Pb and †Laboratório de Farmacologia Cardiovascular, Departamento de Farmacologia, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, MG, Brazil*

Abstract

We have investigated the endothelium-independent vasorelaxant effect of the new flavonoid dioclein (5,2′,5′-trihydroxy-6-7-dimethoxyflavanone) in the rat aorta.

In endothelium-denuded vessels, dioclein induced a concentration-dependent relaxation of aortic rings precontracted with noradrenaline ($IC50 = 3{\cdot}5 \pm 0{\cdot}89 \times 10^{-4}$ M and KCl ($IC50 = 5{\cdot}2 \pm 1{\cdot}2 \times 10^{-4}$ M). In the absence of extracellular calcium, dioclein reduced the contraction induced by noradrenaline (maximal reduction approximately 33%) but not that induced by caffeine. Dioclein also produced a concentration-dependent inhibition of the sustained contractions induced by the phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate in normal ($IC50 = 4{\cdot}0 \pm 0{\cdot}2 \times 10^{-4}$ M) and Ca^{2+}-free solution ($IC50 = 4{\cdot}0 \pm 0{\cdot}3 \times 10^{-4}$ M).

The results indicate that the endothelium-independent vasorelaxant effect of dioclein may be explained by inhibition of contractions dependent on activation of protein kinase C, voltage-dependent Ca^{2+} influx and on the release of intracellular Ca^{2+} stores sensitive to noradrenaline.

Flavonoids constitute a large group of low molecular weight polyphenolic compounds derived from plants and they are consumed in large amounts in the daily diet. Consumption of flavonoids has shown to be inversely associated with morbidity and mortality from coronary heart disease (Hertog et al 1995; Knekt et al 1996). This is probably as a result of their effects as antioxidants (Rice-Evans et al 1997), inhibitors of platelet aggregation (Seigneur et al 1990) and vasodilators (Duarte et al 1993; Herrera et al 1996). Lemos et al (1999) described a nitric oxide- and endothelium-dependent vasorelaxant effect of dioclein (Figure 1), a new flavanone isolated from the roots of *Dioclea grandiflora* Mart. ex Benth. (Bhattacharyya et al 1995), a vine that grows in the coastal plain of northeastern Brazil. However, dioclein also induced a vasorelaxant effect in endothelium-denuded vessels.

Correspondence: V. S. Lemos, Laboratório de Hipertensão, Departamento de Fisiologia e Biofísica, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, MG, Brazil.

In the present work we describe the underlying mechanism involved in the endothelium-independent effect of dioclein in the rat aorta.

Materials and Methods

Experimental procedure

The experiments were performed using rings of rat aorta which had the functional endothelium removed by rubbing the intimal surface with a wooden stick (Lemos et al 1999). Briefly, the tissues were set up in a gassed (95% O_2 and 5% CO_2) Krebs-Henseleit solution (mM): NaCl 118·0, KCl 4·7, $NaHCO_3$ 25·0, $MgSO_4{\cdot}7H_2O$ 1·2, $CaCl_2$ 2·49, KH_2PO_4 1·2 and glucose 11·1, maintained at 37°C under a tension of 1 g. After a 1-h equilibration period, two reproducible submaximal (70–80% maximal) tonic responses were obtained with 3×10^{-7} M noradrenaline. The absence of relaxation in the presence of acetylcholine indicated the deficiency of functional endothelium. Dioclein was

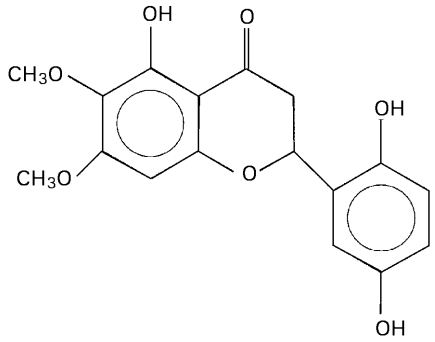

Figure 1. The structure of dioclein (5,2′,5′-trihydroxy-6-7-dimethoxyflavanone).

added cumulatively during the tonic contractions induced by noradrenaline (3×10^{-7} M) and KCl (50 mM) in tissues suspended in normal Krebs–Henseleit or 10^{-6} M 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in normal or Ca^{2+}-free (without $CaCl_2$ plus 1 mM EGTA) solution. The results were expressed as percentage decrease in maximal contraction induced by noradrenaline, considering 100% relaxation as the point when the basal line was reached. Values of inhibitory concentration 50% (IC50) were calculated graphically from concentration–response curves. To investigate the effect of dioclein on the release of intracellular Ca^{2+}, contractile responses to noradrenaline (3×10^{-7} M) and caffeine (20 mM) were performed in Ca^{2+}-free solution (Freitas et al 1996). Under this experimental condition, noradrenaline and caffeine induced transient contractile responses, which were considered as control in the absence of dioclein. Different concentrations of dioclein ($3{\cdot}0\times10^{-6}$–$3{\cdot}0\times10^{-4}$ M) were added 15 min before the addition of noradrenaline or caffeine, and the percentage inhibition was calculated by comparing the mean response obtained in the presence or in the absence of each concentration of dioclein. The experiments with caffeine were performed at 21°C (Freitas et al 1996). Tissue response was recorded using isometric transducers and physiographs (Ugo Basile).

Drugs

Acetylcholine chloride, noradrenaline chloride, caffeine and TPA were purchased from Sigma (USA). The isolation and identification of dioclein has been described in detail by Bhattacharyya et al (1995). Dioclein was solubilized in a mixture of distilled water/chremophor as a 10^{-1} M solution and diluted to the desired concentration with distilled water just before use. The final concentration of chremophor never exceeded 0·1%, which was without effect when exposed to control preparations. The other compounds were freely dissolved in water.

Statistics

Results are expressed as the mean ± s.e.m. of five experiments. Student's *t*-test was used to analyse the results, and was considered significant when $P<0{\cdot}05$. Values of inhibitory concentration 50% (IC50) were calculated graphically from the individual concentration–response curves by non-linear regression equation.

Results

Effects of dioclein on KCl- and noradrenaline-induced contractions

Dioclein ($3{\cdot}0\times10^{-5}$–3×10^{-3} M) relaxed the rat aorta previously contracted with noradrenaline or KCl in a concentration-dependent manner (Figure 2A). The corresponding IC50 values were $3{\cdot}5\pm0{\cdot}89\times10^{-4}$ and $5{\cdot}2\pm1{\cdot}2\times10^{-4}$ M for noradrenaline and KCl, respectively.

Effect of dioclein on contractions induced by noradrenaline and caffeine dependent on the release of intracellular calcium stores

In rat aorta suspended in a Ca^{2+}-free medium, noradrenaline and caffeine induced transient contractions (not shown), which were the result of the release of Ca^{2+} from intracellular stores (Karaki et al 1997). Pre-incubation of tissues with dioclein significantly inhibited noradrenaline transient response in a concentration-dependent manner (Figure 2B). Maximal inhibition of 33·2 ± 3·9% was seen with 3×10^{-5} M dioclein as there was no

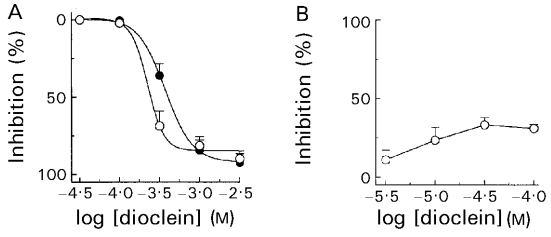

Figure 2. Relaxant effect of dioclein in endothelium-denuded rat aortic rings. A. Effect of dioclein in rings precontracted with noradrenaline (○) or KCl (●) in normal Krebs-Henseleit solution. B. Effect of dioclein in noradrenaline-induced contractions in Ca^{2+}-free solution. The values are mean ± s.e.m. of five experiments.

significant difference between the results after the last two concentrations of the compound. In contrast, dioclein in concentrations up to 10^{-4} M failed to inhibit caffeine-induced contractions. Mean values of transient contractions in control and dioclein (10^{-4} M)-pretreated vessels were $0{\cdot}31 \pm 0{\cdot}08$ and $0{\cdot}28 \pm 0{\cdot}06$ *g*, respectively.

Effect on aortic rings precontracted with the phorbol ester TPA

TPA (10^{-6} M) caused a slowly developing contraction of the rat aorta that was sustained over a 2-h period approximately (not shown) in normal ($1{\cdot}1 \pm 0{\cdot}09$ g) and Ca^{2+}-free ($0{\cdot}7 \pm 0{\cdot}09$ g) Krebs-Henseleit solution. Dioclein ($3{\cdot}0 \times 10^{-5}$–3×10^{-3} M) added cumulatively to the sustained phase of contraction, produced a concentration-dependent relaxation of contractions induced by TPA (10^{-6} M), to a similar extent as observed in sustained contractions evoked by noradrenaline or KCl (Figure 3). There was no difference between IC50 values for dioclein when tissues were stimulated with TPA in normal (IC50 = $4{\cdot}0 \pm 0{\cdot}2 \times 10^{-4}$ M) or Ca^{2+}-free solution (IC50 = $4{\cdot}0 \pm 0{\cdot}3 \times 10^{-4}$ M). The time-courses of the relaxation by dioclein in the presence ($66{\cdot}8 \pm 16{\cdot}8$ min) and in the absence ($46{\cdot}1 \pm 10{\cdot}7$ min) of extracellular calcium were not significantly different.

Discussion

Dioclein exhibited a vasorelaxant effect, which was not dependent on the presence of functional vascular endothelium. This relaxant effect was probably the result of the inhibition of a number of calcium regulatory processes and a decrease in the calcium sensitivity of contractile elements in the rat aorta.

Dioclein relaxed the rat aorta previously contracted with noradrenaline or KCl with the same potency. The inhibitory effects of dioclein on the KCl-induced contractile responses could be attributed partly to the blockade of Ca^{2+} entry through voltage-operated Ca^{2+} channels (VOCs), in as much as KCl-induced contraction in the rat aorta is the result of Ca^{2+} influx through L-type VOCs (Karaki et al 1997). L-type Ca^{2+}-channel blockers such as verapamil and nifedipine are largely known to inhibit the increase in $[Ca^{2+}]_i$ and the accompanying contraction induced by KCl (Baker 2000).

The mechanisms involved in noradrenaline-induced contractions are more complex and are dependent on alteration of the intracellular movement of calcium ions through activation of its influx and release of the intracellular stores, as well as the activation of several enzymes such as protein kinase C (Karaki et al 1997). We designed experiments to investigate a possible effect of dioclein on intracellular Ca^{2+} stores and protein kinase C.

The existence of two different intracellular Ca^{2+} pools has been proposed, one sensitive to inositol trisphosphate and the other sensitive to the plant alkaloid ryanodine and caffeine (Berridge 1993a; Clapham 1995). The initial transient response to noradrenaline is mediated by the release of Ca^{2+} from an intracellular pool sensitive to inositol triphosphate (Irvine 1992; Berridge 1993b). Dioclein significantly inhibited noradrenaline transient response but failed to inhibit caffeine-induced contractions. These results showed that dioclein was able to diminish contractions due to noradrenaline-induced release of intracellular Ca^{2+} in accordance with previous reports for other flavonoids such as luteolin, naringenin, and eriodictyol (Sánchez de Rojas et al 1996). Since dioclein had no effect on contractions dependent on the release of Ca^{2+} stores induced by caffeine, it is likely that dioclein was acting through inhibition of intracellular Ca^{2+} stores sensitive to IP_3.

Protein kinase C has been proposed to play a key role in the maintenance of tonic contraction of vascular smooth muscle by controlling calcium entry and/or Ca^{2+} sensitivity of contractile elements (Karaki et al 1997). This attribution to protein kinase C has been largely due to the observation that phorbol esters produce contractile responses (Sato et al 1992; Nakajima et al 1993). TPA is a phorbol ester that activates protein kinase C and causes a slow-developing sustained contraction of the rat aorta (Spedding 1987; Shimamoto et al 1992, 1993). Inhibitors of protein kinase C such as calfostin C, H-7 and staurosporin are largely reported to relax contractions induced by TPA in the rat aorta (Shimamoto et al 1992, 1993). Here, dioclein relaxed contractions induced by

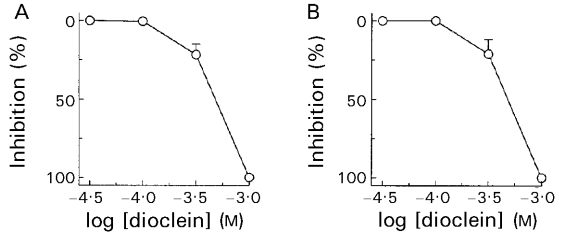

Figure 3. Relaxant effect of dioclein in endothelium-denuded rat aortic rings precontracted with TPA in normal (A) or Ca^{2+} free Krebs-Henseleit solution (B). The results are mean ± s.e.m. of five experiments.

TPA suggesting that dioclein had a vasorelaxant effect, which may be due partly to its inhibitory action on protein kinase C. These results are in agreement with reports showing that other flavonoids displayed similar effects as dioclein over the same concentration range (Ferriola et al 1989; Herrera et al 1996). There was no difference between the IC50 values for dioclein when the tissues were stimulated in normal or Ca^{2+}-free solution, and so it is possible that Ca^{2+} sensitivity of contractile proteins controlled by protein kinase C rather than calcium entry could be inhibited by dioclein.

In conclusion, different mechanisms appear to be involved in the endothelium-independent vasorelaxant effect of dioclein: inhibition of contractions dependent on activation of protein kinase C, inhibition of calcium influx through VOCs and, to a little extent, inhibition of Ca^{2+} release from noradrenaline-sensitive Ca^{2+} stores.

Acknowledgements

V. S. Lemos and S. F. Côrtes were financially supported by CNPq (Consellio Nacional de Desenvolvimento Cientifico e Tecnológies). This work was partially supported by grants from Brazilian Northeast Bank and Pró-Reitonia de Pesguisa, Universidade Federal de Minas Gerais. The authors thank Dr M. F. Agra for identifying the plant and Dr M. M. Teixeira for critical suggestions.

References

Baker, E. H. (2000) Ion channels and the control of blood pressure. Br. J. Clin. Pharmacol. 49: 185–198

Berridge, M. J. (1993a) A tale of two messengers. Nature 365: 388–389

Berridge, M. J. (1993b) Inositol triphosphate and calcium signaling. Nature 361: 315–325

Bhattacharyya, J., Batista, J. S., Almeida, R. N. (1995) Dioclein, a flavanone from the roots of *Dioclea grandiflora*. Phytochemistry 38: 277–278

Clapham, D. E. (1995) Calcium signaling. Cell 80: 259–268

Duarte, J., Vizcaíno, F. P., Utrilla, P., Jiménez, J., Tamargo, J., Zarzuelo, A. (1993) Vasodilatatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. Gen. Pharmacol. 24: 857–862

Ferriola, P. C., Cody, V., Middleton, E. (1989) Protein kinase C inhibition by plant flavonoids: kinetics mechanisms and structure-activity relationships. Biochem. Pharmacol. 38: 1617–1624

Freitas, M. R., Côrtes, S. F., Thomas, G., Barbosa Filho, J. M. (1996) Modification of Ca^{2+} metabolism in the rabbit aorta as a mechanism of spasmolitic action of warifteine, a bisbenzilisoquinoline alkaloid isolated from the leaves of Cissampelos sympodialis Eichl. (Menispermaceae). J. Pharm. Pharmacol. 48: 332–336

Herrera, M. D., Zarzuelo, A., Jiménez, J., Marhuenda, E., Duarte, J. (1996) Effects of flavonoids on rat aortic smooth muscle contractility: structure-activity relationships. Gen. Pharmacol. 27: 273–277

Hertog, M. G. L., Kromhout, D., Aravanis, C., Blackburn, H., Buzina, R., Fidanza, F., Giampaoli, S., Jansen, A., Menotti, A., Nedeljkovic, S., Pekkarinen, M., Simic, B. S., Toshima, H., Feskens, E. J. M., Hollman, P. C. H., Katan, M. B. (1995) Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. Arch. Intern. Med. 155: 381–386

Irvine, R. F. (1992) Inositol phosphate and Ca^{2+} entry: toward a proliferation or a simplification? FASEB J. 6: 3085–3091

Karaki, H., Ozaki, H., Hori, M., Mitsui-Saito, M., Amano, K. I., Harada, K. I., Shigeki, M., Nakazawa, H., Won, K. J., Sato K. (1997) Calcium movements, distribution, and functions in smooth muscle. Pharmacol. Rev. 49: 157–230

Knekt, P., Jarvinen, R., Reunanen, A., Maatela, J. (1996) Flavonoid intake and coronary mortality in Finland: a cohort study. Br. Med. J. 312: 478–481

Lemos, V. S., Freitas, M. R., Muller, B., Lino, Y. D., Queiroga, C. E. G., Côrtes, S. F. (1999) Dioclein, a new nitric oxide- and endothelium-dependent vasodilator flavonoid. Eur. J. Pharmacol. 386: 41–46

Nakajima, S., Fujimoto, M., Ueda, M. (1993) Spatial changes of $[Ca^{2+}]$; and contraction caused by phorbol esters in vascular smooth muscle cells. Am. J. Physiol. 265: C1138–C1145

Rice-Evans, C., Miller, N. J., Paganga, G. (1997) Antioxidant properties of phenolic compounds. Trends Plant. Sci. 2: 152–159

Sánchez de Rojas, V. R., Somoza, B., Ortega, T., Villar, A. M. (1996) Different mechanisms involved in the vasorelaxant effect of flavonoids isolated from *Satureja obovata*. Planta Med. 62: 554–556

Sato, K., Aori, M., Ozaki, H., Takano-Ohmuro, H., Tsuchiya, T., Sugi, H., Karaki, H. (1992) Myosin phosphorylatian-independant contraction induced by phorbal esters in vascular smooth muscle. J. Pharmacol. Exp. Ther. 261: 497–505

Seigneur, M., Bonnet, J., Dorian, B., Benchimol, D., Drouillet, F., Gouverneur, G., Larrue, J., Crockett, R., Boisseau, M., Gayon, R., Bricaus, H. (1990) Effect of the consumption of alcohol, white wine, and red wine on platelets function and serum lipids. J. Appl. Cardiol. 5: 215–222

Shimamoto, H., Shimamoto, Y., Kwan, C. Y., Daniel, E. E. (1992) Participations of protein kinase C in endothelin-1-induced contraction in the rat aorta: studies with a new tool, calphostin C. Br. J. Pharmacol. 107: 282–287

Shimamoto, Y., Shimamoto, H., Kwan, C. Y., Daniel, E. E. (1993) Differential effects of putative protein kinase C inhibitors on contraction of rat aortic smooth muscle. Am. J. Physiol. 264: H1300–H1306

Spedding, M. (1987) Interaction of phorbol esters with Ca^{2+} channels in smooth muscle. Br. J. Pharmacol. 91: 377–384