J. Pharm. Pharmacol. 1995, 47: 30–33
Received April 28, 1994
Accepted August 15, 1994

© 1995 J. Pharm. Pharmacol.

Sapintoxin A and Phorbol 12,13-Dibutyrate: Two Phorbol Derivatives with Contrasting Effects on Rat Blood Vessel Permeability In-vitro

A. M. NORTHOVER, B. J. NORTHOVER, W. J. RYVES* AND F. J. EVANS*

*Department of Pharmacy, School of Applied Sciences, De Montfort University, Leicester LE1 9BH, and *Department of Pharmacognosy, School of Pharmacy, University of London, London WC1N 1AX, UK*

Abstract

Rat isolated small intestine and mesentery were perfused with a gelatin-containing physiological salt solution, and microvascular permeability in the villi was assessed using colloidal carbon as a marker to assess the effect of sapintoxin A in this experimental situation, and to compare it with phorbol 12,13-dibutyrate.

Sapintoxin A (1, 0·25, 0·1 μM) had no effect on colloidal carbon leakage compared with control values, but significantly increased perfusion pressure. Phorbol 12,13-dibutyrate (1 μM) significantly increased both colloidal carbon leakage and perfusion pressure. Pretreatment with the protein kinase C inhibitor Ro 31-8220 (1 μM) significantly increased colloidal carbon leakage in the presence of sapintoxin A, but significantly decreased the phorbol 12,13-dibutyrate-induced leakage of colloidal carbon.

Pretreatment with indomethacin (1 μM) significantly increased colloidal carbon leakage in response to sapintoxin A, but did not affect the response to phorbol 12,13-dibutyrate. Increases in perfusion pressure caused by sapintoxin A (0·25 μM) and phorbol 12,13-dibutyrate (1 μM) were reduced by Ro 31-8220, but neither pressor response was affected by indomethacin. Lower concentrations of phorbol 12,13-dibutyrate (0·25, 0·1 μM) had no effect on colloidal carbon leakage. However, there was a significant increase in perfusion pressure in response to 0·25 μM but not to 0·1 μM phorbol 12,13-dibutyrate.

When rat mesentery alone was perfused using gelatin-free physiological salt solution, sapintoxin A (1 μM) had no effect on perfusion pressure, whereas phorbol 12,13-dibutyrate (1 μM) caused a significant increase over a 15-min period, which was completely abolished by pretreatment with Ro 31-8220.

It may be concluded that the permeability-increasing effects of phorbol 12,13-dibutyrate are dependent on protein kinase C activation.

Phorbol esters have been known for some time to act as tumour promoters (Van Duuren & Orris 1965) and as pro-inflammatory agents (Janoff et al 1970; Evans & Schmidt 1979; Williams et al 1981). Both of these actions are thought to be due to the activation of protein kinase C (Kikkawa et al 1983; Abdel-Latif 1986). Many naturally-occurring phorbol-related compounds have been isolated (Evans 1986). Some of these have been shown to possess pro-inflammatory activity while lacking tumour-promoting activity (Hergenhahn et al 1974; Driedger & Blumberg 1980; Ellis et al 1987). Activation of protein kinase C by one such compound, sapintoxin A, has been shown to be more Ca^{2+}-dependent than that produced by the tumour-promoting compound phorbol myristate acetate (Ryves et al 1991; Merritt et al 1993). Recent work has suggested that leakage of colloidal carbon into the walls of microvessels in rat isolated small intestine can be brought about by the activation of a Ca^{2+}-dependent isoenzyme of protein kinase C (Northover & Northover 1994a). Hence, it was of interest to examine the effect of sapintoxin A in this experimental situation, and to compare it with phorbol 12,13-dibutyrate. Experiments were also performed to compare the effects of sapintoxin A and phorbol dibutyrate on perfusion pressures in isolated mesenteric arterioles from the rat.

Correspondence: A. M. Northover, Department of Pharmacy, School of Applied Sciences, De Montfort University, The Gateway, Leicester LE1 9BH, UK.

Materials and Methods

Chemicals used

Phorbol 12,13-dibutyrate was obtained from Sigma Chemical Co. (Poole, UK); Ro 31-8220 was a gift from Dr G. Lawton (Roche Products, Welwyn Garden City, UK); indomethacin was a gift from Merck, Sharp & Dohme (Hoddesdon, UK); sapintoxin A was prepared in the Department of Pharmacognosy, London University School of Pharmacy. Sapintoxin A, phorbol dibutyrate and Ro 31-8220 were each dissolved in dimethylsulphoxide. Indomethacin was dissolved in alkalinized water and then adjusted to pH 7·4. A dose volume of 0·2 mL was used for each compound. Colloidal carbon (Gunther Wagner, Batch C11/1431a) was obtained from Pelikan Inks (Hanover, Germany). DPX mountant and gelatin were obtained from BDH (Poole, UK).

Initial preparation

Rats, 350–500 g, were killed by inhalation of chloroform vapour. A stainless steel cannula was inserted and tied into the anterior mesenteric artery so that the artery and its branches could be perfused according to one of the following procedures.

Perfusion of isolated mesentery and small intestine

The small intestine with its accompanying blood vessels was ligated and cut at both the caecal and pyloric ends, and

carefully dissected away from the large intestine. The tissue was then transferred to a bath of balanced salt solution (Northover 1993) containing 2% gelatin and maintained at 37°C referred to hereafter as perfusate. Blood was flushed from the vasculature with perfusate from a reservoir, which was connected to the cannula via a peristaltic pump and Condon mercury manometer. This was followed by perfusion at a constant rate of 10 mL min^{-1}, with monitoring of the perfusion pressure throughout. The volumes of perfusate in both the organ bath and the reservoir were adjusted to 50 mL for the duration of the 15-min experimental period. Perfusion fluid was re-circulated to minimize the quantities of test compounds needed. Perfusion for the first 10 min was with perfusate alone or with perfusate containing either Ro 31-8220 (1 μM) or indomethacin (1 μM). Test compounds (sapintoxin A, phorbol dibutyrate) were added to the perfusate in the reservoir for the last 5 min, as described in detail previously (Northover 1993). During this latter 5-min period, perfusion pressures were recorded from the Condon manometer. The vasculature was then perfused with perfusate alone for a further 1 min, after which a bolus of 0·5 mL colloidol carbon suspension was introduced near the cannula. After a further 5-min perfusion, 2 mL rat washed red blood cells was injected to provide a visual check that the blood vessels being studied were patent. The preparation was then removed into a tray of saline.

Preparation of specimens for image analysis

Six pieces of intestine, each approximately 4 cm in length, were cut. After flushing with saline, opening lengthways and stapling to a xylene-resistant coverslip (Thermanox, Nunc Inc., Naperville, USA), specimens were fixed in formal saline (4% formaldehyde in saline) for 1 h, dehydrated in graded alcohols, and cleared overnight in xylene. They were mounted, mucosal surface uppermost, in DPX mountant, and micrographs of villi blood vessels were taken at ×100 magnification from five sites, selected randomly but widely separated, on each specimen, using Ilford FP4 black and white film.

Image analysis

Negative micrographs were analysed using a Seescan system (Cambridge, UK) as previously described (Northover 1993). The amount of colloidal carbon, shown as white areas on the negative, was expressed as % of the total area (frame) being analysed. The mean % value for each piece of intestine was calculated and then used to calculate the mean value for each animal. Results are presented as mean values for all animals in a particular treatment group.

Perfusion of the isolated mesentery

Second-order blood vessels at both the caecal and pyloric ends of the small intestine, and the first-order vessel at the caecal end of the large intestine were ligated. Retaining the maximum possible length of third-order vessels, the mesentery was separated carefully from both large and small intestine. It was then transferred to a bath of physiological salt solution at 37°C (see above). The subsequent experimental procedure was similar to that described above, except that the rate of perfusion was adjusted to give an initial perfusion pressure of between 30 and 35 mmHg. A 15-min settling-in period was allowed.

Statistical analysis

Results were subjected to Bonferroni's test for comparing more than one group of results with a control (Wallenstein et al 1980).

Results

It can be seen from Table 1 that sapintoxin A and phorbol dibutyrate, each at a concentration of 1 μM, produced markedly dissimilar effects on the leakage of colloidal carbon into microvessel walls. Phorbol dibutyrate produced a significant increase in colloidal carbon leakage, which was reduced by pretreatment with the specific protein kinase C inhibitor Ro 31-8220 (1 μM) but not by the cyclo-oxygenase inhibitor indomethacin (1 μM). At the lower concentrations of 0·25 and 0·1 μM, however, phorbol dibutyrate alone elicited colloidal carbon trapping that was no greater than in controls. In contrast, sapintoxin A (1 μM) alone had no effect on colloidal carbon leakage. After pretreatment with either Ro 31-8220 or indomethacin, however, sapintoxin A caused a significant increase in blackening. During the course of these experiments the mean rises in perfusion pressure in response to sapintoxin A (1, 0·25 and 0·1 μM) and phorbol dibutyrate (1 and 0·25 μM) over the 5-min test period were significantly greater than control values (Table 2). Pretreatment with Ro 31-8220 significantly reduced the rise in perfusion pressure in response to 0·25 μM sapintoxin A and also reduced the response to 1 μM sapintoxin A. However, because of the substantial varia-

Table 1. Effects of pretreatment with Ro 31-8220 (1 μM) or indomethacin (1 μM) on the leakage of colloidal carbon in microvessels of villi in rat small intestine in response to phorbol dibutyrate and sapintoxin A, determined by image analysis.

Treatment		Amount of colloidal carbon assessed as % of frame area (± s.e.)					
		n	Perfusate	n	Perfusate + Ro31-8220	n	Perfusate + indomethacin
Control	—	10	0·98 ± 0·16	4	0·96 ± 0·17	4	1·35 ± 0·13
Sapintoxin A	1 μM	5	1·00 ± 0·43	4	2·92 ± 0·59[a]	4	3·59 ± 1·18[a]
	0·25 μM	4	1·42 ± 0·40	4	1·01 ± 0·21		—
	0·10 μM	3	0·87 ± 0·41		—		—
Phorbol dibutyrate	1 μM	11	2·62 ± 0·25[b]	6	1·08 ± 0·36[c]	5	2·74 ± 0·52
	0·25 μM	4	1·17 ± 0·38		—		—
	0·10 μM	4	1·23 ± 0·07		—		—

[a] $P < 0.05$ compared with sapintoxin A alone, [b] $P < 0.05$ compared with control, [c] $P < 0.05$ compared with phorbol dibutyrate alone.

Table 2. Effects of phorbol dibutyrate and sapintoxin A on perfusion pressures in the vasculature of rat isolated mesentery and small intestine recorded via the anterior mesenteric artery, in the presence and absence of Ro 31-8220 (1 μM) or indomethacin (1 μM).

Treatment		Alterations in perfusion pressure (± s.e.) after 5 min perfusion (mmHg)					
		n	Perfusate	n	Perfusate + Ro 31-8220	n	Perfusate + indomethacin
Control	—	10	−2·00 ± 1·16	4	−0·50 ± 0·50	4	0·00 ± 1·29
Sapintoxin A	1 μM	5	29·00 ± 12·07[a]	4	4·00 ± 2·74	4	16·50 ± 2·33
	0·25 μM	4	19·00 ± 4·26[a]	4	3·00 ± 1·87[b]		—
	0·10 μM	3	14·67 ± 1·33[a]		—		—
Phorbol dibutyrate	1 μM	11	21·46 ± 3·28[a]	6	9·83 ± 2·02[c]	5	20·20 ± 4·08
	0·25 μM	4	17·25 ± 6·55[a]		—		—
	0·10 μM	4	0·50 ± 0·66		—		—

[a] $P < 0.05$ compared with control, [b] $P < 0.05$ compared with sapintoxin A alone, [c] $P < 0.05$ compared with phorbol dibutyrate alone.

bility of the pressor effects of 1 μM sapintoxin A, this latter effect did not reach an acceptable level of statistical significance ($P > 0.05$). In contrast, Ro 31-8220 significantly reduced the pressor effects of 1 μM phorbol dibutyrate.

When mesenteric arterioles were perfused after removing the small intestine, sapintoxin A had no effect on perfusion pressure over a 15-min period, whereas phorbol dibutyrate still caused an obvious increase in pressure over the first 5 min, with a small, further increase over the next 10 min (Fig. 1). Pretreatment with Ro 31-8220 virtually abolished this effect.

Discussion

Previously, sapintoxin A has been shown to be a potent activator of protein kinase C (Aitken 1987). Thus, since various inflammatory reactions have been associated with activation of protein kinase C (Abdel-Latif 1986), the lack of effect of sapintoxin A on colloidal carbon leakage was unexpected. Equally surprising was the differing effect of sapintoxin A on perfusion pressure in the mesenteric artery depending on whether the small intestine was still attached to the preparation or was absent (compare Table 2 and Fig. 1). In contrast to the effects of sapintoxin A, the results obtained in the present experiments with phorbol dibutyrate resemble those obtained using a series of other phorbol-related compounds (Northover & Northover 1994a, b). Since the permeability-increasing effects of phorbol dibutyrate were significantly reduced by pretreatment with a specific protein kinase C inhibitor (Tables 1, 2, Fig. 1), it may be concluded that they depended upon protein kinase C activation, as did the effects of 12-deoxyphorbol 13-phenylacetate and thymeleatoxin in earlier experiments (Northover & Northover 1994a, b).

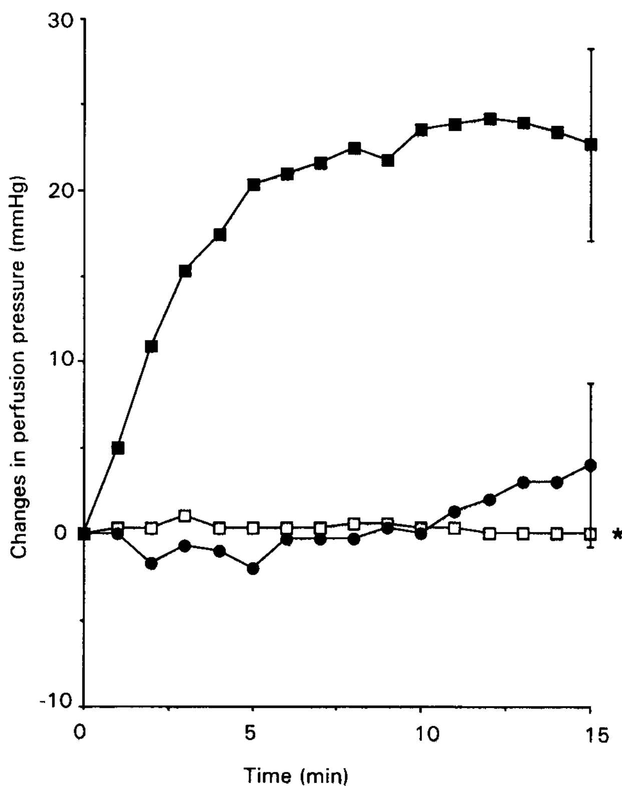

FIG. 1. Changes in perfusion pressure in mesenteric arterioles during perfusion with (●) sapintoxin A (1 μM), or phorbol dibutyrate (1 μM) in the presence (□) and absence (■) of Ro 31-8220 (1 μM). *$P < 0.05$ compared with phorbol dibutyrate alone at 15 min.

The actions of sapintoxin A are complex. Sapintoxin A is a powerful activator of Ca^{2+}-dependent protein kinase C isoenzymes (Ryves et al 1991; Merritt et al 1993), but so also is thymeleatoxin (Ryves et al 1991; Kazanietz et al 1993). However, thymeleatoxin significantly increased colloidal carbon leakage in small intestinal microvessels (Northover & Northover 1994a), but produced relatively small rises in perfusion pressure when the intestine was present, although larger rises when the intestine was absent (Northover & Northover 1994b). Both of these effects of thymeleatoxin are reduced by pretreatment with Ro 31-8220 (Northover & Northover 1994a, b). In contrast, in the present experiments, although pretreatment with Ro 31-8220 reduced the pressor effects of sapintoxin A (1 and 0·25 μM), it increased the leakage of colloidal carbon in response to 1 μM sapintoxin A. This suggests that protein kinase C was important in the pressor response to sapintoxin A. However, any involvement of protein kinase C in sapintoxin A-induced colloidal carbon leakage is probably modified by the release of one or more substances from the small intestine. This may be true also when indomethacin is used, since this too exacerbates sapintoxin A-induced colloidal carbon leakage. It has been shown that leukotrienes, for example, are released in response to stimulation by platelet-activating factor and are secondary mediators of the inflammatory reaction (Hsueh et al 1986). They also may be produced in greater than normal quantities as a result of blockade of cyclo-oxygenase by indomethacin. However,

leukotrienes are vasoconstrictors (Hsueh et al 1986) and, therefore, would be expected to increase perfusion pressure in the presence of indomethacin, rather than produce the observed decrease. Since sapintoxin A had no effect on perfusion pressure in the absence of the small intestine, it is likely that these reactions, if present, would have affected microvessels beyond the mesenteric/intestinal border. Another possible explanation for the unusual effects of sapintoxin A may be that endothelins are being released. These compounds cause vascular contraction and are antagonized by protein kinase C inhibitors (Gray et al 1994). If pre-capillary arterioles and, to a lesser extent, post-capillary venules constrict simultaneously, there would be less flow of perfusate plus colloidal carbon through the vascular bed, and hence no leakage of colloidal carbon, since there has to be substantial intravascular pressure within the microvessels for colloidal carbon leakage to occur (Northover & Northover 1970). Pretreatment with Ro 31-8220 would be likely to prevent this vascular contraction and thus allow flow, and hence colloidal carbon leakage, to occur, assuming that sapintoxin A also activates the release of inflammatory agents such as leukotrienes. However, this is in marked contrast to the combined effects of Ro 31-8220 and phorbol dibutyrate. Of course, sapintoxin A may be acting on kinases that are as yet unknown, and quite different from those activated by phorbol dibutyrate. More work needs to be done on this interesting but complex compound.

References

Abdel-Latif, A. A. (1986) Calcium-mobilizing receptors, polyphosphoinositides, and the generation of second messengers. Pharmacol. Rev. 38: 227–272

Aitken, A. (1987) The activation of protein kinase C by daphnane, ingenane and tigliane diterpinoid esters. Bot. J. Linn. Soc. 94: 247–263

Driedger, P. E., Blumberg, P. M. (1980) Structure-activity relationships in chick embryo fibroblasts for phorbol-related diterpene esters showing anomalous activities in vivo. Cancer Res. 40: 339–346

Ellis, C. A., Brooks, S. F., Brooks, G., Evans, A. T., Morrice, N., Evans, F. J., Aitken, A. (1987) The effects of phorbol esters with different biological activities on protein kinase C. Phytother. Res. 1: 187–190

Evans, F. J. (1986) The phorbol esters. In: Evans, F. J. (ed.) Naturally Occurring Phorbol Esters. CRC Press, Boca Raton, pp 171–215

Evans, F. J., Schmidt, R. J. (1979) An assay procedure for the comparative irritancy testing of esters in the tigliane and daphnane series. Inflammation 3: 215–233

Gray, G. A., Löffler, B.-M., Clozel, M. (1994) Characterization of endothelin receptors mediating contraction of rabbit saphenous vein. Am. J. Physiol. 266: H959–H966

Hergenhahn, M., Kusumoto, S., Hecker, E. (1974) Diterpene esters from 'Euphorbium' and their irritant and cocarcinogenic activity. Experientia 30: 1438–1440

Hsueh, W., Gonzalez-Crussi, F., Arroyave, J. L. (1986) Release of leukotriene C_4 by isolated, perfused rat small intestine in response to platelet-activating factor. J. Clin. Invest. 78: 108–114

Janoff, A., Klassen, A., Troll, W. (1970) Local vascular changes induced by the cocarcinogen, phorbol myristate acetate. Cancer Res. 30: 2568–2571

Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., Blumberg, P. M. (1993) Characterization of ligand and substrate specificity for the calcium-dependent and calcium-independent protein kinase C isozymes. Mol. Pharmacol. 44: 298–307

Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., Nishizuka, Y. (1983) Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. J. Biol. Chem. 258: 11442–11445

Merritt, J. E., Moores, K. E., Evans, A. T., Sharma, P., Evans, F. J., MacPhee, C. H. (1993) Involvement of calcium in modulation of neutrophil function by phorbol esters that activate protein kinase C isotypes and related enzymes. Biochem. J. 289: 919–926

Northover, A. M. (1993) An in vitro method for assessing the effects of pro-inflammatory and anti-inflammatory compounds on microvascular permeability in the rat small intestine. J. Pharmacol. Toxicol. Methods 29: 227–232

Northover, A. M., Northover, B. J. (1970) The effect of vasoactive substances on rat mesenteric blood vessels. J. Path. 101: 99–108

Northover, A. M., Northover, B. J. (1994a) Stimulation of protein kinase C activity may increase microvascular permeability to colloidal carbon via α-isoenzyme. Inflammation 18: 481–487

Northover, A. M., Northover, B. J. (1994b) Vasoconstriction in rat isolated mesentery and small intestine in response to various activators of protein kinase C. Agents Actions In press

Ryves, W. J., Evans, A. T., Olivier, A. R., Parker, P. J., Evans, F. J. (1991) Activation of the PKC-isotypes α, β_1, γ, δ, and ϵ by phorbol esters of different biological activities. FEBS Lett. 288: 5–9

Van Duuren, B. L., Orris, L. (1965) The tumor-enhancing principles of *Croton tiglium* L. Cancer Res. 25: 1871–1875

Wallenstein, S., Zucker, C. L., Fleiss, J. L. (1980) Some statistical methods useful in circulation research. Circ. Res. 47: 1–9

Williams, T. J., Westwick, J., Williamson, E. M., Evans, F. J. (1981) Vascular changes in rabbit skin induced by proinflammatory phorbol and 12-deoxyphorbol esters. Inflammation 5: 29–36