Life Sciences, Vol. 23, pp. 1255-1262
Printed in the U.S.A.

Pergamon Press

THE EFFECT OF PRECURSORS, PRODUCTS, AND PRODUCT ANALOGS OF PROSTAGLANDIN CYCLOOXYGENASE UPON IRIS SPHINCTER MUSCLE

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(Received in final form August 3, 1978)

Summary

Contractions of isolated iris sphincter muscles were measured in response to several free fatty acids, hydroperoxy and hydroxy derivatives of 20:3(n-3), 20:3(n-6) and 20:4, PGH_2, and the epoxymethano analogs of PGH_2. The free acids of prostaglandin precursors elicited comparatively strong contractions, hydroperoxy and hydroxy acids gave intermediate and nonspecific response whereas nonprostaglandin precursor acids elicited little response. PGH_2 was 100 to 1000 times more effective than arachidonic acid or the epoxymethano analogs. The latter compounds inhibited the production of contractions by PGH_2. These results allow an interpretation that the iris sphincter muscle contains an active thromboxane synthase and receptors for endoperoxide and thromboxane that initiate contraction.

Miosis can occur upon contraction of iris sphincter muscle, and can be elicited by intracamerally injected prostaglandins (1,2). Arachidonic acid also elicits miosis in rabbit eye (3), and potentiates contractions produced by transmural stimulation of bovine sphincter muscle (4). The effects of arachidonic acid are reduced by pretreatment with nonsteroidal antiinflammatory drugs (3,4) which are known to inhibit prostaglandin cyclooxygenase (5,6). Such results suggest that prostaglandins may be mediators of the actions of arachidonic acid, and iris tissue has been shown to have the capacity to synthesize prostaglandins (7). Isolated cat iris sphincter muscles also contract in response to low concentrations (0.001 μM) of $PGF_{2\alpha}$, but only to 100-fold higher concentrations of PGE_2 (8). Bovine sphincter muscles also contract (9), but their response to transmural stimulation is potentiated by levels of PGE_2 that were 10-100 fold lower than $PGF_{2\alpha}$ (10).

The oxidative conversion of polyunsaturated fatty acids to prostaglandins also produces numerous associated hydroxy and hydroperoxy lipids (11). The hydroxy and hydroperoxy endoperoxide intermediates in prostaglandin synthesis are recognized as potent stimulators of smooth muscle (12), and various other hydroperoxy acids have been reported to produce strong contractions in a variety of muscles (13,14,15,16). Thus many different oxidized agents are theoretically possible mediators of arachidonic acid action.

Therefore, we have studied the effect on sphincter muscle contraction of various fatty acids that are either precursors or nonprecursors for prostaglandin formation in addition to a prostaglandin metabolic intermediate and its analogs to identify the oxidized acid derivative(s) responsible for the

0300-9653/78/0925-1255$02.00/0

contractile response.

Methods

Eyes were enucleated from adult animals and strips of muscle were dissected as described previously (17). Two strips under 50-75 mg tension were placed in 30 mls of a sugar-salt bathing solution (containing per liter; KCl, 0.35 g; $MgSO_4 \cdot 7H_2O$, 0.29 g; $CaCl_2$, 0.24 g; $NaHCO_3$, 1.25 g; NaCl, 6.95 g; KH_2PO_4, 0.16 g; EDTA, 0.01 g; dextrose, 1.0 g; sucrose, 17 g; pH 7.4) at 37° areated with 95% O_2 - 5% CO_2. The effect of various lipids on the muscle strips was measured under isometric conditions by means of a Grass force displacement transducer (Model FT .03C) and recorded on a Grass polygraph (Model 7C).

Fatty acids were obtained from Nu Check Prep, Elysian, Minn. or Supelco, Inc., Bellefonte, Penn. and prepared as 10 mM solutions in 0.1 M Tris-HCl pH 8.0 or absolute ethanol.

Hydroperoxy and hydroxy acids were prepared in the following manner. 100 ml 0.1 M Tris-HCl (pH 8.5) containing 670 µM phenol and 6 mg (156,500 units/mg) lipoxidase (Sigma Chemical Co., St. Louis, Mo.) was vigorously stirred at room temperature and 3.0 ml ethanol containing 50 mg substrate acid was added dropwise over 5 min. The reaction was stirred an additional 5 min., then 50 ml diethyl ether and 5.0 ml 1 M citric acid was added. After transfer to a separatory funnel, the organic phase was removed and the aqueous phase reextracted twice with 50 ml diethyl ether. The combined ether phases were washed three times with 50 ml H_2O, dried over Na_2SO_4, and the solvent removed *in vacuo*.

The residue was taken up in petroleum ether-diethyl ether (9:1) and applied to a 2 g silicic acid column which was eluted with 40 ml petroleum ether-diethyl ether (9:1) and then 50 ml (8:2). Unreacted acids were eluted in the (9:1) fraction, and the (8:2) fractions containing the hydroperoxy acids were combined, taken to dryness *in vacuo* and stored at -20° in anhydrous diethyl ether. Hydroxy acids were prepared from the corresponding hydroperoxy acids by reduction with triphenylphosphine (1.5 mole triphenylphosphine/mole acid) in diethyl ether and purification by silicic acid chromatography as described above. The excess triphenylphosphine eluted in the 9:1 fraction before the hydroxy acid.

PGH_2 was prepared from 20:4 essentially as previously described (18) and the epoxymethano analogs of PGH_2 U-46619 (15S)-hydroxy-11α,9α-(epoxymethano) prosta-5Z,13E-dienoic acid (I) and U-44069, (15S)-hydroxy-9α,11α-(epoxymethano) prosta-5Z,13E-dienoic acid (II) were generous gifts from Dr. G. L. Bundy, of the Upjohn Company (19).

Results

The isolated sphincter muscle from the iris of cat or rabbit contracted after addition of the prostaglandin precursors 20:4 and 20:3 (n-6), but there was no response to any of the solvents and little or no response to the acids that were not prostaglandin precursors, 20:3 (n-3) or 18:1 (n-9) (Table I). Concentrations are expressed in terms of µM and µg/ml for convenient comparison with other results. Because the response of the rabbit muscle was much more variable and required a 30-fold higher concentration of acid to elicit contractions comparable to the cat, further studies were conducted on the cat sphincter muscle.

TABLE I

Response of the Ocular Sphincter Muscle From Cat and Rabbit to Various Fatty Acids

Acid	Cat 3.3 μM (ca. 1 μg/ml)	Rabbit 100 μM (ca. 30 μg/ml)
20:4 (n-6)	52 ± 7 (24)	40 ± 10 (18)
20:3 (n-6)	21 ± 5 (10)	41 ± 17 (6)
20:3 (n-3)	0.5 ± 0.5 (10)	11 ± 2 (5)
18:1 (n-9)	-1.5 ± 3 (12)	1 ± 1 (2)

Isolated muscles were incubated as described in Methods and the fatty acids added to give the concentrations indicated. Contractions are expressed in mg tension at the plateau ± the standard error for the number of determinations noted in parenthesis.

The cat sphincter responded to 20:4 and 20:3 (n-6) in a dose-dependent manner as shown in Figure 1, and small contractions were observed at 100-fold higher concentrations of 20:3 (n-3) and 18:1 (n-9).

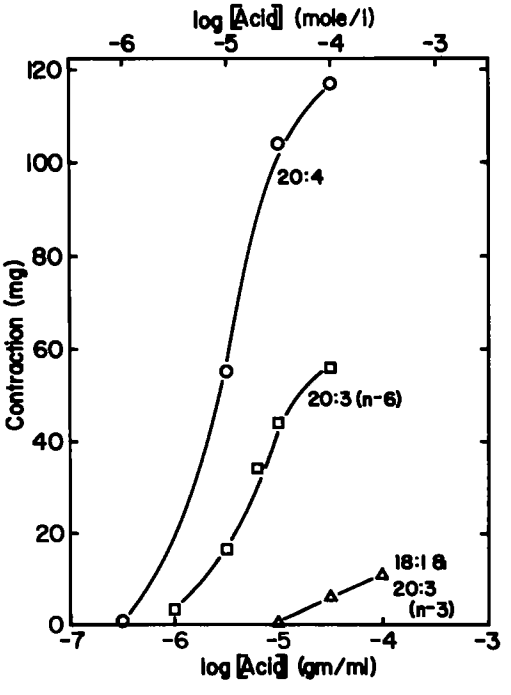

FIG. 1

Cumulative dose response curves of cat sphincter muscle to various fatty acids. Dose-dependent contractions were obtained by cumulative addition of the indicated acids. Values are the mean of at least 4 determinations.

Table II shows the magnitude of contractions produced by the hydroperoxy and hydroxy forms of the 20:4 and 20:3 (n-6), which caused contraction as the free acids; and of 20:3 (n-3) which did not. All six derivatives elicited contractions which were approximately equal and about 20% of the response produced by the same concentration of arachidonic acid.

TABLE II

Response of Cat Sphincter Muscle to Various Hydroperoxy and Hydroxy Acids

Acid Precursor 3.3 μM (ca. 1 μg/ml)	ROOH	ROH
20:4 (n-6)	10 ± 3	13 ± 5
20:3 (n-6)	15 ± 4	10 ± 4
20:3 (n-3)	13 ± 3	8 ± 5

Conditions the same as in Table I

The cat sphincter contracted in dose-dependent manner to the prostaglandin endoperoxide, PGH_2, and the two epoxymethano derivatives, I and II, as shown in Figure 2.

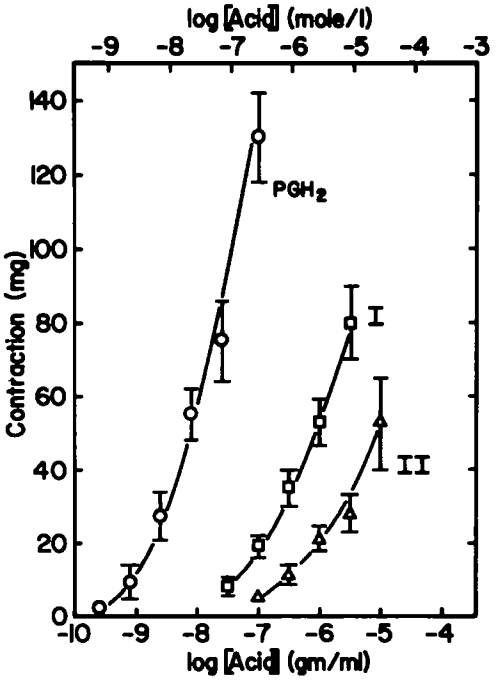

FIG. 2

Cumulative dose response curves of cat sphincter muscle to endoperoxide and endoperoxide analogs. Experimental conditions the same as in Figure 1. Bars indicate standard error of the mean values.

When cat sphincter muscle was bathed in .067 μM flurbiprofen, the contractile response to 3 μM arachidonic acid was blocked (Table III), however, higher amounts of fatty acid allowed a measurable contraction that could, in turn, be blocked by increased amounts of flurbiprofen.

TABLE III

Flurbiprofen Inhibition of Sphincter Muscle Response to 20:4

20:4 μM	Flurbiprofen			
	-0-	0.067 μM	0.67 μM	6.7 μM
3	135	-0-	-	-
10	-	13	1	-
30	-	-	18	2
100	-	-	-	18

Muscles were preincubated 10 min with flurbiprofen before addition of arachidonic acid then washed 3x before increasing inhibitor concentration. Contractions are expressed in mg as the mean of two determinations.

Although preincubation of the muscle with 13 μM flurbiprofen blocked contraction to either 5 μM 20:4 or 10 μM hydroperoxy or hydroxy derivatives of 20:4 (results not shown), it had no effect on the contractions produced by PGH_2 or the epoxymethano analogs. However, if dose response curves were determined by first measuring the contractions produced by the PGH_2 analogs II and I, the muscle did not respond to PGH_2 whereas prior incubation with PGH_2 did not block the muscle's response to the analogs (Table IV).

TABLE IV

Inhibition of PGH_2 Contractions by Pretreatment with PGH_2 Analogs I and II

Series	PGH_2 0.024 μM	II 10 μM	I 1 μM	PGH_2 0.024 μM	I 1 μM
A	61 ± 10 (6)	62 ± 20 (4)	59 ± 10 (5)	-0-	-
B	-	40 ± 6 (6)	35 ± 8 (6)	4 ± 4 (6)	37 ± 7 (2)

All determinations were made sequentially from left to right in both series and the muscle strips were washed 3x before addition of the next agent. Contractions are expressed in mg ± the standard error for the number of determinations noted in parenthesis.

Discussion

The selective responses to 20:3 (n-6) and 20:4 (n-6) indicate that the contractions were dependent upon some selective event such as that caused by a receptor or an enzyme, and was unlikely to be due to a physical effect (e.g., detergent) of the acid. This selectivity was also similar to that generally recognized for the prostaglandin cyclooxygenase (20). Therefore the observed contractions could have been elicited by oxidative products of this reaction. Since the prostaglandins that are derived from 20:4 seem more potent than those derived from 20:3 in producing contractions of the cat muscle (8), the greater observed response to 20:4 compared to 20:3 agrees with the concept that the

contractions may be due to products of cyclooxygenase action, but does not indicate which of the products are the active agents.

Fatty acid hydroperoxides have been reported to evoke numerous physiological events including pain and erythema (21), increase in the rate of contraction of murine portal vein (22) and contraction of various muscles (13,14, 15,16). Nonsteroidal antiinflammatory drugs have been reported to reduce the effects of arachidonic hydroperoxide (16,22), but the effects of these drugs on the actions produced by nonprostaglandin precursor hydroperoxides has not been determined (15,22). From one point of view, the contractions produced by the hydroxy and hydroperoxy acids can appear to be unrelated to cyclooxygenase activity, since derivatives of nonprecursors were as effective as those of prostaglandin precursors. On the other hand, the blockade by flurbiprofen of the weak contractile activity of the hydroxy and hydroperoxy acids allows the suggestion that the oxidized fatty acids may have produced contraction by stimulating prostaglandin synthesis in situ. If this interpretation is correct, the various hydroxy or hydroperoxy acids used in this study may provide a general nonspecific stimulus that provokes the release and subsequent oxidation of endogenous acids. Such a general effect could be the basis for the action of the hydroperoxy forms of linolenic, linoleic, 20:3 (n-3) or 20:4 (n-6) which are reported to cause contractions in rabbit aorta, rat stomach strip or rat colon (15).

Prevention of contraction by added flurbiprofen indicates that the principal agent mediating contraction by the fatty acids was derived from action of the prostaglandin-forming cyclooxygenase. Although the blockage of cyclooxygenase action by flurbiprofen in vitro has been reported to be time-dependent and irreversible (23), we observed that the inhibition of the sphincter muscle contraction could be partially overcome by higher concentrations of 20:4. In this regard, other known cyclooxygenase inhibitors, aspirin and paracetamol, at concentrations of 100 µg/ml were shown to be ineffective in blocking prostaglandin biosynthesis in rabbit ocular tissue microsomes (24). Other inhibitors, such as indomethacin and phenylbutazone were less effective compared with their ability to inhibit spleen or seminal vesicle enzymes. These different tissue selectivities may represent limited penetration of the drug to the location of the cyclooxygenase within the ocular tissue (24) leaving a small but significant portion of the enzyme still uninhibited.

Other oxidized derivatives of arachidonate have been shown to be active in eliciting contractions of iris sphincter muscle. $PGF_{2\alpha}$ was as potent as PGH_2 and the contractile activity of PGH_2 could be attributed to $PGF_{2\alpha}$ if all the PGH_2 were converted to $PGF_{2\alpha}$. Such a total conversion seems unlikely since PGH decomposes in aqueous medium (25,26) to a mixture of PGD, PGE and PGF. Furthermore, the epoxymethano analogs inhibited the PGH-induced contractions, and this would require them to inhibit the reduction of PGH_2 to $PGF_{2\alpha}$; an unlikely event if the formation of PGF is non-enzymic as recently proposed (27).

PGH_2 and its epoxymethano analogs (I and II) have been shown to produce contractions of rabbit aorta (28) and constriction of bronchi (29) with the analog I approximately 6-fold more potent than PGH_2 in eliciting contractions of the aorta (28). The greater reported biological activity of the epoxymethano (28,29) and azo (30) analogs has been attributed to their increased stability allowing continued stimulation of an endoperoxide-responsive receptor. In contrast to these results, we found PGH_2 was 100-fold more potent than the analogs in eliciting cat sphincter muscle contractions. Thromboxane A_2, which is a metabolite of the endoperoxide, was reported to be 10 to 300-times more potent than PGH_2 in contracting rabbit aorta (31,32). The marked contractile activity with small amounts of PGH_2 suggests that the endoperoxide may be enzymatically converted to thromboxane in this tissue, and that it is the

thromboxane rather than the endoperoxide that is causing the major contraction. Pretreatment of the sphincter muscle with epoxymethano analogs (Table IV) did not affect the subsequent muscle response to the analogs while it greatly reduced the response to PGH_2. This indicates that the principal action of PGH_2 was not directly upon an endoperoxide-responsive receptor, but rather a process that can be inhibited by low levels of the analogs. The analogs have recently been shown to be effective inhibitors of the conversion of PGH_2 to thromboxane in platelet microsomes (26). The lack of contraction with PGH_2 following treatment with the analogs is then further support for thromboxane synthase action mediating the contraction by PGH_2. Our findings indicate that PGH_2 may be less effective than the analogs in directly stimulating the endoperoxide-sensitive receptor (as it is in the aorta), and that the structurally-related analogs can prevent contractions with PGH by inhibiting formation of thromboxane.

The high sensitivity of the sphincter muscle to PGH_2 leads to the suggestion that thromboxane synthase activity may be much greater in cat iris sphincter muscle than in rabbit aorta. Furthermore, there may be different types of receptor (or one receptor with different classes of affinity) for contraction; an endoperoxide-type that may be of minor significance in this tissue, and thromboxane and PGF types that may be major ones. Our results indicate that the endoperoxide analogs could exert opposing influences upon muscle contraction, a direct effect upon endoperoxide-responsive receptors and an indirect effect of inhibiting the transformation of endoperoxide to other potent agents such as TXA_2 and possibly PGI_2. In summary, hydroxy and hydroperoxy acids stimulated iris sphincter muscle contraction by a mechanism that was dependent upon cyclooxygenase formation of the endoperoxide and blocked by inhibitors of cyclooxygenase. The endoperoxide, PGH_2, in turn stimulated contraction by a mechanism blocked by inhibitors of thromboxane synthase.

Acknowledgments

This work was supported in part by a grant from the Rackham Foundation, the National Science Foundation (BMS75-13157), and a Fellowship from the Medical Research Council of Canada (H.W.C.).

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