Biochemical Pharmacology, Vol. 24, pp. 1115–1119. Pergamon Press, 1975. Printed in Great Britain.

INHIBITION OF PROSTAGLANDIN BIOSYNTHESIS BY 2-ARYL-1,3-INDANDIONES

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(*Received* 5 *July* 1974; *accepted* 12 *November* 1974)

Abstract—The effect of several 2-aryl-1,3-indandiones on the biosynthesis of prostaglandin E_2 from arachidonic acid by bovine seminal vesicle microsomes was examined. 2-Phenyl-1,3-indandione appeared to be a weak inhibitor but by introducing electron-withdrawing substituents into the phenyl ring and augmenting lipophilicity, very active compounds were obtained. Activity, however, disappeared entirely on substitution in the *ortho* position. Inhibition was not related to the presence of an acidic group and was irreversible in some cases. The inhibition of prostaglandin biosynthesis by 2-aryl-1,3-indandiones showed no clear relationship with anti-inflammatory action.

It is now generally assumed that prostaglandins (PGs) play a major role in the inflammatory process. PGE_2 and $PGF_{2\alpha}$ have been demonstrated in the exudate [1, 2], and recent results indicate that they function as potentiators of other inflammatory mediators [3, 4]. Since under normal conditions the tissue concentration of prostaglandins is low, release must be due to biosynthesis. The observation by Vane and co-workers [5–7] that non-steroidal anti-inflammatory drugs inhibit this biosynthesis in clinically attainable concentrations is the most significant contribution in recent years to a better understanding of the mechanism by which these drugs act.

This inhibition has been demonstrated in many tissues, such as lung [5], spleen [6, 7], sheep [8–10] and bovine [11, 12] seminal vesicles, skin [13, 14] and tumour cells [15, 16], and also *in vivo* [17, 18] (see ref. 19 for an extensive review of this subject). In addition, correlations between the relative potencies as prostaglandin synthetase inhibitors *in vitro* and anti-inflammatory activity *in vivo* have been reported [7, 10, 11]. Inhibition by indomethacin is time-dependent [8, 20] and irreversible [9].

The present paper describes the effects of some anti-inflammatory 2-aryl-1, 3-indandiones on the biosynthesis of PGE_2 by bull seminal vesicle microsomes. Lipophilicity and in particular the electronic effects of the substituents were found to determine the inhibitory activities of these compounds.

MATERIALS AND METHODS

Chemicals. 2-Aryl-1,3-indandiones were synthesized as described by Bruynes [21]. Salicylic acid was obtained from E. Merck AG and phenylbutazone from Geigy-Ciba. Indomethacin was kindly supplied by Merck Sharpe & Dohme, Nederland B.V. Drugs were dissolved in ethanol and the solutions were used within 1 hr after preparation.

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) was purchased from Sigma Chemical Co. (grade 1, 99%), dissolved in Tris–HCl, pH 8·3, and stored under nitrogen at −20°. GSH (glutathione) was obtained from Boehringer-Mannheim and L-adrenaline from E. Merck AG.

Prostaglandin E_2 was a gift from the Gist-Brocades N.V. Research Department, Delft.

Preparation of bovine seminal vesicle microsomes. Bovine seminal vesicles were obtained from a slaughter house, cooled in ice, and stored at −40°. The microsomal fraction was prepared as described by Takeguchi *et al.* [22]. The material was homogenized in 2 vol of 0·1 M phosphate buffer, pH 8·0, using a Waring Blendor (2 min at maximal speed). The homogenate was centrifuged at 12,000 *g* for 10 min and the supernatant was recentrifuged at 100,000 *g* for 1 hr. The microsomes from 7 animals were pooled, lyophilized and stored at −40°. This enzyme preparation was used in all experiments; 392 g of vesicles yielded 8·6 g of microsomes containing about 43 per cent protein (determined as described by Lowry *et al.* [23]).

Incubations. Unless otherwise stated, incubations were carried out in medium (3 ml) containing 50 mM Tris–HCl (pH 8·3), 1 mM GSH, 1 mM adrenaline, 0·167 mM arachidonic acid and about 2 mg of microsomal protein at 37° for 5 min. Drugs were added in 0·1 ml of ethanol and the same amount of ethanol was added to the controls. The reaction was stopped by adding 0·3 ml of 1 N HCl and the reaction mixture extracted three times with 3 ml of diethyl ether. The combined extracts were extracted with 2 ml of water and evaporated to dryness. The residue was dissolved in 0·2 ml of absolute ethanol and analyzed by the Zimmerman reaction.

Assay. The Zimmerman reaction was performed as described by Takeguchi *et al.* [22]. Absorbance was measured at 620 nm, thus avoiding errors arising from the absorbance of arylindandiones. Recovery after the extraction procedure was about 75 per cent and a good linearity was found. The presence of 0·1 μmole PGE_2 in the incubation mixture resulted in an absorbance of 0·092.

RESULTS

Prostaglandin synthetase activity. Biosynthesis of PGE_2 by bovine seminal vesicles was measured as

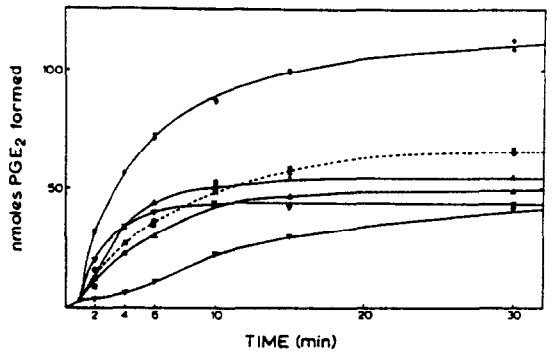


Fig. 1. Time-course of the conversion of arachidonic acid into PGE_2. ○ ●, control; □ ■, 2-(3-*tert*-butylphenyl)-1,3-indandione, 15 (7.5×10^{-5} M); △ ▲, 2-(3,5-dichlorophenyl)-1,3-indandione, 23 (5×10^{-6} M); ▽ ▼, indomethacin (10^{-5} M). In the case of closed symbols, the enzyme was pre-incubated in the absence of substrate at 37° for 5 min. The numbers 15 and 23 refer to the compounds in Table 1.

described in Methods. Figure 1 shows the time-course of this synthesis and indicates that pre-incubation in the absence of substrate had no effect. Prostaglandin synthetase activity between the 1st and the 5th min was 9.5 ± 0.3 nmoles of PGE_2/min × mg protein (mean ± S.D. of 10 experiments).

Inhibition by drugs. Inhibitory effects of 2-aryl-1,3-indandiones and some other compounds on the biosynthesis of PGE_2 were measured at three suitable drug concentrations and the concentration producing a 50 per cent inhibition was determined graphically (ID_{50}). These values are listed in Tables 1 and 2.

A number of *ortho*-substituted 2-phenyl-1,3-indandiones, tested at concentrations up to 3×10^{-4} M, had no effect on PGE_2 synthesis. An attempt to

Table 2. Inhibitory potencies of various compounds on the biosynthesis *in vitro* of PGE_2

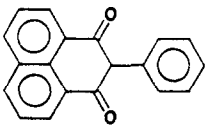
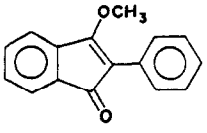
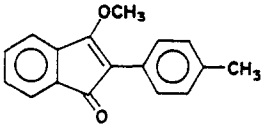
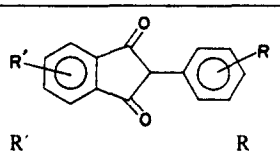
Compound No.	Compound	$-\log ID_{50}$ (M^{-1})
28		3·62
29		4·41
30		3·99
	Salicylic acid	<3·00
	Phenylbutazone	3·40
	Indomethacin	5·00

Table 1. Inhibitory potencies ($-\log ID_{50}$) and physicochemical properties of 2-aryl-1,3-indandiones



Compound No.	R′	R	π*	σ†	$-\log ID_{50}$ (M^{-1}) Obsd	Calcd‡
1	H	H	0·00	0·000	3·22	3·47
2	H	4-methyl	0·56	−0·170	3·60	3·42
3	H	4-ethyl	1·02	−0·151	3·84	3·63
4	H	4-isopropyl	1·53	−0·150	3·84	3·84
5	H	4-*t*-butyl	1·98	−0·200	3·89	3·93
6	H	4-*n*-octyl	4·03	−0·250	4·68	4·67
7	H	4-phenyl	1·89	−0·010	4·73	4·21
8	H	4-chloro	0·71	0·227	4·38	4·13
9	H	4-bromo	0·86	0·232	4·44	4·19
10	H	4-trifluoromethyl	0·88	0·540	4·94	4·71
11	H	4-methoxy	−0·02	−0·268	3·37	3·03
12	H	3-methyl	0·56	−0·069	3·49	3·58
13	H	3-ethyl	1·02	−0·070	3·52	3·76
14	H	3-isopropyl	1·53	−0·050	3·83	4·00
15	H	3-*t*-butyl	1·98	−0·100	4·09	4·10
16	H	3-chloro	0·71	0·373	4·16	4·37
17	H	3-trifluoromethyl	0·88	0·430	4·47	4·53
18	H	3-methoxy	−0·02	0·115	3·50	3·65
19	H	3,5-dimethyl	1·12	−0·138	3·42	3·69
20	H	3,5-diethyl	2·04	−0·140	3·80	4·06
21	H	3,5-diisopropyl	3·06	−0·100	4·17	4·53
22	H	3,5-di-*t*-butyl	3·96	−0·200	4·95	4·72
23	H	3,5-dichloro	1·42	0·746	5·19	5·26
24	H	3,5-dimethoxy	−0·04	0·230	3·78	3·83
25	5-*t*-butyl	H			3·95	
26	5-trifluoromethyl	3-methyl			4·70	
27	H	3,4-$(CH)_4$			4·58	

* From ref. 24. † From ref. 25. ‡ Calculated by using the regression equation.

Table 3. Reversibility of inhibition of prostaglandin synthetase activity

	Activity after pre-incubation in the presence of inhibitor* at 37° for 10 min and removal of the inhibitor by dilution and centrifugation.†					
	Control	Control	Comp. 15	Comp. 22	Comp. 23	Indomethacin
Activity‡ (nmoles/min)	68·5 ± 0·6	30·3 ± 1·1	49·8 ± 1·7	40·0 ± 2·0	16·3 ± 0·5	6·3 ± 1·2
% Activity (vs control)	100	44	73	58	24	9
% Activity (vs pre-incubation control)	—	100	165	132	54	17

* Inhibitor concentration was as follows: comp. 15, 3×10^{-4} M; 22, 5×10^{-5} M; 23, $2{\cdot}5 \times 10^{-5}$ M; indomethacin, 5×10^{-6} M. Inhibition was more than 90% at this concentrations.

† Medium was diluted with 5 vol of buffer; the enzyme was centrifuged at 100,000 *g* for 1 hr and incubated in a medium as described under Methods.

‡ Results are the means of three experiments with S.D.

correlate the ID_{50} values from Table 1 with π and σ (hydrophobic and electronic parameters, respectively) was made using a least square analysis, affording the following equation:

$$-\log (ID_{50}) = 3{\cdot}474 + 0{\cdot}397\,\pi + 1{\cdot}635\sigma$$
$$(8{\cdot}404) \quad (8{\cdot}304)$$
$$n = 24,\ r = 0{\cdot}912,\ s = 0{\cdot}239,\ F = 52{\cdot}023$$

where n is the number of compounds, r the regression coefficient, s the standard error of the estimate and F the overall statistical significance of the equation. The figures in parentheses are the Student's t-test values. For a study of the mechanism of inhibition, three compounds were selected, viz. 2-(3,5-dichlorophenyl)-1,3-indandione (23), a powerful inhibitor, 2-(3-*tert*-butylphenyl)-1,3-indandione (15), a moderate inhibitor, and indomethacin, a well-known anti-inflammatory agent. 2-(3,5-Di-*tert*-butylphenyl)-1,3-indandione (22) was used in part of this study.

Effects of incubation time and pre-incubation. Figure 1 shows that inhibition of PGE_2 synthesis by compound 15 was not time-dependent. On the other hand, compound 23 and indomethacin inhibited synthesis completely within 10 and 6 min, respectively. With compound 15 no difference was found when enzyme and inhibitor were pre-incubated in the absence of substrate at 37° for 5 min. The two other compounds were obviously more inhibitory after pre-incubation.

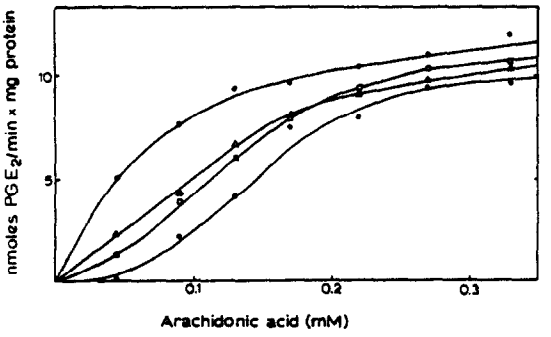


Fig. 2. Effect of substrate concentration on rate of PGE_2 synthesis. ○, control; ●, 2-(3-*tert*-butylphenyl)-1,3-indandione, 15 (3×10^{-5} M); △, 2-(3,5-dichlorophenyl)-1,3-indandione, 23 ($2{\cdot}5 \times 10^{-6}$ M); □, indomethacin (5×10^{-6} M). The numbers 15 and 23 refer to the compounds in Table 1.

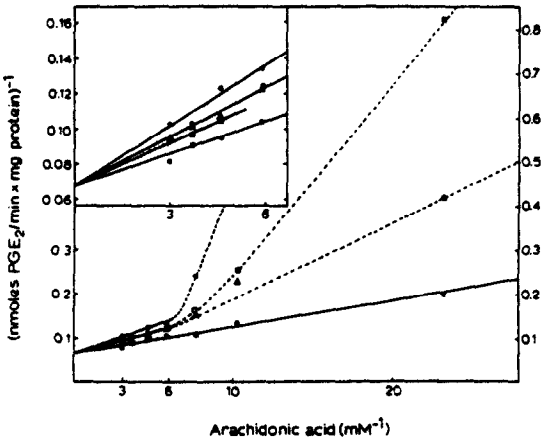


Fig. 3. Lineweaver–Burk plot of inhibition of PGE_2 synthesis. ○, control; ●, 2-(3-*tert*-butylphenyl)-1,3-indandione, 15 (3×10^{-5} M); △, 2-(3,5-dichlorophenyl)-1,3-indandione, 23 ($2{\cdot}5 \times 10^{-6}$); □, indomethacin (5×10^{-6} M). The numbers 15 and 23 refer to the compounds in Table 1.

Irreversibility of inhibition. The following experiment was carried out to establish any irreversibility of inhibition. Enzyme and excess inhibitor (about 4 times ID_{50}) were pre-incubated in the absence of substrate at 37° for 10 min. Inhibition by this concentration was more than 90 per cent. The inhibitor was removed by adding 5 vol of buffer and centrifuging the enzyme at 100,000 *g* for 1 hr. The pellet was suspended in the usual medium, and incubated in the presence of substrate without further addition of inhibitors. The results are shown in Table 3.

In the period of 3 hr between the first and the second incubation, 56 per cent of the enzyme activity was eliminated by denaturation. In the presence of compounds 15 or 22, there was less denaturation and activity could be largely recovered. After pre-incubation with indomethacin, inhibition remained above 90 per cent. Inhibition by compound 23 was only partially offset.

Effect of substrate concentration on inhibition. Determination of PGE_2 synthesis at different substrate concentrations afforded a saturation curve normal for enzymes; see Fig. 2. In the presence of an inhibitor, however, the curve assumed a sigmoid shape. Inhibition was most overt at low substrate concentrations, and the Lineweaver–Burk plot in

Fig. 3 shows inhibition at high substrate concentrations to be competitive.

DISCUSSION

The biosynthesis of prostaglandins is catalyzed by a barely defined multistage enzyme complex [12, 22, 26]. Under the conditions employed in the present experiment, only PGE_2 can be formed from arachidonic acid [22, 26]. The enzyme reaction shows the normal Michaelis–Menten kinetics (see Figs. 2 and 3) with $K_m = 8{\cdot}2 \times 10^{-5}$ M (Takeguchi *et al.* [22] found, however, $K_m = 4 \times 10^{-5}$ M). PGE_2 production with time shows two phenomena also described by Lands *et al.* [20] for the oxygenase reaction of a sheep vesicular gland preparation, namely a period of latency before the onset of reaction and termination of the reaction before all substrate has been converted. Lands *et al.* [20] believe that the latter phenomenon is due to a self-catalyzed inactivation of the enzyme. Normal denaturation is out of the question, as pre-incubation has no effect on activity (Fig. 1). Addition of a similar amount of enzyme after 10 min incubation results in a minimal increase in PGE_2 synthesis (unpublished results), indicating that one or more reaction products inhibit biosynthesis. This is in agreement with the observations of Wallach and Daniels [27]. In another experiment (unpublished) the presence of 114 nmoles of PGE_2 in the incubation medium had no influence on biosynthesis, hence a by-product must have been responsible for the inhibition mentioned above.

As seen from Tables 1 and 2, several 2-aryl-1,3-indandiones can inhibit the biosynthesis of PGE_2. There is also a distinct relationship between structure and activity. Thus, 2-phenyl-1,3-indandione is a weak inhibitor but by introducing electron-withdrawing substituents into the phenyl ring and augmenting lipophilicity, active compounds are obtained, as has also been substantiated by the regression equation derived from Table 1. Activity, however, disappears entirely by substitution in the *ortho* position. This suggests that activity is largely determined by steric conformation of the molecule. The results presented in Table 2 show that activity is not related to the presence of an acidic group; 3-methoxy-2-phenylindone (29) is even more active than the parent compound (1). Ham *et al.* [10] also described two non-acidic inhibitors of prostaglandin synthesis. The question, however, whether the two ethers (29 and 30) act in the same way as acidic compounds, has not been examined.

Whilst many publications have been devoted to the inhibition of prostaglandin synthesis by non-steroidal anti-inflammatory drugs, little attention has been paid to the underlying mechanism. In our work, the mechanism of action was studied in more detail for only three compounds. Inhibition by compounds 15 and 22 proved to be fully reversible (see Table 3) and thus not time-dependent. Pre-incubation in the absence of substrate, therefore, did not affect activity (Fig. 1). Indomethacin, on the other hand, caused irreversible inhibition; inhibition increased with time, and pre-incubation enhanced it considerably (Fig. 1). These findings are in accordance with already reported observations [8, 9, 20]. Many unsaturated fatty acids likewise produce irreversible inhibition [28, 29], which, according to Lands *et al.* [20, 30], is the result of irreversible enzyme destruction. These authors believe, however, that indomethacin has another site of inhibition [20]. Compound 23 occupies an intermediate position between indomethacin and 15 or 22, inhibition being only partially irreversible. The reversibility of inhibition is not connected with the potency of the compound.

Vane's first publication in this field [5] speculated on a possible competition between arachidonic acid and non-steroidal anti-inflammatory drugs, which are also acidic compounds. Although it has meanwhile become clear that non-acidic compounds can also inhibit prostaglandin synthesis (Table 2 and ref. 10), inhibition proves competitive [10]. This is also the case with inhibition by compounds 15 and 23 and indomethacin. At high substrate concentrations, inhibition is purely competitive, as can be seen in the double reciprocal plot of Fig. 3. Conversely, low substrate concentrations imply an inhibition much stronger than that expected from the normal Michaelis kinetics, and the curves in Fig. 2 are somewhat sigmoid. Such a discrepancy has also been reported by Ham *et al.* [10]. It should be noted, however, that inhibition by unsaturated fatty acids is purely competitive [31]. This difference might point to another site of inhibitory action.

Our previous publication [32] dealt with the anti-inflammatory activities of 2-aryl-1,3-indandiones, determined in the carrageenan oedema test as described by Winter *et al.* [33]. The anti-inflammatory activities of only 18 compounds listed in Table 1 were available and these ED_{50} values were plotted against the inhibitory potencies of Table 1 (Fig. 4). Various parallels have thus been found, as indicated below.

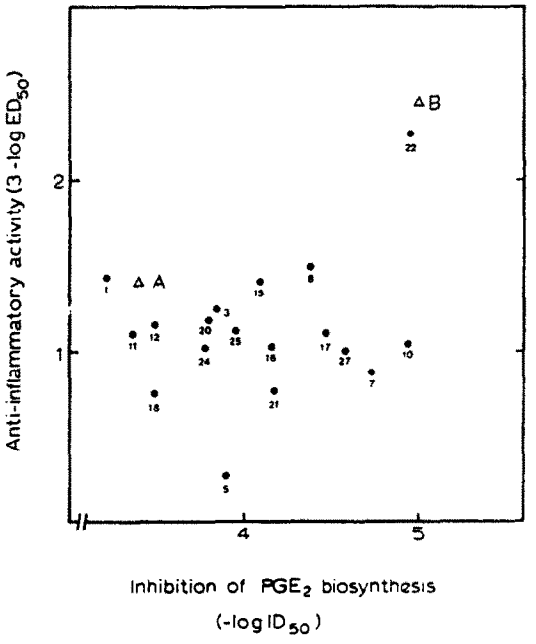


Fig. 4. Correlation between anti-inflammatory activity and inhibition of PGE_2 biosynthesis by 2-aryl-1,3-indandiones (●), phenylbutazone (A) and indomethacin (B). The numbers refer to the compounds in Table 1.

The *ortho*-substituted 2-aryl-1,3-indandiones usually have no anti-inflammatory activity and are inactive as inhibitors of the biosynthesis of PGE_2. Phenylbutazone and 2-phenyl-1,3-indandione (1) are equipotent in both tests, and so are indomethacin and compound 22. There are also several compounds for which such a correlation is absent. Thus, compound 23 is the most active inhibitor of PGE_2 biosynthesis but is virtually without anti-inflammatory activity.

In general, it may be concluded that in the indandione series there is no clear relationship between the inhibition of prostaglandin biosynthesis by bovine seminal vesicles *in vitro* and anti-inflammatory activity *in vivo*.

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