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INHIBITION OF PROSTAGLANDIN BIOSYNTHESIS IN RAT SMALL INTESTINE BY SL-573*

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Abstract—The 20,000 *g* supernatant of rat small intestine homogenates converted [1-^{14}C]arachidonic acid into several compounds, including prostaglandin $F_{2\alpha}$ (20.0 per cent), E_2 (16.1 per cent), D_2 (45.2 per cent), and other unknown products (18.7 per cent). The identity of each prostaglandin, which was isolated by Sephadex LH-20 column chromatography and subsequent preparative thin-layer chromatography (t.l.c.), was performed by t.l.c., bioassay (contraction of rat stomach fundus strip), sodium borohydride reduction and mass spectrometry. The predominant prostaglandin biosynthesized by rat small intestine was prostaglandin D_2. 1-Cyclopropylmethyl-4-phenyl-6-methoxy-2-(1H)-quinazolinone (SL-573) and indomethacin inhibited the prostaglandin biosynthesis by the rat small intestinal enzymes. The IC_{50} values (concentration of inhibitor resulting in 50 per cent inhibition) were 9.1 μg/ml for SL-573 and 5.6 μg/ml for indomethacin. The inhibition of prostaglandin biosynthesis by either drug was reversible. The reversible inhibition of indomethacin in this case may be due to the presence of endogeneous arachidonic acid in the enzyme preparation, since arachidonic acid protected against the irreversible and time-dependent inhibition of the bovine seminal vesicle enzymes by indomethacin.

Prostaglandins and their synthesizing enzymes are widely distributed in animal tissues, and considered to be closely concerned with the physiological and pathological processes in these tissues [1, 2]. The inhibition of prostaglandin biosynthesis, therefore, may result in undesirable side effects in such tissues.

Nonsteroidal anti-inflammatory agents such as indomethacin are known to inhibit the prostaglandin cyclo-oxygenase in many biological systems [3–6]. These drugs cause gastrointestinal irritation in animals and man [7–10]; the inhibition of prostaglandin biosynthesis is postulated to be involved in this side effect [11]. This hypothesis is strongly supported by the data that indomethacin- and flufenamic acid-induced intestinal ulcers in rats were prevented by various prostaglandins and their analogs [12].

The prostaglandin biosynthesis in gut, however, has not been well established thus far, although there are several papers concerned with it [13, 14]. The purpose of the present study is to assess the effect of 1-cyclopropylmethyl-4-phenyl-6-methoxy-2-(1H)-quinazolinone (SL-573) and indomethacin on prostaglandin biosynthesis in rat small intestine, and to discuss the possible relationship between prostaglandin biosynthesis inhibition and ulcer formation. SL-573 is a novel anti-inflammatory agent which inhibits prostaglandin biosynthesis by bovine seminal vesicle microsomes [15], but it does not induce intestinal lesions in rats [16]. Indomethacin, on the other hand, induces severe intestinal perforations at a single oral dose of 10 mg/kg. The rat small intestinal enzymes, therefore, may exhibit different drug specificity for SL-573 and indomethacin.

* SL-573 = 1-cyclopropylmethyl-4-phenyl-6-methoxy-2-(1H)-quinazolinone.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]arachidonic acid (sp. act. 58 mCi/m-mole, Radiochemical Centre, Amersham, England), unlabeled arachidonic acid (grade: 99 per cent pure, Sigma Chemical Co., St. Louis, MO), and prostaglandin $F_{2\alpha}$ and E_2 (Fujiyakuhin Kogyo Co., Tokyo) were purchased from commercial sources. Prostaglandin D_2, SL-573 and indomethacin were synthesized in this laboratory.

Preparation of enzymes. HLA-Wistar strain male rats (Nihon Dobutsu, Osaka) weighing from 160 to 220 g were allowed free access to food and water, and were killed by decapitation. Small intestines were immediately excised, washed with Tyrode's solution, and placed on ice. All subsequent operations were done at 0–5°. The small intestines were cut into small pieces, suspended in 3 vols of 1.15% KCl containing 2 mM noradrenaline and 2 mM potassium phosphate buffer (pH 7.4), and homogenized in a glass homogenizer (Ikemoto Rikakogyo Co., Tokyo). The homogenates were centrifuged at 20,000 *g* for 20 min. The supernatant was filtered through cotton packed in a glass column and used as the enzyme source within 1 hr. The enzyme activities in small intestine homogenates were almost completely recovered in the supernatant fluid. Further purification of the enzymes has not been achieved, because their activities disappeared after ultracentrifugation (65,000 *g*, 90 min). The reconstitution of supernatant and precipitate resulted in the restoration of enzyme activities.

Assay of prostaglandin biosynthesis activity. Unless otherwise stated, prostaglandin biosynthesis was carried out in 1.0 ml of reaction medium consisting of [1-^{14}C]arachidonic acid (0.082 mM, 0.05 μCi), enzymes (0.25 ml of 20,000 *g* supernatant), noradrenaline (2 mM), and pH 7.4 potassium phosphate buffer (50 mM). Arachidonic acid, SL-573 and indomethacin

were dissolved in ethanol and added to the reaction medium. Ethanol was added not to exceed 10 μl/ml of medium. Noradrenaline enhanced enzyme activities two to three times. Other co-factors such as hydroquinone, reduced glutathione, serotonin, tryptophan and hemoglobin were less effective than noradrenaline.

The reaction was started by addition of enzymes at 30°, and 10 min later stopped by addition of 1.0 ml ethanol. The reaction medium was then mixed with 1.0 ml of pH 3.0 citrate buffer (200 mM) containing 10% (w/v) NaCl and 5.0 ml ethyl acetate, and centrifuged at 2500 rev/min for 10 min. Radioactivities were quantitatively extracted into the organic phase (97.8 ± 1.2 per cent). Five ml of the organic phase was neutralized with 1.0 ml of 0.025 M ammonium–ethanol solution, and evaporated *in vacuo*.

The resultant residue was re-dissolved in 0.5 ml ethyl acetate, and shaken with 1.0 ml of pH 7.4 potassium phosphate buffer (200 mM) and 3.0 ml *n*-hexane. After standing for 15 min, an *n*-hexane phase was removed with aspiration. This procedure was repeated three times. An aqueous phase was then mixed with methanol, and the radioactivity was assayed with a liquid scintillation spectrometer (Packard Tri-Carb model 3375 or 3385). As reported previously [15], arachidonic acid and its non-polar derivatives were selectively extracted into the organic phase under neutral pH conditions (pH 7.4), while protaglandins remained in the aqueous phase.

Isolation of prostaglandins. Prostaglandin biosynthesis was undertaken in a large scale (100 ml of reaction medium) as described above to prepare prostaglandins in quantities sufficient for characterization.

The reaction medium was extracted with 2 vols of *n*-hexane–ethyl acetate (2:1, v/v) twice. The organic phase, in which most of arachidonic acid was selectively extracted, was discarded. The aqueous phase was acidified by the addition of 1 vol. of pH 3.0 citrate buffer (200 mM) containing 10% (w/v) NaCl, and shaken with 3 vols ethyl acetate to extract prostaglandins. Extraction was repeated twice. The extracts were combined, neutralized with 1.0 M ammonium–ethanol solution, and evaporated *in vacuo*. The resultant residues were dissolved in about 3 ml chloroform and submitted to Sephadex LH-20 column chromatography.

Sephadex LH-20 column chromatography was carried out by the modified method of Christensen and Leyssac [17]. Prostaglandins were separated into three fractions. Prostaglandin D_2 was eluted in fraction 1, prostaglandin E_2 in fraction 2, and prostaglandin $F_{2\alpha}$ in fraction 3. Each fraction was evaporated *in vacuo* and submitted to preparative thin-layer chromatography (t.l.c.) [solvent system (II) described below]. The radioactive bands on t.l.c. plates were located by a t.l.c. radio-scanner (Aloka, model 202B), scraped from the plates, and shaken with 1.0 ml of pH 3.0 citrate buffer (200 mM) and 5.0 ml ethyl acetate. After centrifugation (2500 rpm, 10 min), the ethyl acetate phase was evaporated *in vacuo*. The resultant residues were dissolved in ethanol and stored at −25° until used.

Thin-layer chromatography. Thin-layer chromatography was carried out on precoated Silica gel plates (HF_{254}, thickness 0.25 mm, E. Merck A.-G. Darmstadt, West Germany). The chromatogram was developed with two solvent systems as follows: (I) chloroform–methanol–acetic acid (18:1:1, v/v) and (II) an upper phase of ethyl acetate–acetic acid–*iso*-octane–H_2O (90:20:50:100, v/v). Radioactive spots on t.l.c. were located by the t.l.c. radio-scanner. Unlabeled arachidonic acid, prostaglandin $F_{2\alpha}$ and E_2 on t.l.c. were visualized with iodine.

Assay of biological activity. The rat stomach strip was prepared according to the method of Vane [18] from HLA-Wistar male rats weighing 200–250 g which had been fasting overnight. Rat stomach strips were suspended with 0.6 g tension in 10 ml Tyrode's solution at 35°. Contractions were recorded by an isotonic transducer (TD-111S and JD-111S, Nihon Koden Kogyo, Tokyo) and a recticorder (RJG-3028, Nihon Koden Kogyo). Prostaglandins were dissolved in 100 mM Tris–HCl buffer (pH 7.4) and added to the tissue bath not to exceed 100 μl. The dose–response curve was obtained by the cumulative contraction.

Sodium borohydride reduction. A prostaglandin (10–50 μg) was dissolved in 1.0 ml of absolute methanol containing 5 mg sodium borohydride. After standing at 0° for 1 hr, the reaction mixture was shaken together with 5.0 ml ethyl acetate and 1.0 ml of pH 3.0 citrate buffer (200 mM). The extracts were evaporated *in vacuo*, and submitted to t.l.c. and bioassay. Authentic prostaglandin E_2 was reduced to a 1:1 mixture of prostaglandin $F_{2\alpha}$ and $F_{2\beta}$.

Mass spectrometry. Mass spectra were obtained with a Shimadzu-LKB 9000 instrument, using direct probe introduction with an ion source temperature of 100°, electron potential of 12 eV, and an ionizing current of 60 μA.

Assay of prostaglandin biosynthesis activity by bovine seminal vesicle microsomes. Preparation of bovine seminal vesicle microsomes and assay of their prostaglandin biosynthesis activity were performed as described in a previous paper [15].

RESULTS

Prostaglandin biosynthesis. As illustrated in Fig. 1, [1-^{14}C]arachidonic acid was converted into several compounds, including compound I (11.4 per cent), II (20.0 per cent), III (16.1 per cent), and IV (45.2 per cent), by the 20,000 *g* supernatant of rat small intestine homogenates. Compound V represents arachidonic acid.

R_f values and biological activities of compounds II, III, IV, and sodium borohydride-reduced compound IV are listed in Table 1. R_f values of compounds II and III coincided precisely with those of prostaglandin $F_{2\alpha}$ and E_2, respectively, but their biological activities were more potent than those of their corresponding authentic compounds. Probably, this discrepancy is due to the fact that prostaglandins were not only synthesized from exogeneously added arachidonic acid, but also from endogeneous substrate contained in the 20,000 g supernatant of rat small intestine homogenates. As shown in Table 2, the prostaglandin levels in rat small intestine were very low, but a 10-min incubation of the 20,000 *g* supernatant at 30° resulted in an increase of compounds II and III even if arachidonic acid was not exogeneously added to the medium.

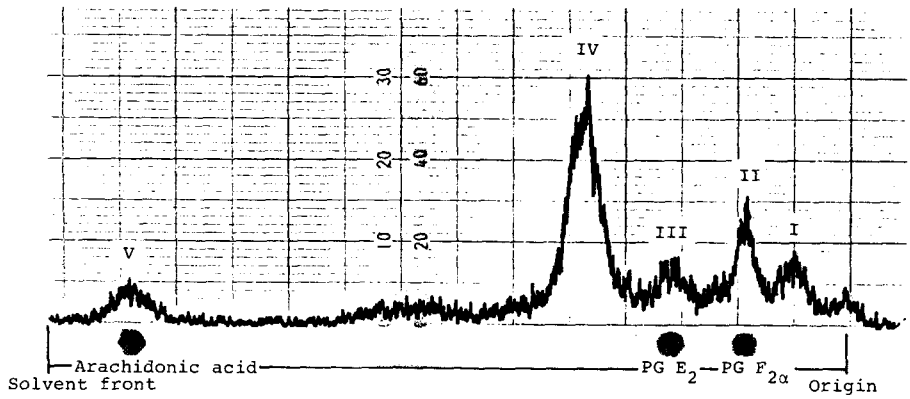

Fig. 1. Prostaglandin biosynthesis by 20,000 *g* supernatant of rat small intestine homogenates. The chromatogram was developed with an upper phase of ethyl-acetate–acetic acid–*iso*-octane–H_2O (90:20:50:100, v/v).

Compound IV represented 45.2 per cent of total products. It was chromatographically consistent with prostaglandin D_2 and biologically inactive, as shown in Table 1. When compound IV was treated with sodium borohydride, it was reduced to the compound coinciding with prostaglandin $F_{2\alpha}$ on t.l.c. The biological activity of reduced compound IV was about 1.6 times more potent than that of authentic prostaglandin $F_{2\alpha}$. Probably, this is also explained by the presence of endogeneous substrate in the enzyme preparation as described above.

Figure 2 shows the mass spectra of compound IV, prostaglandin D_2 and E_2. The mass spectrum of compound IV was very similar to that of prostaglandin D_2. Compound IV exhibited fragment ions at the same m/e values as prostaglandin E_2, although their intensity differed from each other. Additionally, the fragmentation pattern of compound IV was very similar to that of prostaglandin D_1 as reported by Foss *et al.* [19]. From these data, compound IV was estimated to be prostaglandin D_2.

Prostaglandin E_2 is well known to be reduced to two isomers, prostaglandin $F_{2\alpha}$ and $F_{2\beta}$, in ratio of 1:1 by sodium borohydride. Prostaglandin D_2, however, was almost quantitatively reduced to prostaglandin $F_{2\alpha}$. Foss *et al.* [19] have also reported that the reduction of prostaglandin D_1 with sodium borohydride affords a 10:1 mixture of prostaglandin $F_{1\alpha}$ and 11-epi-prostaglandin $F_{1\beta}$. D-type prostaglandins, therefore, may possess the steric hindrance against the introduction of β-hydroxyl group at C-11 position.

Inhibition of prostaglandin biosynthesis by SL-573 and indomethacin. Figure 3 shows that SL-573 and indomethacin caused the concentration-dependent inhibition of prostaglandin biosynthesis by 20,000 *g* supernatant of rat small intestine homogenates. The concentrations of inhibitors used were as follows: SL-573, 5.0, 7.0, 10, 14 and 20 μg/ml; and indomethacin, 2.0, 4.0, 6.0, 9.0 and 14 μg/ml. The IC_{50} values (concentration of inhibitor resulting in 50 per cent inhibition) were 9.1 μg/ml for SL-573 and 5.6 μg/ml for indomethacin. SL-573 and indomethacin inhibited the synthesis of all products from arachidonic acid to the same degree. The data, therefore, suggest that these drugs inhibit the cyclo-oxygenase.

The effect of pre-incubation of SL-573 and indomethacin with rat small intestinal enzymes on prostaglandin biosynthesis was investigated as follows. SL-573 (10 μg/ml) and indomethacin (5.0 μg/ml) were added to the reaction medium, and pre-incubated at 30° for 2 min under anaerobic conditions using Tunberg-type test tubes. The reaction was then initiated

Table 1. R_f values and biological activities of compounds produced from [1-^{14}C]arachidonic acid by 20,000 *g* supernatant of rat small intestine homogenates

Compound	R_f value (I)*	R_f value (II)†	Biological activity equipotent molar ratio (PG E_2 = 1)‡
Compound II	0.11	0.088	5.1
Compound III	0.23	0.15	0.75
Compound IV	0.28	0.22	>50
Reduced compound IV	0.11	0.088	7.3
Prostaglandin $F_{2\alpha}$	0.11	0.088	11
Prostaglandin E_2	0.23	0.15	1.0
Prostaglandin D_2	0.28	0.22	
Arachidonic acid	0.69	0.72	

* Solvent system (I): chloroform–methanol–acetic acid (18:1:1, v/v).

† Solvent system (II): an upper phase of ethyl-acetate–acetic acid–*iso*-octane–H_2O (90:20:50:100, v/v).

‡ The equipotent molar ratios for each compound were calculated from their radioactivities on the basis of the specific radioactivity of [1-^{14}C]arachidonic acid.

Table 2. Biosynthesis of prostaglandins from endogenous and exogenous substrate by 20,000 g supernatant of rat small intestine homogenates

	Radiochemical assay*	Bioassay*	Bioassay†	Bioassay‡
Compound II	2.0	4.5§	2.4§	0.25§
Compound III	1.6	2.2‖	1.2‖	0.16‖
Compound IV	4.6			
Reduced compound IV		7.1§		

* Concentration of exogenous substrate, 0.082 mM; incubation time, 10 min.
† No exogenous substrate added; incubation time, 10 min.
‡ No exogenous substrate added; supernatant was not incubated.
§ Amounts of compound II and reduced compound IV were calculated by the dose–response curve of prostaglandin $F_{2\alpha}$.
‖ Amounts of compound III were calculated by the dose–response curve of prostaglandin E_2.

by aeration for 10 min. The inhibitory activities of SL-573 and indomethacin under pre-incubation conditions were compared with those under no pre-incubation conditions. As shown in Fig. 4, the inhibition of prostaglandin biosynthesis by SL-573 and indomethacin was not significantly affected by their pre-incubation with enzymes. Additionally, the dilution of the drugs in a pre-incubation medium was found to result in the reversible restoration of enzyme activity. These results indicate that SL-573 and indomethacin reversibly inhibit the rat small intestinal enzymes, unlike the bovine seminal vesicle enzymes, which were inhibited by indomethacin in a time-dependent and irreversible manner [15].

As the rat small intestinal enzymes contained considerable amounts of endogeneous arachidonic acid (about 0.3 μmole/ml of 20,000 g supernatant of rat small intestine homogenates), the following experiment was performed to assess how arachidonic acid modifies the time-dependent and irreversible inhibition of indomethacin on bovine seminal vesicle enzymes.

The inhibitory action of SL-573 and indomethacin on prostaglandin biosynthesis by bovine seminal vesicle microsomes was compared under the following three different conditions: (1) no pre-incubation, (2) pre-incubation in the absence of arachidonic acid and (3) pre-incubation in the presence of arachidonic acid.

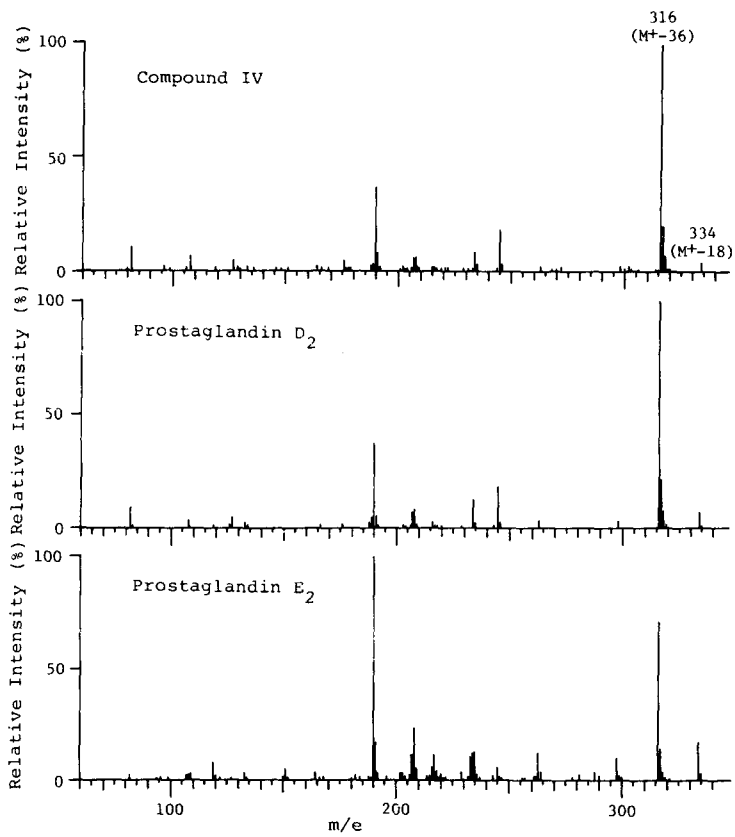

Fig. 2. Mass spectra of compound IV, prostaglandin D_2 and E_2. Mass spectra were obtained with a Shimadzu-LKB 9000 instrument, using direct probe introduction with an ion source temperature 100°, electron potential of 12 eV, and an ionizing current of 60 μA.

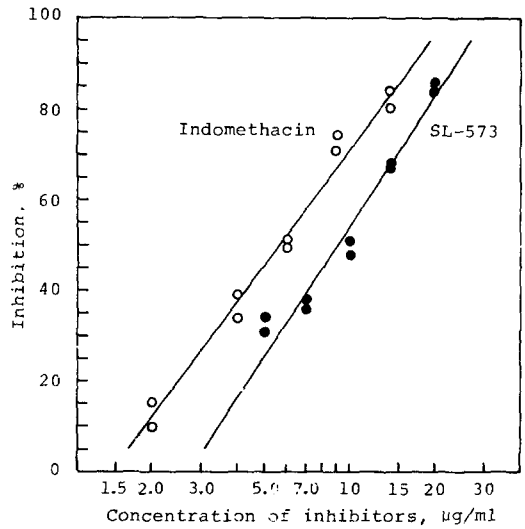

Fig. 3. Inhibition of prostaglandin biosynthesis by 20,000 *g* supernatant of rat small intestine homogenates by SL-573 and indomethacin.

The reaction was carried out at 37° for 10 min in 1.0 ml medium consisting of [1-^{14}C]arachidonic acid (0.082 mM), bovine seminal vesicle microsomes (1.0 mg protein/ml), hydroquinone (1.0 mM), ethanol (1.0 per cent) and pH 7.4 potassium phosphate buffer (50 mM). Ethanol was used to dissolve arachidonic acid and inhibitors. The concentrations of inhibitors used were 0.5 μg/ml for SL-573 and 0.1 μg/ml for indomethacin. In no pre-incubation, the reaction was started by addition of an aliquot of enzymes. The pre-incubation, on the other hand, was done at 37° for 2 min in the absence or presence of arachidonic acid under anaerobic conditions using Tunberg-type test tubes, and the reaction was initiated by aeration. In the case of pre-incubation in the absence of arachidonic acid, arachidonic acid was added simultaneously with aeration.

As seen in Fig. 5, the inhibitory activity of indomethacin was markedly enhanced by its pre-incubation with enzymes in the absence of arachidonic acid, but its enhancement was counteracted by the presence of arachidonic acid in the pre-incubation medium. The data indicate that arachidonic acid prevented the time-dependent and irreversible inhibition of indomethacin on bovine seminal vesicle enzymes. The inhibitory activities of SL-573, on the other hand, did

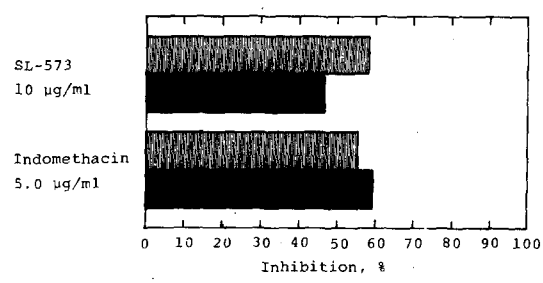

Fig. 4. Effect of pre-incubation of SL-573 and indomethacin with enzymes on prostaglandin biosynthesis by 20,000 *g* supernatant of rat small intestine homogenates. Key: □, no pre-incubation; and ■, 2-min pre-incubation.

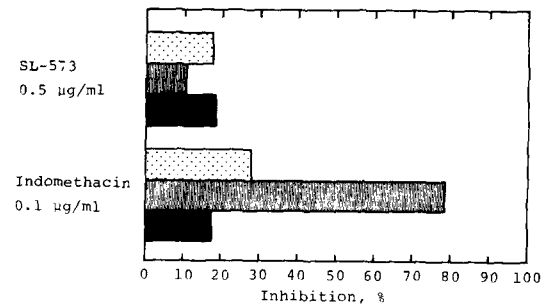

Fig. 5. Effect of pre-incubation of SL-573 and indomethacin with enzymes on prostaglandin biosynthesis by bovine seminal vesicle microsomes in the absence and presence of arachidonic acid. Key: ▨, no pre-incubation; □, 2-min pre-incubation in the absence of arachidonic acid; and ■, 2-min pre-incubation in the presence of arachidonic acid.

not significantly vary under three different experimental conditions.

DISCUSSION

The 20,000 *g* supernatant of rat small intestine homogenates was shown to convert arachidonic acid into prostaglandin $F_{2\alpha}$, E_2, D_2 and other unknown products. It was characteristic of these enzymes that prostaglandin D_2 was preferentially produced (45.2 per cent of total products).

The biosynthesis of E-type prostaglandins has been reported to be catalyzed by two enzymes, namely cyclo-oxygenase [20] and prostaglandin endoperoxide E isomerase [21]. Arachidonic acid is converted into prostaglandin endoperoxides (prostaglandin G_2 and H_2) by cyclo-oxygenase, and subsequently cleaved into prostaglandin E_2 by prostaglandin endoperoxide E isomerase. Prostaglandin endoperoxides are also postulated to be converted into prostaglandin $F_{2\alpha}$ and D_2 by prostaglandin endoperoxide F reductase and prostaglandin endoperoxide D isomerase respectively. In rat small intestine, prostaglandin endoperoxide D isomerase appears to be the predominant enzyme, because the data indicate that prostaglandin D_2 was produced at levels two to three times those of prostaglandin $F_{2\alpha}$ and E_2. Since little is known about the tissue distribution of D-type prostaglandins and their synthesizing enzymes, the rat small intestine is an unusual organ.

The biological significance of D-type prostaglandins has not been thoroughly studied so far, although there are several works concerned with their production [22–24]. They have been regarded as biologically inactive substances and by-products in pathways of F- and E-type prostaglandin biosynthesis. However, the biological activity of prostaglandin D_2 has recently attracted attention. For example, prostaglandin D_2 showed powerful inhibition of platelet aggregation [25, 26] and significant biological activity on several smooth muscle preparations [27]. Prostaglandin D_2, therefore, may play some important physiological functions in rat small intestines.

As reported in a previous paper [15], SL-573 inhibited the bovine seminal vesicle enzyme in a reversible manner, while indomethacin did in a time-

dependent and irreversible manner. The rat small intestinal enzymes, on the other hand, were reversibly inhibited by both drugs. The reversible inhibition of indomethacin in this case is probably due to the presence of endogeneous arachidonic acid in the enzyme preparation. Bovine seminal vesicle microsomes, in contrast, did not contain any free arachidonic acid available for prostaglandin production. The addition of arachidonic acid to bovine seminal vesicle microsomes, therefore, resulted in the prevention of time-dependent and irreversible inhibition of indomethacin.

The inhibitory activity of indomethacin was only about two times more potent than that of SL-573. The IC_{50} values were 9.1 μg/ml for SL-573 and 5.6 μg/ml for indomethacin. The rat small intestinal enzymes, therefore, did not exhibit markedly different drug specificity for SL-573 and indomethacin to provide an explanation for the lack of an adverse effect of SL-573 on rat small intestines, based on the hypothesis that the prostaglandin deficiency may result in ulcer formation.

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