Biochemical Pharmacology, Vol. 27, pp. 2157–2161.

0006-2952/78/0901-2157$02.00/0

THE INFLUENCE OF SOME PYRAZOL–PYRIDINE DERIVATIVES ON PROSTAGLANDIN SYNTHETASE ACTIVITY *IN VITRO*

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(*Received* 18 *November* 1977; *accepted* 25 *January* 1978)

Abstract—Four pyrazol–pyridine derivatives were found to increase about 3-fold the oxygen consumption by microsomes of ram seminal vesicles in the presence of arachidonic acid (100 μM) and 19 times prostaglandins generation. They had no influence on malondialdehyde formation. Radiochemical studies showed that mainly PGE_2 generation was increased. Stimulation of PG generation was observed also in bovine seminal vesicle microsomes but only in the absence of hydroquinone and glutathione. Tested compounds stimulate oxygen consumption in renal medulla but not in renal cortex. They had no influence on soybean lipoxidase activity.

Some pyrazol–pyridine derivatives (Fig. 1) were synthesized as potential antimitotic drugs [1]. Two of these compounds (III and IV) possess similar chemical structure as herbicide paraquat (1,1'-dimethyl-4,4'-dipiridylium chloride). Paraquat was found [2] to generate superoxide radicals (O_2^-), which produce hydrogen peroxide under the influence of superoxide dismutase. Since hydrogen peroxide stimulates prostaglandin biosynthesis [3] it has been interesting to test the influence of the title compounds on the prostaglandin synthetase activity.

MATERIALS AND METHODS

Enzymic preparations. Homogenates of the rabbit kidney were prepared separately from renal cortex and renal medulla. The tissues were homogenized in a 0.05 M phosphate buffer pH 7.0 to yield the final concentration of 100 mg wet tissue/ml in the medulla, and 20 mg wet tissue/ml in the cortex. Microsomes of ram (RSVM) and bull (BSVM) seminal vesicles were prepared by the method of Takeguschi [4]. RSVM were lyophylized and resuspended just before experiments in 0.1 M Tris–HCl buffer pH 8.2 at concentrations of 2.5 or 5 mg/ml. Freshly prepared BSVM were resuspended in 0.067 M phosphate buffer pH 8. Lipoxidase (linoleate–oxygen oxidoreductase EC 1.13.1.13) from soybean was dissolved in 0.1 M borate buffer pH 9 at the concentration of 2 μg/ml.

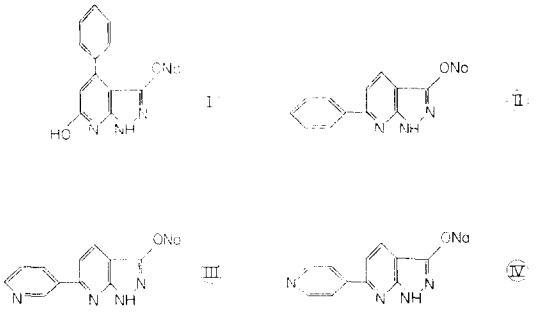

Fig. 1. Chemical structure of tested compounds.

Measurement of enzymic activities. Determination of malondialdehyde (MDA) formation [5] was used as a measure of total lipid peroxidation both by cyclo-oxygenase and by non-specific reactions [6]. The method was applied for RSVM suspensions incubated with 100 μM of sodium arachidonate at 37° for 10 min. The results were expressed in nmoles of MDA/1 mg of microsomes Measurement of oxygen consumption by enzymic preparation refers to oxygen which was used for peroxidation of linolenate by lipoxidase and peroxidation of arachidonate by RSVM or by kidney homogenates. Oxygen consumption was measured using Clark's electrode and YSI Oxygen Monitor model 53 in 3 ml samples. Before inserting the electrode each sample was stirred in air for 5 min with or without tested compounds. Oxygen consumption was measured 10 min for RSVM, 15 min for lipoxidase, 1 min for renal medulla and 5 min for renal cortex after addition of substrates. The substrates used were 1 mM sodium linolenate (for lipoxidase) or 0.1 mM sodium arachidonate (for microsomal PG synthetase and kidney homogenates). The temperature of the incubation was 25° for lipoxidase or 37° for RSVM and kidney homogenates. In some experiments cyclo-oxygenation was suppressed by indomethacin [7] at a concentration of 10 μM. The results were expressed in nmoles of oxygen consumed by an enzymic preparation during given period of time. Prostaglandin-like activity was bioassayed by the method of Vane [8] after the incubation of microsomal preparations with arachidonic acid. RSVM were incubated for 10 min at 37° without exogenous cofactors in the presence of 100 μM of sodium arachidonate. BSVM were incubated for 20 min at 37° without or with 45 μM of hydroquinone and 165 μM of glutathione. In both cases 33 μM of sodium arachidonate was present. After incubation the mixture was boiled and bioassayed without extraction. Blank samples contained microsomes boiled before incubation. The results were

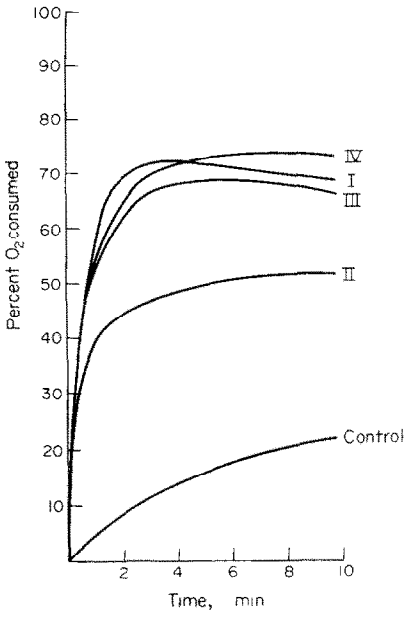

Fig. 2. The influence of 1000 μM of compounds I–IV on the oxygen consumption by RSVM. RSVM "B" (2.5 mg/ml) were incubated with tested compounds for 10 min before 100 μM arachidonic acid was added. Ordinate: time measured starting from arachidonic acid addition. Abscissa; per cent of oxygen consumed by a sample in time taking the amount of the oxygen in sample saturated with air as 100 per cent.

expressed in nmoles of PGE_2-like activity/mg of microsomal protein. Protein content in BSVM was determined by biuret method [9]. In one experiment RSVM were incubated with 1.64 nmole (0.1 μCi) of [1-^{14}C]sodium arachidonate. The ethyl acetate extract of this mixture was chromatographed on a thin layer plate in a system A1 [10]. After drying the autoradiograph was developed and the spots on a film were compared with R_f values for PGE_2, $PGF_{2\alpha}$, PGD_2 and 6-keto-$PGF_{1\alpha}$ which were described in the paper [10].

Reagents. Arachidonic acid (Sigma, U.S.A.) was purified by column chromatography on silica gel (Serva, 200–300 mesh). [1-^{14}C]arachidonic acid (Amersham, U.K.). Lipoxidase, Trisma–HCl and Trisma–base were obtained from Sigma and indomethacin (Metindol) from Polfa. The pyrazol–pyridine derivatives were obtained from the Department of Organic Chemistry Polish Academy of Sciences, Warsaw.

RESULTS

The influence of tested pyrazol–pyridine derivatives on the enzymic activity in RSVM was determined in two batches of RSVM. Batch A, 5 mg/ml consumed 9.1 ± 0.2 (mean ± S.E. $n = 4$) and batch B, 2.5 mg/ml consumed 17.8 ± 0.6 ($n = 14$) nmoles of oxygen/mg of microsomes. Each of four tested compounds at a concentration of 1 mM increased oxygen consumption in RSVM (Fig. 2). Compound II was the weakest in this respect while the other three compounds were more or less equipotent (Fig. 2 and Table 1). The maximal effect was observed 2 min after addition of arachidonic acid. Later the oxygen consumption reached a plateau, or in some cases the amount of oxygen in the mixture increased (Fig. 2).

The amount of oxygen consumed during 10 min in the samples containing 1 mM of tested compounds was highly significant as compared with the control (Table 1). In this series of experiments none of the tested compounds significantly influenced MDA formation (Table 1).

Compound I increased significantly PG generation by RSVM (Table 1). Compound I was chosen for more detailed studies. The stimulation of oxygen consumption by I was dose-dependent (Fig. 3). In this batch of RSVM (A) a stimulation of MDA formation by I was observed. It was highly significant only at a concentration of 100 μM whereas at a concentration of 1000 μM it was significant only at the level of $0.01 < P < 0.05$ (Fig. 3).

Indomethacin at the concentration of 10 μM inhibited in about 65% oxygen consumption, MDA and PG generation (Table 2). The residual enzymic activity was stimulated by I in the respect of oxygen consumption and PG generation. The stimulation of PG generation was much stronger than stimulation of oxygen consumption. Radiochromatogram of the incubation mixture (Fig. 4) shows that I stimulated PGE_2 and PGD_2 formation by about 400 per cent, 6-keto-$PGF_{1\alpha}$ generation by 150 per cent and had

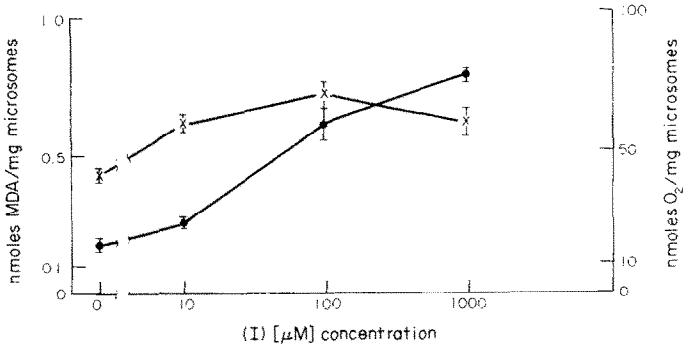

Fig. 3. Relationship between the concentration of I and oxygen consumption (circles) and MDA generation (crosses) by RSVM. RSVM "A" (5 mg/ml) were incubated with I 10 min before 100 μM arachidonic acid was added. Oxygen consumption was measured for 10 min and then MDA was estimated in the sample. Ordinate; I concentration. Abscissa; nmoles of O_2 consumed or MDA generated by 1 mg of microsomes.

Table 1. The influence 1000 μM of compounds I, II, III and IV on oxygen consumption, MDA and PG generation by RSVM

	Oxygen consumption	MDA generation	PGE_2-like activity
Control	17.75 ± 0.57 (14)	0.694 ± 0.047 (6)	1.34 ± 0.235 (6)
I	*59.14 ± 4.01 (10)	0.581 ± 0.036 (4)	†25.2 ± 9.79 (5)
II	*45.31 ± 3.26 (4)	0.824 ± 0.089 (4)	NT
III	*59.14 ± 2.85 (3)	0.729 ± 0.089 (3)	NT
IV	*65.12 ± 2.41 (3)	0.770 ± 0.095 (3)	NT

RSVM "B" (2.5 mg/ml) were incubated with tested compounds in 37° for 10 min before 100 μM of arachidonic acid was added. Oxygen consumption was measured for 10 min and then the sample was taken for MDA or PG estimation. Results are expressed as nmoles/mg of microsomes. Results were compared with the control using Student's 't' test.

* $P < 0.001$, † $0.01 < P < 0.05$. NT = not tested. The numbers of experiments in the parentheses.

Table 2. The influence of indomethacin and I + indomethacin on oxygen consumption, MDA and PG generation by RSVM

	RSVM + 100 μM arachidonic acid + 10 μM of indomethacin			+ 10 μM indomethacin + 1000 μM of I		
	O_2	MDA	PG	O_2	MDA	PG
nmoles/mg of microsomes	6.7 ± 0.3 (11)	0.21 ± 0.06 (3)	0.52 ± 0.17 (5)	29.3 ± 5 (7)	0.21 (1)	9.0 ± 4.6 (5)
Inhibition (as compared with values on Table 1)	62%	70%	61%	—	—	—
Increase of the residual activity	—	—	—	337%	0%	1630%

RSVM "B" (2.5 mg/ml) were preincubated with indomethacin (10 μM) for 5 min at room temperature, then 5 min with or without I (1000 μM) in 37°. Oxygen consumption was measured for 10 min after addition 100 μM of arachidonic acid and then MDA and PG were determined in the sample.

no influence on $PGF_{2\alpha}$ production. The same mode of action was observed both in non-inhibited and in partially by indomethacin inhibited samples.

Compound I, at the concentration of 1 mM stimulated PG-generation by BSVM (Fig. 5). The maximal effect was observed at the concentration of 350 μM. Enrichment of the homogenate by 45 μM of hydroquinone and 165 μM of glutathione increased the activity of microsomes from 1.25 ± 0.16 ($n = 3$) nmoles to 3.29 ± 0.3 ($n = 5$) nmoles of PGE_2-like activity/mg of protein. Exogenous cofactors abolished completely the stimulatory effect of I on PG synthetase activity in BSVM (Fig. 5).

Compound I had no significant influence on lipoxidase activity (measured as nmoles of O_2 consumed by 1 μg of the enzyme during 15 min). In control samples it was 28.4 ± 3.9 ($n = 3$) and in samples containing I (1000 μM) 38.3 ± 4.36 ($n = 3$), $P > 0.1$.

Compound I increased oxygen consumption by homogenates of rabbit kidney medulla from 0.2 ± 0.0003 nmoles O_2/1 mg of tissue ($n = 3$) to 0.43 ± 0.02 nmoles O_2/1 mg of tissue, $P < 0.001$ ($n = 3$). It diminished oxygen consumption in homogenates of rabbit kidney cortex [3.3 ± 0.2 nmoles O_2/1 mg of tissue ($n = 5$) in control homogenates and 2.2 ± 0.26 nmoles O_2/1 mg of tissue ($n = 5$) in homogenates containing 1 mM I, $0.001 < P < 0.01$].

DISCUSSION

Autoradiographic studies revealed that 47 per cent of arachidonic acid (100 μM) was converted by RSVM to numerous more polar products, with the chromatographic mobility of 6-keto-$PGF_{1\alpha}$ (3.5 per cent), $PGF_{2\alpha}$ (3 per cent), PGE_2 (9 per cent), PGD_2 (2.7 per cent), a product with R_f 0.57 (7.6 per cent) and hydroxyacids (11 per cent). This is in a good agreement with the finding that 15 per cent of oxygen which was consumed during incubation of arachidonic acid with RSVM was used to generate PG-like substances with the contractile activities of the bioassay organ as follows: $PGE_2 \geqslant PGF_{2\alpha} \gg$ 6-keto-$PGF_{1\alpha} \geqslant PGD_2$.

Compound I stimulated the conversion of arachidonic acid by RSVM to radioactive products and only 7.5 per cent of the substrate remained unreacted. This stimulation was the most prominent in case of PGE_2 which comprised 46 per cent of total radioactivity spotted on a plate. The corresponding values for other PGs were: 6-keto-$PGF_{1\alpha}$ (7.2 per cent), $PGF_{2\alpha}$ (1.4 per cent), PGD_2 (11.2 per cent). Again there was a correlation between the data obtained by radioassay and bioassay. We calculated that in the presence of I, 85 per cent of oxygen which was consumed during incubation of arachidonic acid with RSVM was used for production of PG-like substances.

Compounds II, III and IV stimulated similarly to I the oxygen consumption by RSVM, however neither compound I nor its analogs stimulated the formation of MDA. Therefore it seems that I specifically stimulates biosynthesis of PGE_2 in RSVM. Renal medulla and renal cortex constitute a good model for differentiation between the routes

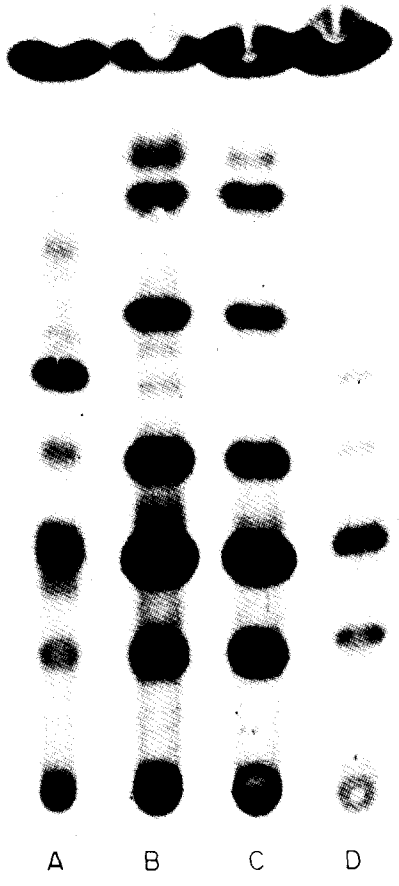

Fig. 4. Radiochromatogram of the extract from the incubation mixture containing RSVM and 100 μM of arachidonic acid + 1.64 nmoles of [1-^{14}C]arachidonic acid. Experimental conditions were as described in Fig. 2. A—RSVM (2.5 mg/ml) + 100 μM arachidonic acid, B—RSVM + arachidonic acid + 1000 μM I, C—RSVM + arachidonic acid + 10 μM indomethacin + 1000 μM I, D—RSVM + arachidonic acid + 10 μM indomethacin.

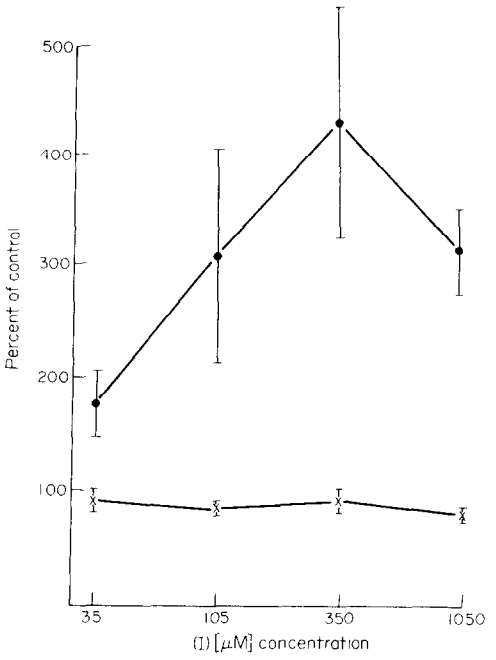

Fig. 5. The influence of various concentration of I on PG generation in BSVM in the absence (circles) or in the presence of 45 μM of hydroquinone and 165 μM of glutathione (crosses). PGE_2-like activity was bioassayed after 20 min of incubation.

of arachidonic acid oxidation either to form PG endoperoxides (medulla) or linear lipid peroxides (cortex) [6]. We have found that I stimulates oxygen consumption only in renal medulla but not in renal cortex. The final proof that the I is specific stimulator of PG synthetase system is that I does not stimulate oxygen consumption in the system; linolenic acid–soybean lipoxidase. The stimulatory effect of I on PG synthetase was abolished by the presence of hydroquinone and glutathione in the incubation mixture (BSVM + arachidonic acid). We have described a similar phenomenon for paracetamol [11].

Compound I and paracetamol increase oxygen consumption and PG generation. This effect may be due to a direct stimulation of cyclooxygenase. Alternatively I and paracetamol may activate both PGG_2/PGH_2 peroxidase or PGE_2 isomerase. However, glutathione known as a cofactor for PGE_2 isomerase [12] does not stimulate oxygen consumption by RSVM incubated with arachidonic acid [11]. On the other hand, phenol and its derivatives being recognized as the stimulators of PGG_2/PGH_2 peroxidase [13, 14] stimulate oxygen consumption and PG generation in the same system [15].

Therefore we believe that compounds I–IV either stimulate cyclooxygenase or activate PGG_2/PGH_2 peroxidase. We have not enough data to confirm any of these possibilities. A long list of PG-system inhibitors [7] was supplemented here by a new class of compounds namely: phenol, 2-aminomethyl-4-*t*-butyl-6-iodophenol (MK-447), paracetamol and pyrazol–pyridine derivatives which stimulate this enzyme.

The effect of PG-synthetase stimulators on inflammation is not elucidated. Phenol and MK-447 [14] were claimed to be anti-inflammatory, paracetamol was found to be inactive [16] or to possess only weak anti-inflammatory activity [17]. Our preliminary results show that I has no influence on the carrageenin oedema in rats.

Acknowledgement—We would like to express our gratitude to Professor P. Nantka-Namirski for supplying of pyrazol–pyridine derivatives.

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