Biochemical Pharmacology, Vol. 33, No. 4, pp. 692–695, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Identification of a serotonin type 2 receptor linked to prostacyclin synthesis in vascular smooth muscle cells

(*Received* 19 *February* 1983; *accepted* 23 *August* 1983)

Receptor-mediated regulation of arachidonic acid metabolism has been examined in cultured vascular endothelial and smooth muscle cells. Receptors for angiotensin [1], bradykinin [2], norepinephrine [3], and histamine [4] have been reported on vascular endothelial cells. Receptors for bradykinin and angiotensin [5], platelet-derived growth factor [6] and serotonin [7], among others [8], have been reported on smooth muscle cells. Recently, two subspecies of serotonin (5-HT) receptors have been described in brain and other tissues and designated types 1 (5-HT_1) and 2 (5-HT_2) [9–11]. In this report, we provide evidence that a type 2 serotonin receptor mediates prostacyclin synthesis by cultured bovine aortic smooth muscle cells and that this response is calcium dependent. Bovine aortic smooth muscle cells were cultured as previously described [7, 12, 13]. For all experiments, cells were used between passages one and three. Forty-eight hours prior to the start of an experiment, cells were plated at a density of approximately 10^5 cells/well (16 mm diameter) in Dulbecco's Modified Eagle Medium supplemented with 10% calf serum, 2 mM 1-glutamine, 20 μg/ml ascorbate, and antibiotics [7, 12]. To begin an experiment, each well was washed once with 1.0 ml of M199 (Medium 199 (Gibco) plus 2 mM L-glutamine, 1 μM pargyline, 100 μg/ml ascorbate, and antibiotics [7]), and then filled with 1.0 ml of M199 containing vehicle or treatment and incubated at 37° in an atmosphere of 5% CO_2/95% air. Medium 199 contains 2 mM calcium and 0.8 mM magnesium. Pargyline and ascorbate were added to the incubation medium to reduce enzymatic and oxidative degradation of serotonin. This was found necessary because, in the absence of these two drugs, levels of serotonin (as determined by high performance liquid chromatography with electrochemical detection [14]) decreased to 5% of original concentrations after 24 hr; in the presence of pargyline and ascorbate, levels decreased to 50–80% of initial amounts.

Incubation periods were 24 hr unless otherwise specified. To terminate an experiment, indomethacin was added to each well to achieve a final concentration of 4 μM. The medium was then removed for determination of prostaglandin (PG) concentration. Prostacyclin production was measured in the incubation medium by specific radioimmunoassay (RIA) of 6-keto $PGF_{1\alpha}$ [7, 12, 15].

Cell number per well was assessed at the end of a 24-hr test incubations as previously reported [7]. None of the compounds tested produced significant changes in cell number. Data are thus expressed simply as nanograms of prostaglandin per well.

Pitzotifen was provided by Sandoz (Basel, Switzerland) and cyproheptadine by Merck, Sharpe & Dohme (West Point, PA); other serotonin antagonists were provided by Janssens Pharmaceutica (Beerse, Belgium). All other drugs were purchased from the Sigma Chemical Co. (St. Louis, MO). Stock solutions (20 mM) of serotonin receptor blockers were made up in dimethyl sulfoxide (DMSO) and stored at −20°. For use, stocks were initially diluted 1/100 with absolute ethanol and then with M199. The final concentrations of DMSO and ethanol in the cell cultures never exceeded 0.005 and 0.5%, respectively, amounts which had no effect on cell morphology or prostaglandin production. Serotonin hydrochloride stocks (20 mM) were made up in M199 immediately before use.

As previously reported [7], the absolute amounts of 6 keto-$PGF_{1\alpha}$ varied between primary cultures and cell passages. Nevertheless, in each experiment, responses to the addition of serotonin were highly reproducible and statistically significant when compared to levels in control wells. Thus, 5-HT-induced increases varied from 1.1 ng/well to 40 ng/well (Figs. 1 and 2); both values differed significantly from basal levels in their respective experiments ($P < 0.01$).

Serotonin elicited a dose-dependent increase in prostacyclin production when added to smooth muscle cells in culture. This response was detected at concentrations above 100 nM and reached a plateau at 10 μM and above. The response to the addition of serotonin was linear with time when tested between 2 and 24 hr (Fig. 1). Other aminergic agonists (isoproterenol, phenylephrine, dopamine, and histamine) did not elicit a similar response when added to smooth muscle cell cultures at concentrations ranging from 10 nM to 100 μM [6]. The concentration of serotonin required to elicit a 50% maximal response was 0.45 ± 0.07 μM (mean ± S.E.M. of eleven experiments using serotonin concentrations of 100 nM to 100 μM in half-log increments). According to classical receptor theory, the EC_{50} for an agonist-induced biological response must be equal to or less than the K_a for agonist binding to its receptor [16]. Serotonin type 1 receptors have a thousand-fold higher affinity for serotonin (3 nM) than type 2 receptors (3 μM) [9]. Although the experimental conditions in cultured smooth muscle cells differ significantly from those used to determine K_a by isotopic ligand binding methods, an EC_{50} of 0.45 μM is suggestive but not definitive for a type 2 receptor-mediated response.

The serotonin receptor blockers pizotifen, cyproheptadine, methysergide, spiroperidol, ketanserin, mianserin, pipamperone, and haloperidol inhibited serotonin-induced increases in smooth muscle cell prostacyclin synthesis with the apparent K_i values shown in Table 1. Differences

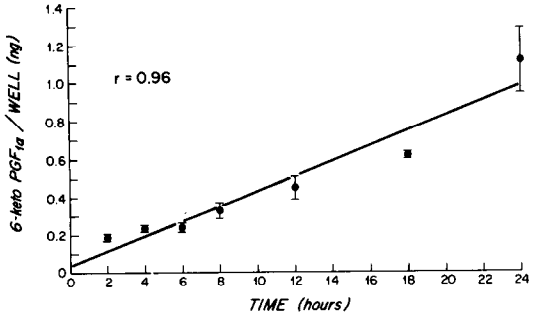

Fig. 1. Time-dependent changes in prostacyclin synthesis following the addition of serotonin. Smooth muscle cells were seeded as described in the text. Serotonin (10 μM) was added to separate wells and incubated for 2, 4, 6, 8, 12, 18 and 24 hr. Levels of 6-keto $PGF_{1\alpha}$ represent the means ± S.E. of six wells. Data were analyzed by linear regression, and a correlation coefficient of 0.96 was calculated.

Table 1. Correlation between the reported affinities for 5-HT_2 receptors with the inhibition of serotonin-induced prostacyclin synthesis by serotonin receptor blockers*

	K_i (PGI_2) (nM)	K_i (ligand binding) (Ref. 17) (nM)							K_i (ligand binding) (nM) (Ref. 10)
		5-HT_2	5-HT_1	Histamine	$Alpha_1$	$Alpha_2$	Dopamine	Muscarinic	5-HT_2
Pizotifen	0.32	6.5	1500	1.9	120	480	99	23	
Cyproheptadine	0.36	6.5	700	2.7	100	760	31	19	2.4
Methysergide	0.43	12	99	I	2300	2600	200	I	3.1
Spiroperidol	1.0	1.2	160	I	10	I	0.16	I	0.7
Ketanserin	1.5	2.1	I	10	10	I	220	I	
Mianserin	1.8	13	1100	2.9	82	60	620	I	5.0
Pipamperone	2.3	5.3	5000	I	54	610	120	I	6.6
Haloperidol	28	48	I	I	8	I	1.2	I	40
r		0.96	0.52	0.39	−0.20	−0.46	−0.28	0.27	1.00
P		<0.001	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	<0.001

* The relative abilities of receptor blocking drugs to inhibit serotonin-induced increases in smooth muscle protacyclin synthesis (K_i values) were derived from Schild plots [18] obtained as follows. Responses to serotonin were determined at half-log increments from 100 nM to 10 μM in the presence or absence of antagonists. Each antagonist was employed at three concentrations equaling 1, 3.16, and 10 times its approximate K_i as determined from preliminary experiments. In all experiments, points were performed in quadruplicate; standard errors were generally less than 10% of the mean. Schild plots were constructed from the above described dose–response curves, and values for K_i were determined using the formula:

$$\log\left[\frac{C_B}{C_0} - 1\right] = \log B - \log K_i$$

Each K_i represents the mean of three experiments. For all serotonin receptor blockers reported here, the slope of the Schild plot did not differ significantly from one. Ligand binding data were taken from Peroutka *et al.* [10] and Leysen *et al.* [17]. For linear regression analysis, those compounds listed as inactive (I) were assigned a K_i of 5000 nM.

C_B = concentration of serotonin required to elicit a 50% maximal response in the presence of serotonin receptor blocker at concentration B.
C_0 = concentration of serotonin to elicit 50% maximal response in the absence of a receptor blocker.

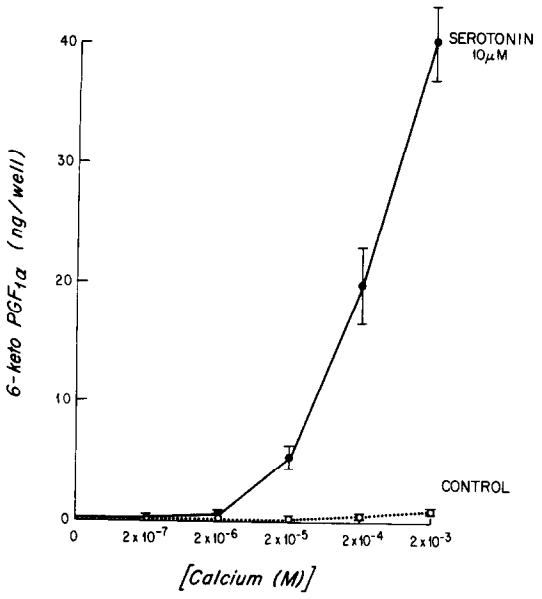

Fig. 2. Effect of calcium on serotonin-induced increases in smooth muscle prostacyclin synthesis. Smooth muscle cells were seeded as described in the text. After a 5-day growth period, wells were washed four times with calcium-free Krebs–Henseleit solution and then incubated for 24 hr in 1 ml of calcium-free Krebs-Henseleit containing the indicated concentration of added calcium chloride plus serotonin or vehicle. Medium was then harvested for determination of prostaglandin concentration. No significant cells loss was observed during the incubation period. Each point represents the mean ± S.E.M. of eight replicate wells. This experiment was replicated five times.

between these values and the affinities determined by ligand binding techniques may be explained by differences inherent in the two methodologies (whole cells vs tissue homogenates or membrane preparations). Even using similar ligand binding methods on identical tissues, 3- to 4-fold differences in affinities were measurable (e.g. Table 1; [10, 17]). A positive correlation was found between the ability of an antagonist to inhibit serotonin-induced increases in prostacyclin synthesis and its reported affinity for 5-HT_2 receptors as determined by ligand binding (Leysen *et al.* [17] and Peroutka *et al.* [10]; Table 1). No correlation was obtained with the affinity of these antagonists for the other known transmitter receptors for which binding data are available (Table 1). It is also clear that beta-adrenergic receptors are not involved in mediating the response to serotonin, since isoproterenol had no effect on smooth muscle prostacyclin synthesis at concentrations ranging from 10 nM to 100 μM, and propranolol (500 nM) did not attenuate the prostacyclin response to serotonin [7]. These data strongly suggest that the effect of serotonin on smooth muscle prostacyclin synthesis is mediated by a serotonin type 2 receptor, as defined by Leysen *et al.* [17] and Peroutka *et al.* [10].

Serotonin-induced increased in smooth muscle prostacyclin synthesis were neither mimicked nor potentiated by dibutyryl cAMP or isobutyl-methyl-xanthine (IBMX), a phosphodiesterase inhibitor. The addition of 100 μM dbcAMP and/or IBMX to smooth muscle cell cultures did not influence prostacyclin synthesis in the presence or absence of serotonin. Wells containing smooth muscle cells and dibutyryl cyclic AMP (100 μM) and/or IBMX (100 μM) contained 0.02 ± 0.01 ng of 6-keto $PGF_{1\alpha}$. These compounds did not raise or lower the amount of 6-keto $PGF_{1\alpha}$ synthesized in the presence of serotonin (10 μM) regardless of the incubation period utilized (3, 6 or 24 hr). Serotonin type 1, but not type 2, receptors are reported to be coupled to adenylate cyclase activity [9, 10, 19, 20], and 5-HT-induced increases in cyclic AMP formation have been reported in cultured smooth muscle cells [21]. Based on our own observations, however, this receptor is not apparently coupled in any direct way to prostacyclin formation. Thus, the PGI-inducing effect of serotonin in these cells is consistent with a 5-HT type 2 receptor mediated response.

Omission of calcium from the incubation medium abolished the ability of serotonin to stimulate prostacyclin production (Fig. 2). In each of three separate experiments, the addition of the calcium ionophore A23187 to the incubation medium (0.3 μg/ml) elicited 2- to 5-fold increases in smooth muscle prostacyclin synthesis, an effect that could be progressively decreased by decreasing the calcium concentration in the incubation medium. In the same experiments, arachidonic acid (5 μM) elicited 3- to 5-fold increases in prostacyclin synthesis, thereby suggesting that these cells were capable of forming prostacyclin from arachidonic acid. The omission of calcium attenuated arachidonate-induced increases by 20% in some experiments and did not decrease significantly the number of cells in culture after 24 hr. We therefore suggest that calcium ions play a role in the transduction of the signal from 5-HT_2 receptor stimulation to arachidonate release and increased prostacyclin synthesis. Calcium may also be important in the transduction of other receptor-mediated phenomena coupled to arachidonic acid metabolism.

In vivo, serotonin is a vasoconstrictor in certain vascular beds [22, 23], and this effect appears to be mediated by 5-HT_2 receptors [24]. In other vessels, serotonin is a vasodilator [25]; it has been reported to dilate blood vessels that have been constricted by other agents [26]. 5-HT-induced vasodilation may be due to the ability of serotonin to stimulate prostacyclin synthesis by vascular smooth muscle. The constricting and/or vasodilating effects of serotonin may depend upon which of the above mechanisms predominates in a given vessel. In a blood vessel contracted by serotonin, the synthesis of prostacyclin may also serve to attenuate or terminate its constrictor effect. Hence, treatment with a cyclooxygenase inhibitor, such as indomethacin or aspirin, might be expected to enhance serotonin-induced smooth muscle contraction.

We have reported previously that serotonin and platelet-derived growth factor (PDGF) act synergistically to stimulate smooth muscle prostacyclin synthesis [7]. This synergism also appears to be mediated by a serotonin type 2 receptor inasmuch as serotonin type 2 selective drugs such as cyproheptadine completely block synergism at concentrations that would not block a 5-HT_1-mediated response [7]. Moreover, the EC_{50} for serotonin in the presence of PDGF does not change; rather, the maximum response increases dramatically [7]. As discussed above, an EC_{50} in the micromolar range is consistent with 5-HT_2, not 5-HT_1, mediated responses. The possible consequences of this serotonin receptor-mediated synergy on the biological activity of PDGF (e.g. effects on cell division [27, 28], migration [29] and metabolism [12, 29–31]), or serotonin itself, deserves further study.

In summary, our data suggest the existence of a serotonin type 2 receptor which is linked to prostacyclin synthesis by a calcium-dependent mechanism in cultured vascular smooth muscle. Serotonin stimulated smooth muscle prostacyclin synthesis in a dose-dependent, saturable manner with an EC_{50} of 0.45 μM. The effect of serotonin was inhibited by the serotonin receptor blockers pizotifen, cyproheptadine, methysergide, spiroperidol, ketanserin, mianserin, pipamperone, and haloperidol (listed in order of decreasing potency). A highly significant positive correlation was found between the ability of an antagonist to inhibit serotonin-induced prostacyclin synthesis and its reported affinity for type 2, but not type 1, serotonin

receptors. The effect of serotonin was neither mimicked nor potentiated by dibutyryl cAMP or isobutyl-methylxanthine, consistent with a serotonin type 2 mediated effect. Omission of calcium from the incubation medium abolished both serotonin and A23187 induced increases in prostacyclin synthesis, suggesting the involvement of calcium ions in the transduction of this response.

Acknowledgements—The authors acknowledge the excellent technical assistance of Mrs. Georgina Curtis and Mr. Peter Barley. We also wish to thank Ms. Kathi DeFilippo for her secretarial assistance. Shaun Coughlin was supported by the Insurance Medical Science Scholarship Fund (Prudential). Michael Moskowitz is an Established Investigator of the Americal Heart Association. Lawrence Levine is an American Cancer Society Research Professor of Biochemistry (Award PRP-21). This work was supported by National Institutes of Health Grant NS 19038-01.

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Biochemical Pharmacology, Vol. 33, No. 4, pp. 695–697, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Calcium-dependent conversion of procollagen to collagen and its inhibition by other divalent cations

(*Received* 28 *April* 1983; *accepted* 24 *August* 1983)

Collagens, a family of closely-related, yet genetically distinct proteins, are synthesized as a precursor, procollagen, that contains noncollagenous extensions at both the N- and C-terminal ends of the molecule. These extensions are removed by two specific enzymes, procollagen N-proteinase and procollagen C-proteinase, respectively (for review on collagen, see Refs. 1–5). Previous studies [6, 7] had shown that the activity of procollagen N-proteinase is inhibited by EDTA, suggesting that the enzyme requires calcium or another divalent cation for its activity. Similarly, the activity of partially purified procollagen C-proteinase is inhibited by EDTA [8]. Our previous studies, employing a pulse-chase technique, had also indicated that the extracellular conversion of type II procollagen to collagen is inhibited by EDTA *in vitro*, and that the inhibition can be reversed by the addition of calcium [9]. In the present study, we