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THE EFFECT OF CHRONIC RITANSERIN AND CLORGYLINE ADMINISTRATION ON 5-HT$_2$ RECEPTOR LINKED INOSITOL PHOSPHOLIPID HYDROLYSIS

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Abstract—We have previously shown that chronic administration of the 5-hydroxytryptamine (5-HT) receptor antagonist, ritanserin (10 mg/kg/day) or the monoamine oxidase type A inhibitor (MAOI), clorgyline (2 mg/kg/day), results in a reduction in 5-HT$_2$ receptor number in rat cerebral cortex. This study investigates the effects of acute and chronic ritanserin administration, on 5-HT$_2$ receptor linked inositol phospholipid hydrolysis in rat cortical slices and compares it with the effect of a chronic clorgyline regimen. [^{3}H]Myo-inositol (50 μCi) was used to label inositol phospholipids. Their subsequent hydrolysis in the presence or absence of 5-HT was determined by the accumulation of [^{3}H]myoinositol monophosphate ([^{3}H]InsP). Addition of 5 nM ritanserin to slices had no effect on basal or 5-HT stimulated [^{3}H]InsP accumulation whereas 100 nM ritanserin blocked the stimulated response by 65%. Acutely, ritanserin (15 mg/kg i.p.) completely blocked 5-HT stimulated [^{3}H]InsP accumulation. Chronic ritanserin or clorgyline treatment had no effect on basal levels of [^{3}H]InsP accumulation compared to controls (mean value 3125 ± 298 dpm/mg protein). Ritanserin increased 5-HT stimulated [^{3}H]InsP accumulation at 1 μM, 100 μM and 1 mM 5-HT and this effect was significant at 100 μM 5-HT. Clorgyline had no significant or consistent effect on 5-HT stimulated [^{3}H]InsP accumulation at 1 μM, 100 μM and 1 mM 5-HT. Thus the effects of both chronic clorgyline and ritanserin administration on 5-HT$_2$ linked inositol phospholipid hydrolysis do not correlate with their effects on 5-HT$_2$ receptor number (B_{max}). The situation is further complicated since ritanserin significantly increases phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) labelling whereas clorgyline significantly increases PtdIns and PtdIns4P labelling. The implications of this are discussed.

5-Hydroxytryptamine (5-HT‡) receptors can be divided into at least three major subtypes, 5-HT$_1$, 5-HT$_2$ and 5-HT$_3$, based on biochemical, pharmacological and electrophysiological criteria [1, 2]. 5-HT$_2$ receptors, which are the subject of this study, have a low affinity (μM) for 5-HT and a high affinity (nM) for 5-HT antagonists, e.g. ketanserin and ritanserin [3].

5-HT$_2$ receptors appear to be homologous and are found mainly in the cortex and also in the striatum, mesolimbic areas [4] and on blood platelets [5, 6]. Recently it has been reported that there may be different 5-HT$_2$ receptor subtypes in rat cortex compared to bovine brain [7]. 5-HT$_2$ receptor stimulation causes inositol phospholipid hydrolysis [8–10] with the formation of diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (Ins(1,4,5)P$_3$). These two second messengers cause activation of protein kinase C and the mobilization of calcium from intracellular stores, respectively [11]. Ins(1,4,5)P$_3$ feeds into the inositol phosphate cycle where it is converted into inositol by a series of dephosphorylations. The final step, converting inositol monophosphate (InsP) to inositol, can be blocked by lithium which allows measurement of receptor-mediated inositol phospholipid breakdown [12].

Various antidepressants, e.g. imipramine, iprindole and mianserin, reduce 5-HT$_2$ receptor number [13–15] and chronic imipramine and iprindole administration produce parallel reductions in 5-HT induced inositol phospholipid hydrolysis [16]. Chronic administration of 5-HT antagonists, e.g. ritanserin and ketanserin, have also been reported to down-regulate 5-HT$_2$ receptors [17–20] which is an unusual phenomenon compared with the regulation of, for example, dopaminergic receptors where chronic antagonist administration increases receptor number [21].

Previously, we have shown that chronic administration of both ritanserin and clorgyline (a specific monoamine oxidase type A inhibitor, MAOI) [22], reduce 5-HT$_2$ receptor number in rat cerebral cortex [20]. The aim of this study was to determine whether the same chronic ritanserin regimen causes a parallel change in the 5-HT$_2$ receptor linked second messenger system or whether its "unusual" down-regulating effect occurs only at the receptor level. The effect of acute ritanserin, both *in vivo* and *in vitro*, was examined to confirm that it does inhibit 5-HT

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‡ Abbreviations: 5-HT, 5-hydroxytryptamine; MAOI, monoamine oxidase inhibitor; DAG, diacylglycerol; Ins(1,4,5)P$_3$, inositol 1,4,5-trisphosphate; InsP, inositol monophosphate; B_{max}, receptor number; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P$_2$, phosphatidylinositol 4,5-bisphosphate; CDP-DAG, cytidine diphosphate-diacylglycerol.

stimulated hydrolysis of inositol phospholipid. The effect of clorgyline was also examined to compare the effect of ritanserin with a drug which, by increasing 5-HT levels, effectively acts like an agonist.

MATERIALS AND METHODS

Animals. Adult, Wistar rats (350 g) received ritanserin (10 mg/kg/day), clorgyline (2 mg/kg/day) or vehicle (50 mM tartaric acid, pH 5) in drinking water, for 28 days followed by a 3-day drug-free period. These regimens are non-toxic and induce 5-HT_2 receptor down-regulation [19, 20, 23]. The 3-day drug-free period was chosen to ensure that all residual drug was removed from the system. This is particularly important in the case of ritanserin since it has a slow rate of dissociation from the receptor site [19]. It has previously been shown that B_{max} values are still reduced after 3 days drug-free, whereas K_d values have returned to control levels [19, 20]. In acute studies, animals received either ritanserin (5 mg/kg i.p.) or saline, 2 hr prior to killing. Following decapitation, brains were rapidly removed and cerebral cortices dissected on ice.

Measurement of inositol monophosphate accumulation and inositol phospholipid labelling. 5-HT_2 receptor linked inositol phospholipid hydrolysis was measured by the method of Berridge *et al.* [12]. Cerebral cortices were cross-chopped into 350 × 350 μm slices using a McIlwain chopper and washed three times by resuspension and centrifugation (150 *g* for 20 sec) in warm Tyrodes buffer (pH 7.4). Slices were preincubated for 1 hr with myo-[2-^{3}H]inositol (50 μCi/mL packed slices) in a shaking water bath at 37° in a humified atmosphere of 95% O_2:CO_2. They were then washed (4 times) with warm Tyrodes buffer to remove free [^{3}H]myo-inositol before being incubated in Tyrodes buffer containing LiCl (10 mM) and chlorimipramine (1 μM) to block 5-HT uptake. Approximately 100 mg tissue (wet weight) was used per tube and it was incubated with or without 5-HT for 1 hr as before. When appropriate, ritanserin or ketanserin was added 15 min before 5-HT. The reaction was terminated using trichloroacetic acid (15%), tubes were centrifuged (130 *g* for 10 min) and the supernatant removed and washed (3 times) with diethylether. [^{3}H]InsP was extracted and separated by either HPLC [24] or by Dowex-1 ion exchange columns (formate form, 100–200 mesh) according to the method of Berridge *et al.* [12]. Radioactivity was determined by scintillation counting using Optiphase Hisafe III. Pellets were washed (3 times) with water and the lipids extracted according to the method of Downes and Wusterman [25]. The lipid extract was dried under a stream of nitrogen and redissolved in chloroform. Inositol phospholipids were separated by thin layer chromatography in conjunction with phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) standards, according to the method of Gonzalez-Sastre and Folch-Pi [26]. The lipid zones were identified using iodine vapour and marked. The plates were decolourized and the marked zones scraped and mixed with 4 mL Optiphase Hisafe III. Radioactivity was determined by liquid scintillation counting.

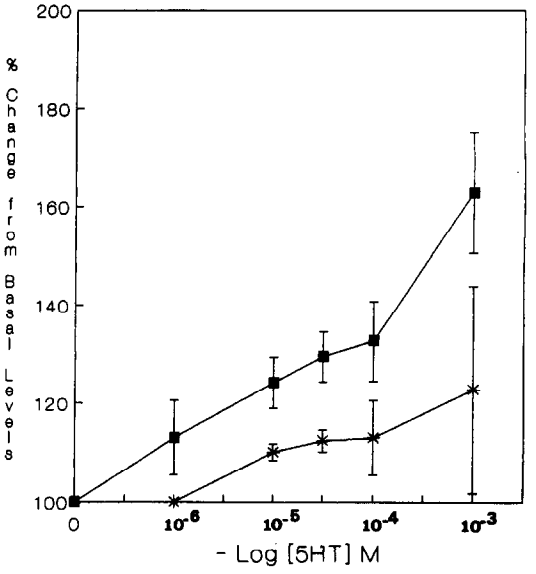

Fig. 1. Effect of increasing concentrations of 5-HT on [^{3}H]InsP accumulation in rat cerebral cortical slices. Prelabelled slices were incubated with varying concentrations of 5-HT plus LiCl (10 mM) in the absence [■——■) or presence (*——*) of ketanserin (30 nM). Each value is the mean of at least four separate experiments, expressed as the percentage change ± SE in [^{3}H]InsP accumulation relative to basal levels, i.e. in the absence of 5-HT (100%).

Protein levels in the samples were determined using the method of Lowry *et al.* [27] as modified by Peterson [28] using bovine serum albumin as standards.

Chemicals and drugs. Myo-[2-^{3}H]inositol (20 Ci/mmol) was obtained from Amersham International (Bucks, U.K.). Ritanserin was a gift from Janssen Pharmaceutica (Beerse, Belgium) and clorgyline a gift from May and Baker Pharmaceuticals (Dagenham, U.K.). Chlorimipramine was obtained from CIBA-Geigy Pharmaceuticals (Horsham, U.K.) and ketanserin tartrate was obtained from Janssen Pharmaceutica (Beerse, Belgium).

RESULTS

Effect of 5-hydroxytryptamine on inositol phospholipid turnover

Increasing concentrations of 5-HT (10^{-6} M to 10^{-4} M) caused a linear increase in the accumulation of [^{3}H]InsP in rat cortical slices (Fig. 1). Addition of 10^{-3} M 5-HT resulted in a disproportionate increase in [^{3}H]InsP accumulation in a seemingly non-specific manner. The increases in [^{3}H]InsP accumulation at 10^{-5} M, 5×10^{-5} M, 10^{-4} M and 10^{-3} M 5-HT were all significantly above unstimulated levels ($P < 0.05$ at 10^{-5} and 5×10^{-5} M, $P < 0.005$ at 10^{-4} M and $P < 0.001$ at 10^{-3} M) using unpaired *t*-tests. Ketanserin (30 nM) partially blocked 5-HT stimulated [^{3}H]InsP accumulation at all 5-HT concentrations used (Fig. 1), whilst at the higher dose of 1 μM

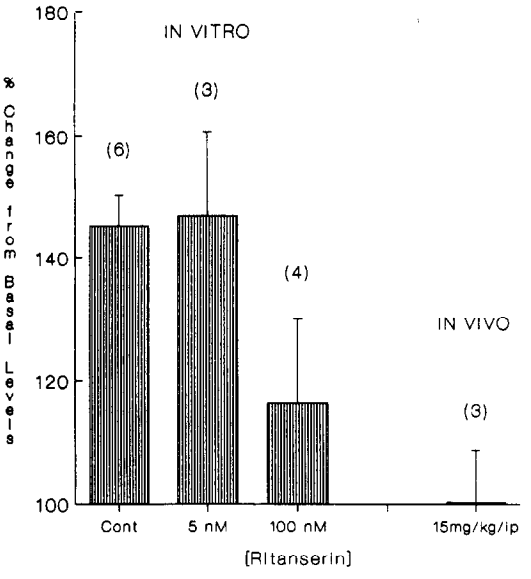

Fig. 2. Effect of acute ritanserin administration *in vivo* and *in vitro* on 5-HT induced [^{3}H]InsP accumulation in rat cortical slices. In the *in vivo* studies, animals were injected with either ritanserin (15 mg/kg i.p.) or saline, 2 hr prior to killing. Cortical slices were incubated with 100 μM 5-HT in the presence of LiCl (10 mM). In the *in vitro* studies, cortical slices were incubated with 5 or 100 nM ritanserin and 100 μM 5-HT in the presence of LiCl (10 mM). In both cases results are expressed as percentage change ± SE in [^{3}H]InsP accumulation relative to basal levels, i.e. in the absence of 5-HT (100%). The number of experiments at each concentration of 5-HT are shown in parentheses.

it completely blocked [^{3}H]InsP accumulation when slices were stimulated with 1 mM 5-HT.

Effect of acute in vivo *and* in vitro *ritanserin administration on 5-hydroxytryptamine-stimulated inositol phospholipid turnover*

[^{3}H]InsP was measured after incubating slices from control or acute ritanserin treated rats (15 mg/kg i.p.) with and without 5-HT (100 μM). In the absence of 5-HT, there was no difference in [^{3}H]InsP accumulation between the two groups; however in the presence of 5-HT there was a 46% increase in [^{3}H]InsP accumulation above basal levels in the control group whereas in the ritanserin group there was no significant increase (Fig. 2). In the *in vitro* studies, preincubation with 5 nM ritanserin had no significant effect on [^{3}H]InsP accumulation when slices were stimulated with 100 μM 5-HT, however 100 nM ritanserin caused a reduction in [^{3}H]InsP accumulation of approximately 65% (Fig. 2).

Effect of chronic ritanserin and clorgyline administration on 5-hydroxytryptamine-stimulated inositol phospholipid turnover

There was no significant difference, as indicated by unpaired *t*-tests, in the basal levels of [^{3}H]InsP accumulation between the control and the two chronically drug treated groups. The mean level of [^{3}H]InsP in these groups was 3125 ± 298 dpm/mg protein.

In the ritanserin treated group, there was a 27% increase in [^{3}H]InsP accumulation over basal levels at 1 μM 5-HT compared to a 22% increase in controls. With 100 μM 5-HT, there was a significant ($P < 0.05$) 51% increase in [^{3}H]InsP accumulation in the ritanserin group compared to a 31% increase in the control group. At 1 mM 5-HT, [^{3}H]InsP accumulation increased to 156% in the ritanserin group compared to 33% in the control group and even though this was the biggest increase, it failed to reach significance due to the large SE (Fig. 3a). The overall increases in [^{3}H]InsP accumulation produced by 5-HT were small; however, the response in the ritanserin group at 1 μM 5-HT was in fact 32% greater than in controls and at 100 μM it was 67% higher. In addition, although some of the results did not show significant changes, overall, there was an increase in [^{3}H]InsP accumulation in the ritanserin group. In the clorgyline treated group there was no significant difference in [^{3}H]InsP accumulation compared to controls when slices were stimulated with either 1 μM, 100 μM or 1 mM 5-HT and in contrast to the ritanserin group there was no overall pattern of change (Fig. 3b).

Effect of chronic ritanserin and clorgyline administration on inositol phospholipid labelling

Inositol phospholipid labelling in cortical samples from control and drug treated animals with and without 5-HT stimulation was examined. 5-HT had no significant effect on the labelling of PtdIns, PtdIns4P or PtdIns(4,5)P_2 (data not shown) and so the results for the 5-HT stimulated and non-stimulated samples in each of the two drug treatments were combined. Both chronic drug regimens significantly increased PtdIns and PtdIns4P levels and ritanserin also significantly increased PtdIns(4,5)P_2 (Fig. 4). Ritanserin increased the inositol phospholipid levels in the order PtdIns4P < PtdIns(4,5)P_2 < PtdIns, whereas clorgyline increased the inositol phospholipids in the order PtdIns < PtdIns4P < PtdIns(4,5)P_2. Thus ritanserin had a greater effect on PtdIns4P and PtdIns(4,5)P_2 whereas clorgyline had a greater effect on PtdIns.

DISCUSSION

5-HT stimulated inositol phospholipid hydrolysis occurs in a number of tissues. It is mediated by the 5-HT_2 receptor in rat cerebral cortex [8, 16], rat aorta [29] and human and rabbit blood platelets [9, 30] and by the 5-HT_{1C} receptor in the choroid plexus [10]. We have shown that inositol phospholipid hydrolysis in rat cortical slices, as measured by [^{3}H]InsP accumulation, increases with increasing concentrations of 5-HT up to 1 mM. This increase is biphasic and has been reported by others [8, 16]. Thirty nM ketanserin partially blocks this response whereas 1 μM ketanserin is completely inhibitory even when 1 mM 5-HT is used to stimulate [^{3}H]InsP accumulation; this confirms that the response is 5-HT_2 receptor mediated. Other studies of 5-HT mediated inositol phospholipid hydrolysis, however, have shown that some 5-HT_2 receptor antagonists (e.g. clozapine) inhibit hydrolysis in brain slices and aorta [8, 29] while others are weak

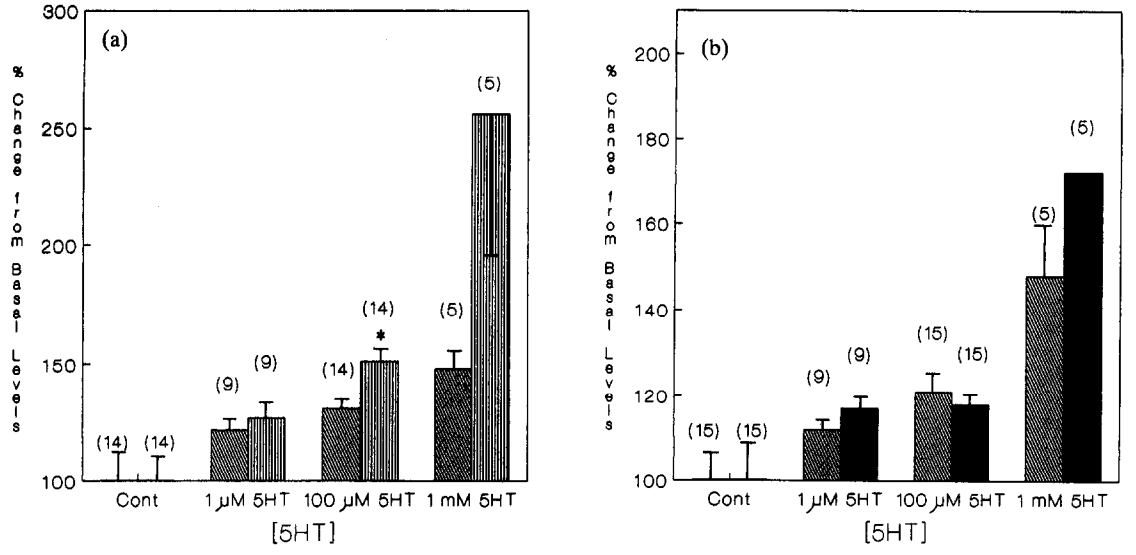

Fig. 3. (a) Effect of chronic ritanserin administration on 5-HT induced [^{3}H]InsP accumulation. Animals were pretreated with ritanserin (10 mg/kg/day) or vehicle orally for 28 days followed by a 3-day drug-free period. Cortical slices were incubated with 1 μM, 100 μM or 1 mM 5-HT in the presence of LiCl (10 mM). The results are expressed as percentage change ± SE in [^{3}H]InsP accumulation relative to basal levels, i.e. in the absence of 5-HT (100%). (▥) Ritanserin administration, (▨) vehicle administration. The number of experiments at each concentration of 5-HT are shown in parentheses. Unpaired t-tests were used to obtain a measure of significance (*$P < 0.05$). (b) Effect of chronic clorgyline administration on 5-HT induced [^{3}H]InsP accumulation. Animals were pretreated with clorgyline (2 mg/kg/day) or vehicle orally for 28 days followed by a 3-day drug-free period. Slices were incubated with 1 μM, 100 μM or 1 mM 5-HT in the presence of LiCl (10 mM). The results are expressed as the percentage change ± SE in [^{3}H]InsP accumulation relative to basal levels, i.e. in the absence of 5-HT (100%). (▦) Clorgyline administration, (▨) vehicle administration. The number of experiments at each concentration of 5-HT are shown in parentheses.

or ineffective [16], suggesting that there could be 5-HT_2 receptor subtypes.

The chronic clorgyline and ritanserin regimens used in this study have been shown to cause a 21 and 29% reduction in [^{3}H]ketanserin binding, respectively, with no effect on K_d [20]. The aim of this study was to determine whether these chronic drug regimens caused similar decreases in 5-HT_2 receptor stimulated inositol phospholipid hydrolysis. It was found that changes in the second messenger system did not correlate with the decreases in receptor number elicited by the two drug regimens. The chronic clorgyline regimen had no significant effect on 5-HT stimulated [^{3}H]InsP accumulation relative to corresponding controls whereas the ritanserin regimen increased rather than decreased the effect: in unstimulated tissue, both drugs had no significant effect on [^{3}H]InsP levels. Thus, the clorgyline regimen, whilst significantly decreasing 5-HT_2 receptor number, produces no apparent change in the effector mechanism whereas the ritanserin regimen decreases receptor number but increases the response.

It is presently unclear why there is a lack of correlation between receptor number and response. However, the chronic drug regimens have also been shown to increase the levels of [^{3}H]PtdIns, [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 compared to controls. It has been shown that drugs, for example barbiturates, which cause proliferation of the endoplasmic reticulum, the primary site of phospholipid synthesis, can increase the incorporation of radioactive precursors into phospholipids [31, 32]. In addition, amphiphilic cationic drugs such as fenfluramide, propranolol and phenothiazines have been shown to alter the physical properties of membrane lipids and their turnover. The rate of conversion of phosphatic acid to DAG is inhibited whereas the formation of cytidine diphosphate-diacylglycerol (CDP-DAG) is stimulated. This leads to redirection of phospholipid synthesis away from major membrane lipids such as phosphatidylcholine and phosphatidylethanolamine and towards minor, acidic lipids, e.g. phosphatidylinositol [33]. The inositol phospholipid pool increased by the chronic drug regimens may or may not be involved in receptor mediated effects since phosphoinositides have been shown to exist in metabolically distinct and therefore functionally different pools [34–36]. Ritanserin has a relatively greater effect than clorgyline on the levels of PtdIns(4)P and PtdIns(4,5)P_2, which are the phospholipids known to be involved in receptor mediated effects. This explains the findings reported here, i.e. 5-HT_2 receptor stimulation results in an increase in [^{3}H]InsP accumulation in the ritanserin group compared to controls but in the absence of receptor stimulation there is no change in [^{3}H]InsP accumulation. Clorgyline, on the other hand, has a greater effect on PtdIns and no effect on [^{3}H]InsP accumulation; therefore, it appears that clorgyline increases an inositol phospholipid pool which is not linked to agonist-induced hydrolysis.

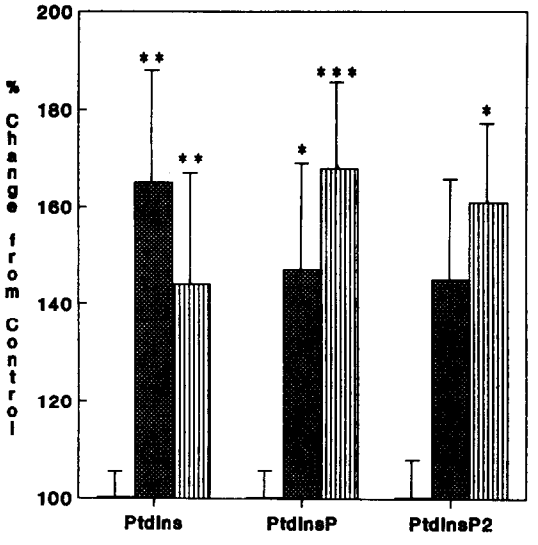

Fig. 4. Effect of chronic ritanserin and clorgyline administration on inositol phospholipid labelling. Cortical slices from animals pretreated with either ritanserin (10 mg/kg/day) or clorgyline (2 mg/kg/day) were preincubated with [^{3}H]inositol (50 μCi) (60 min) and then ± 5-HT (60 min). Reactions were terminated with TCA, slices were homogenized, centrifuged and the phospholipids from the resultant pellet extracted, separated by TLC and counted by liquid scintillation counting. The data is expressed as the % change in inositol phospholipid labelling compared to control (i.e. 100%). (□) Control, (▩) clorgyline, (▥) ritanserin. Each value is the mean ± SE of six values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (unpaired t-tests).

In conclusion, it is possible that chronic ritanserin and clorgyline administration affect different inositol phospholipid pools. Ritanserin may have a greater effect on the receptor linked pool and so, even though receptor number is decreased, the overall effect of chronic antagonist treatment is a supersensitivity of the system. Clorgyline, however, may increase inositol phospholipids which play no part in receptor mediated effects. The decrease in 5-HT$_2$ receptor number following both chronic drug regimens could be a decrease in spare 5-HT$_2$ receptors in the cortex, i.e. receptors not linked to inositol phospholipid hydrolysis. As ritanserin and clorgyline have very different acute effects, it is unclear why they both increase the formation of PtdIns, PtdIns4P and PtdIns(4,5)P$_2$. Furthermore, the changes in the inositol phospholipids and in their hydrolysis do not provide an explanation for the fact that chronic ritanserin administration results in a decrease in 5-HT$_2$ receptor number.

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