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SEROTONERGIC, ADRENERGIC AND HISTAMINERGIC RECEPTORS COUPLED TO PHOSPHOLIPASE C IN CULTURED CEREBELLAR GRANULE CELLS OF RATS

JIAN XU and DE-MAW CHUANG*

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, St. Elizabeths Hospital, Washington, DC 20032, U.S.A.

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Abstract—Phosphatidylinositol (PtdIns) turnover has been studied in the primary culture of granule cells dissociated from the cerebellum of postnatal rat. Addition of serotonin (5-hydroxytryptamine, 5-HT) caused an increase (300–600% of control) of [^{3}H]inositol monophosphate (IP_1) accumulation in the presence of LiCl in cells prelabeled with [^{3}H]myo-inositol. The EC_{50} and saturation concentrations of 5-HT were about 0.1 and 10 μM respectively. Some nonselective 5-HT receptor agonists, MK212, 5-methoxytryptamine, tryptamine and quipazine, were capable of stimulating IP_1 accumulation; a selective $5\text{-}HT_{1A}$ receptor agonist, 8-OH-DPAT, was ineffective. The 5-HT-induced response was potently blocked by several $5\text{-}HT_2$ receptor antagonists such as ketanserin, mianserin, spiroperidol and pyzotyline. The 5-HT-induced accumulation was dependent on the culturing time of granule cells with the maximal response seen in an 8-day culture. Norepinephrine (NE) also promoted an increased IP_1 accumulation (300% of the control) with an EC_{50} of about 1 μM, while prazosin inhibited this NE-induced response with a K_i of about 0.2 nM. The responses induced by NE and 5-HT appeared to be additive. In addition, histamine in a dose-dependent manner enhanced the accumulation by about 100%; this histamine effect was blocked by triprolidine, an H_1 receptor antagonist, but not by cimetidine, an H_2 receptor antagonist. These results suggest that 5-HT, NE and histamine could be part of the neurotransmitter substances present in mossy fibers or other afferent nerve endings which innervate granule cells *in vivo* and that PtdIns turnover regulated by their selective receptors on granule cells may play a role in modulating the excitatory function of these neurons.

The brain tissue is particularly active in metabolizing phosphatidylinositol (PtdIns) and its phosphorylated derivatives, leading to the formation of two key metabolites, diacylglycerol which is an endogenous protein kinase C activator and inositol trisphosphate which mobilizes calcium from the non-mitochondrial pool [1, 2]. Activation of a variety of neurotransmitter receptors in the brain results in enhanced turnover of PtdIns and subsequent activation of protein kinase C [1, 2], which is present in high concentrations in the CNS. Thus, receptor-mediated activation of PtdIns hydrolysis may be a signal transduction mechanism involved in synaptic transmission and is likely to play a major role in the regulation of neuronal function.

The study of receptor-mediated metabolism of PtdIns in brain slices has been complicated by the presence of an extremely heterogenous cell population and sometimes hampered by a relatively small signal of activation, especially in the case of serotonergic receptors [3]. Cerebellar granule cells, which are the most numerous and the only known excitatory neurons in the cerebellar cortex, can be dissociated from the tissue of postnatal rats and cultured *in vitro* with a high degree of homogeneity (>90%) [4, 5]. Moreover, during *in vitro* culturing, these cells express a stimulus-coupled release of the transmitter glutamate [4, 5]. In this study, we used cultured cerebellar granule cells to demonstrate that 5-HT, NE and histamine increase turnover of PtdIns in a receptor-mediated process.

MATERIALS AND METHODS

Materials. [^{3}H]Myo-inositol (16.5 Ci/mmol) was a product of New England Nuclear (Boston, MA). Basal modified Eagle's medium, glutamine, gentamycin and fetal calf serum were purchased from GIBCO (Grand Island, NY). The following compounds were gifts from pharmaceutical companies: spiroperidol and ketanserin tartrate were from Janssen Pharmaceutical (Beerse, Belgium); mianserin hydrochloride was from Organon (Oss, Holland); pizotyline was from Sandoz Pharmaceutical (East Hanover, NJ); MK-212 was from Merck Sharp & Dohme Research Laboratory (Rahway, NJ); and prazosin was from Pfizer Chemical Inc. (Ridgefield, NJ). Quipazine maleate and 8-hydroxy-2-(di-*n*-propylamino) tetralin (8-OH-DPAT) were purchased from Research Biochemicals Inc. (Wayland, MA). All other chemicals were products of the Sigma Chemical Co. (St. Louis, MO).

Preparation of primary culture of cerebellar granule cells. Cerebellar granule cells were prepared from 8-day-old postnatal Sprague–Dawley rats according to the procedures of Levi and coworkers [4, 5]. Briefly, the tissue was chopped into 0.4-mm cubes and then placed immediately into Krebs–Ringers bicarbonate

* To whom correspondence should be addressed.

(KRB) buffer. The suspension was centrifuged at 200 *g*, and the resulting pellet was digested with 0.025% trypsin in KRB buffer for 15 min at 37°. The digestion was stopped by addition of soybean trypsin inhibitor in the presence of deoxyribonuclease. The dissociated cells were cultured in basal modified Eagle's medium containing 10% heat-inactivated fetal calf serum, glutamine (2 mM), gentamycin (50 μg/ml) and 25 mM KCl. The cells were seeded to 35-mm tissue culture dishes (Costar 3035) precoated with poly-L-lysine at a density of 3×10^6 cells per dish and cultured at 37° in a humidified CO_2 (6%) incubator. Cytosine arabinoside (10 μM) was added 18–24 hr later to arrest replication of non-neuronal cells. For routine experiments, the dissociated cells were used for PtdIns turnover studies on day 8 after culturing. The morphology of cultured granule cells on days 2, 4 and 8 was indistinguishable from that reported previously [4, 5]. Progressive aggregation of cells and the concomitant outgrowth of fibers were seen during the culturing. Cells with morphology characteristic of astroglials were less than 2% of the total population in the 8-day culture.

Measurement of PtdIns hydrolysis. The turnover of PtdIns in cultured granule cells was measured as the lithium-induced accumulation of inositol monophosphate (IP_1) essentially as described by Chuang [6]. It should be noted, however, that the concentration of LiCl used in this study was 20 mM. Briefly, the cultured medium was aspirated from the dish on day 8 after culturing and replaced with the growth medium containing 2.5 μCi/ml of [^{3}H]myo-inositol to label the endogenous inositol phospholipids. After labeling for 16–18 hr, the cells were washed twice with physiological saline solution (PSS) comprised of 118 mM NaCl, 4.7 mM KCl, 3.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.5 mM EDTA, 10 mM glucose and 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) at pH 7.4. Following preincubation with 20 mM LiCl in PSS for 20 min at 37°, receptor drugs as indicated were added, and the reaction mixtures were further incubated for 30 min (which was in the linear range of IP_1 accumulation). The reaction was terminated by the addition of ice-cold methanol, and the cells were scraped off the dish. The accumulation of [^{3}H]IP_1 was measured by using Bio-Rad AG 1 × 8 column chromatography according to the procedures of Berridge *et al.* [7].

RESULTS

Characterization of 5-HT-induced [^{3}H]IP_1 accumulation in cerebellar granule cells. Addition of 5-HT in the presence of 20 mM LiCl to cultured granule cells prelabeled with [^{3}H]myo-inositol induced a dose-dependent increase of [^{3}H]IP_1 accumulation (Fig. 1). This activation was saturable and, depending on the preparation of granule cell culture used, maximal stimulation was between 300 and 600% of the control. From the data in Fig. 1, the EC_{50} and saturation concentration of 5-HT were found to be 0.1 and 10 μM respectively. The data in Table 1 show that some nonselective 5-HT receptor agonists, MK-212. 5-methoxytryptamine, tryptamine and quipazine, were also capable of stimulating [^{3}H]IP_1 accumulation. The efficacy of MK-212 and

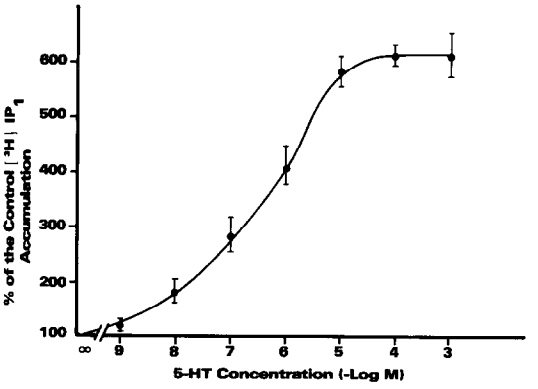

Fig. 1. Dose–response relationship for 5-HT-induced accumulation of [^{3}H]IP_1 in granule cells. Labeled granule cells from the day 8 culture were exposed to different concentrations of 5-HT for 30 min, and the accumulation of IP_1 wss measured as described. The data presented are mean ± SEM of the percent of control measured in the absence of 5-HT from three independent experiments. The 100% values were 3530 ± 850 dpm/dish.

5-methoxytryptamine was similar to that of 5-HT, whereas that of tryptamine and quipazine was less. 8-OH-DPAT, a prototypic 5-HT_{1A} receptor agonist [8], was ineffective in causing the hydrolysis of PtdIns. Several 5-HT_2 receptor antagonists were examined for their ability to inhibit the [^{3}H]IP_1 accumulation induced by 10 μM 5-HT (Fig. 2). Ketanserin, mianserin, spiroperidol and pyzotyline blocked this response in nanomolar concentrations with a rank order potency of spiroperidol > ketanserin ≥ pyzotyline > mianserin. It should be noticed that the shape of the inhibition curve elicited by spiroperidol was markedly different from those of other antagonists used. Atropine, a nonselective muscarinic acetylcholine receptor antagonist, and prazosin, a selective α_1-adrenergic receptor antagonist, did not alter the response at concentrations up to 10^{-5} M (Table 2).

Table 1. Effects of various serotonergic receptor agonists on the accumulation of [^{3}H]IP_1 in granule cells

Agonist	EC_{50} (μM)	Efficacy (% of the control)
5-HT	0.15	324 ± 7
MK-212	0.53	316 ± 16
5-Methoxytryptamine	0.62	303 ± 1
Tryptamine	0.70	237 ± 16
Quipazine	0.15	233 ± 11
8-OH-DPAT		113 ± 17

Labeled cells were stimulated with various concentrations (10^{-8}–10^{-4} M) of receptor agonists for 30 min in the presence of lithium, and the accumulations of [^{3}H]IP_1 were then determined. The efficacies presented are mean ± SEM of a triplicate experiment that was reproduced three times with similar results. The 100% values were 3160 ± 770 dpm/dish. The EC_{50} of 8-OH-DPAT was not given because this drug failed to induce a significant increase above the basal accumulation.

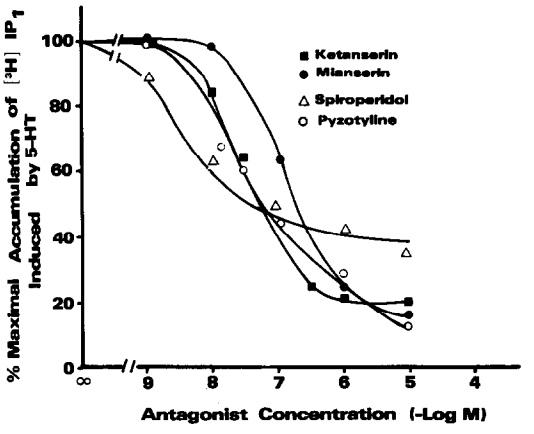

Fig. 2. Effects of various 5-HT antagonists on the 5-HT-induced IP_1 accumulation in granule cells. Labeled cells were preincubated for 5 min with indicated concentrations of antagonists prior to the addition of 10 μM 5-HT. The data present were the mean of a triplicate experiment that was reproduced four times with similar results. The SEMs were omitted for clarity but were generally between 10 and 20% of the means. The 100% values were the maximal stimulation measured in the presence of 5-HT and were 16,730 ± 1860 dpm/dish.

The 5-HT-induced accumulation of $[^3H]IP_1$ was dependent on the culturing time of granule cells (Table 3). The maximal stimulation by 5-HT was seen in an 8-day culture, whereas the stimulation was considerably less in 3- and 12-day cultures. The effect of lithium concentrations on the $[^3H]IP_1$ accumulation is shown in Fig. 3. In the presence of 5-HT, the accumulation was increased 9-fold over the range of lithium from 0 to 60 mM with a maximal accumulation seen at 20 mM, whereas the basal accumulation was increased 5-fold over this concentration range. At 20 mM lithium, 5-HT elicited an accumulation which was about 400% of the control.

NE and histamine-induced $[^3H]IP_1$ *accumulations in granule cells.* NE in a dose-dependent manner increased the accumulation of IP_1 with an EC_{50} of about 1 μM (Fig. 4). The maximal stimulation occurred between 10 and 100 μM NE and was about 300% of the control. In the presence of 10 μM NE, the increased accumulation of IP_1 was potently inhibited by prazosin with an IC_{50} of 2 nM or a K_i of about 0.2 nM (Fig. 5). Ketanserin and atropine at

Table 2. Effects of various receptor antagonists on the 5-HT-induced accumulation of $[^3H]IP_1$

Addition	Concn (μM)	$[^3H]IP_1$ (dpm/dish)
None		3,950 ± 130
5-HT	10	14,090 ± 330
5-HT + atropine	0.1	14,080 ± 600
5-HT + atropine	1	14,120 ± 410
5-HT + prazosin	10	15,790 ± 910
5-HT + ketanserin	1	4,230 ± 160

Experimental conditions are described in the text. When used, cells were preincubated for 5 min with the receptor antagonist before addition of 10 μM 5-HT. The data presented are mean ± SEM of a triplicate experiment that was reproduced three times with similar results.

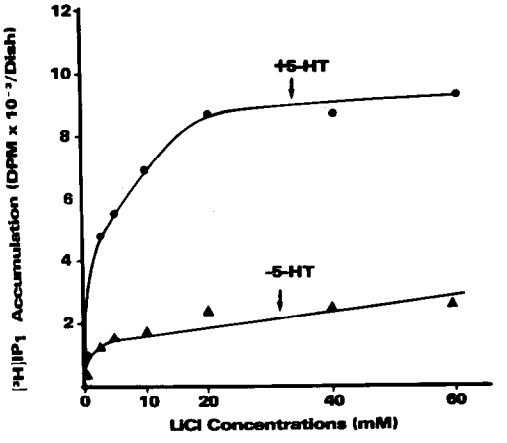

Fig. 3. Effects of lithium chloride on basal and 5-HT-induced IP_1 accumulation. Cells were preincubated with different concentrations of LiCl for 20 min, and the basal and 5-HT (10 μM)-induced accumulations were measured as described. The data presented are mean ± SEM of a triplicate experiment that was reproduced three times with similar results.

10^{-5} M were ineffective in blocking this event (data not shown). The results in Table 4 show that the accumulations of IP_1 increased by a saturating concentration (100 μM) of NE and 5-HT appeared to be additive in nature.

Histamine in a dose-dependent manner enhanced the accumulation by about 100% (Table 5). The

Table 3. 5-HT-induced accumulation of $[^3H]IP_1$ in granule cells from different days of cultures

Days in culture	$[^3H]IP_1$ (dpm/dish) Control	5-HT (10 μM)	Fold stimulation
3	4,350 ± 140	7,710 ± 610	1.8
6	3,670 ± 30	12,180 ± 1,070	3.3
8	3,830 ± 20	14,030 ± 1,150	3.6
12	1,850 ± 90	4,860 ± 590	2.6

Experimental conditions are described in the text except that cells were cultured for different days as indicated. Data presented are mean ± SEM of a triplicate experiment that was reproduced three times with similar results.

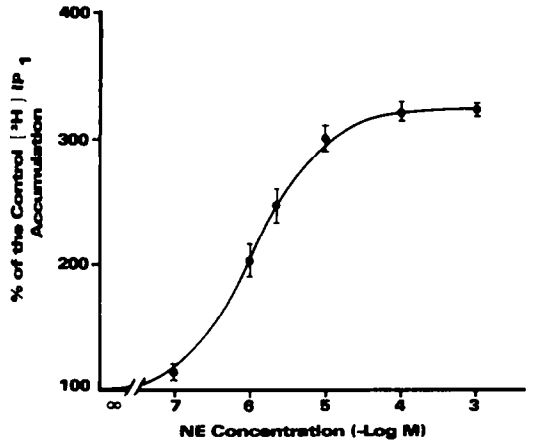

Fig. 4. Dose–response relationship for NE-stimulated IP_1 accumulation in granule cells. Labeled cells were exposed to different concentrations of NE, and the accumulations of IP_1 were measured as described. Data were expressed as percent of the basal accumulation and were mean ± SEM of three independent experiments. The basal accumulations were 2250 ± 490 dpm/dish (N = 3).

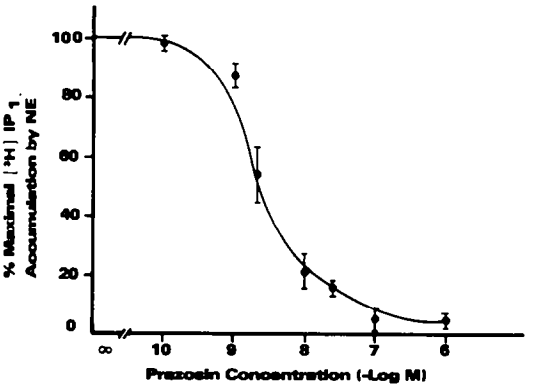

Fig. 5. Effects of prazosin on the NE-induced IP_1 accumulation in granule cells. Cells were preexposed to different concentrations of prazosin prior to addition of 10 μM NE, and the accumulations of IP_1 were measured as described. Data presented were percent of the accumulation stimulated by NE and were mean ± SEM of three independent experiments. The basal accumulations were subtracted from the total accumulations measured in the presence of 10 μM NE. The maximal $[^3H]IP_1$ accumulations elicited by NE were 5930 ± 700 dpm/dish (N = 3). The basal accumulations were unaffected by prazosin in the concentration ranges used in the study.

Table 4. Effects of the co-presence of NE and 5-HT on the accumulations of $[^3H]IP_1$ in granule cells

	$[^3H]IP_1$	
Addition	Accumulation (dpm/dish)	Stimulated accumulation (dpm)
None	2,650 ± 190	0
NE (100 μM)	8,470 ± 640	5,820
5-HT (100 μM)	9,250 ± 420	6,600
NE + 5-HT	13,970 ± 560*	11,320

Experimental conditions are described in the text. Data presented are mean ± SEM of a triplicate experiment that was reproduced three times with similar results.

* $P < 0.05$ when compared with NE or 5-HT alone using Student's *t*-test.

Table 5. Effects of histamine and histamine receptor antagonists on the accumulation of $[^3H]IP_1$ in granule cells

Addition (μM)	Percent of basal IP_1 accumulation
Histamine (1)	131 ± 11
(10)	162 ± 21
(100)	204 ± 20
(1000)	198 ± 20
Histamine (100) + triprolidine (0.1)	135 ± 9
Histamine (100) + triprolidine (1)	114 ± 8
Histamine (100) + cimetidine (0.1)	199 ± 8
Histamine (100) + cimetidine (1)	192 ± 16

Experimental conditions are described in the text. When used, cells were preincubated for 5 min with the receptor antagonist prior to addition of 100 μM histamine. The data presented are mean ± SEM of three independent experiments. The basal value was 2120 ± 420 dpm/dish.

enhanced accumulation could be blocked completely by 1 μM triprolidine, a histamine H_1 receptor antagonist, whereas cimetidine, a histamine H_2 receptor antagonist, was ineffective.

A few receptor agonists were found to have no effect on IP_1 accumulation. These included γ-aminobutyric acid (GABA) (10^{-6} to 10^{-3} M), (±)-baclofen (10^{-4} M), morphine (10^{-5} M), enkephaline (10^{-5} M), and D-ala-D-leu-enkephaline (10^{-5} M) (results not shown).

DISCUSSION

The cerebellar cortex has been a focus of intense neuroscience research because of its well defined neuroanatomy. It has been shown that there exists in the cerebellar cortex receptor binding sites for a variety of neurotransmitters including NE [9, 10], acetylcholine [11, 12], GABA [13, 14] and neuropeptides [15, 16]. In this study, we used cultured granule cells to demonstrate the presence of serotonergic, α_1-adrenergic and histamine H_1 receptors that are linked to Ptdlns-specific phospholipase C. Granule cells in the cerebellum relay the input of mossy fibers (whose cell bodies lie in the spinal cord and various nuclei in the brain stem) and send out axons to the molecular layer of cerebellar cortex [17]. The bulk of the information from both the periphery and higher brain centers reaches the cerebellum via the mossy fiber system. The neurotransmitters present in the mossy fibers are still unclear. However, the present results strongly suggest that 5-HT, NE and histamine are part of the neurotransmitter substances of the mossy fibers or other afferent nerves and that Ptdlns turnover, mediated by their selective receptors on the granule cells, may play a key role in modulating the glutamate-mediated excitatory function of granule cells.

The 5-HT-induced activation of Ptdlns turnover has been characterized. 5-HT, as well as some non-selective 5-HT receptor agonists, were capable of producing this activation, while the selective 5-HT_{1A} agonist, 8-OH-DPAT, was ineffective. Moreover, addition of 5-HT to granule cell membranes failed to activate adenylate cyclase activity (data not shown). These results suggest that granule cells are devoid of 5-HT_{1A} receptors linked to adenylate cyclase or phospholipase C. The potencies of spiroperidol, ketanserin, pyzotyline and mianserin in blocking the serotonin response are consistent with the notion that this is a 5-HT_2 receptor-mediated event, analogous with findings in rat aorta [18, 19] and cerebral cortex [3]. The potency, however, of 5-HT in the granule cell Ptdlns system was at least 10-fold greater than that in those two systems and the extent of stimulation was much greater than that detected in the cerebral cortical slices. It is interesting to notice that spiroperidol failed to inhibit completely the 5-HT-increased accumulation even up to a 10 μM concentration of the drug. Recently, Conn *et al.* [20] reported that a novel serotonin receptor, 5-HT_{1C}, in the choroid plexus is linked to Ptdlns turnover. This receptor response is highly sensitive to classical 5-HT_2 receptor antagonist with the exception of spiroperidol which has a K_i in the micromolar range. It remains to be characterized whether the spiroperidol-insensitive minor component in the granule cell Ptdlns system is due to involvement of the 5-HT_{1C}-Ptdlns system.

The effect of 5-HT on the Ptdlns hydrolysis in granule cells was dependent on the time of culturing, with a maximal response seen in an 8-day culture (Table 3). It is interesting to notice that the time effect of this 5-HT response is similar to that for the appearance of morphological differentiation and the expression of depolarization-dependent release of glutamate from granule cells in culture [4, 5]. The response induced by 5-HT and NE in cultured granule cells appears to be additive in nature. This may suggest that 5-HT receptors and α_1-adrenergic receptors are either situated on different loci on the same cell utilizing a different pool of Ptdlns–phospholipase C system or located on different populations of cells in the culture. Further experiments are required to differentiate these two possibilities. In any event, the Ptdlns turnover in cultured granule cells is subject to regulation by 5-HT, NE and histamine. Moreover, stimulation of muscarinic acetylcholine receptors in cultured cerebellar granule cells led to a robust increase (30-fold) in the hydrolysis of Ptdlns [21]. Thus, the granule cell system should be useful for studying the physiological role of this receptor-mediated process as well as the molecular mechanisms underlying the coupling of these receptors to phospholipase C.

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