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A M_3 MUSCARINIC RECEPTOR COUPLED TO INOSITOL PHOSPHATE FORMATION IN THE RAT COCHLEA?

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Abstract—Various neuroactive substances, including excitatory and inhibitory amino acids, biogenic amines and neuropeptides, were tested for their ability to stimulate the inositol phosphate (IPs) cascade in the presence of lithium in the rat cochlea. Among them, only the muscarinic agonists (carbachol and oxotremorine M) were able to stimulate the IPs formation in 12-day-old rat cochleas. The carbachol-elicited IPs formation was inhibited by muscarinic antagonists with the following relative order of potency: atropine > 4-DAMP ≫pirenzepine > methoctramine = AF-DX 116. This pharmacological profile suggests that the activation of the M_3 muscarinic receptor subtype is responsible for the increase in IPs synthesis in the rat cochlea. However, an interaction with a m5 receptor subtype could not be completely excluded. The unusual link of only one receptor subtype with the phosphoinositide breakdown in the cochlea, as opposed to the usual existence of several receptors coupled to this transduction system in other organs such as the brain, suggest a unique role for muscarinic agonists in the cochlea.

The mammalian cochlea contains two types of hair cells: namely the inner hair cells (IHCs‡) and the outer hair cells (OHCs). The auditory transmission between IHCs and the first auditory neurons (type I afferent neurons) is thought to be assumed by an excitatory amino acid, likely glutamate [1–4]. This transmission may be modulated by the efferent lateral olivocochlear fibers which synapse at the afferent dendrites of the type I auditory neurons. Several neurotransmitters including dopamine, GABA, acetylcholine have been identified in these fibers [5]. The OHCs receive neuronal information via the medial efferent system which is mainly cholinergic [6]. In addition, the OHCs contact the dendrites of type II neurones, which represent the other afferent system of the cochlea. This afferent system, for which the neurotransmitter remains unknown, represents only 5% of the whole cochlear afferent system.

The OHCs have been postulated to indirectly modulate IHCs activity [7, 8] probably due to their motile properties. Motility (shortening and elongation) of isolated OHCs has been shown to occur in response to intracellular current administration (fast motility) [9] or in response to neuroactive substances such as potassium or acetylcholine (slow motility) [9, 10]. The molecular mechanisms, which underlie such OHCs motility, may originate from phosphoinositide metabolism and the subsequent intracellular calcium mobilization [11, 12], which in turn controls actin polymerization and the formation of microfilament assemblies [13].

Despite the importance of such molecular mechanisms in the understanding of cochlear functioning, no direct measurement of inositol phosphate formation has been performed in this organ. In the present paper, we examine the accumulation elicited by neuroactive substances of these second messenger molecules (inositol mono-, bis- and tris-phosphate) in the rat cochlea.

MATERIALS AND METHODS

Incorporation of [^{3}H]*inositol.* Wistar rats of age 12 days were killed by decapitation. After dissecting out the bony capsule, whole cochleas were rapidly removed and immediately placed in Krebs–Ringer buffer, pH 7.4. The Krebs–Ringer buffer used contained 125 mM NaCl; 3.5 mM KCl; 1.25 mM KH_2PO_4; 1.2 mM $MgSO_4$; 1.5 mM $CaCl_2$; 10 mM glucose and 25 mM $NaHCO_3$. Before use, this buffer was equilibrated to pH 7.4 by saturation with a gaseous mixture of 95% O_2 and 5% CO_2 (v/v). *Myo*-[2-^{3}H]Inositol incorporation in 75 cochleas was carried out at 37°, under gassing of 95% O_2/5% CO_2, for 75 min in 5 mL Krebs–Ringer buffer containing 1 mM cytidine and 50 μCi of the radioactive inositol (sp. act. 17 Ci/mmol, CEA Saclay, France). The labelled cochleas were then washed four times in 5 mL Krebs–Ringer buffer before being individually distributed in plastic tubes containing 500 μL Krebs–Ringer buffer. The tubes were then transferred to a water bath maintained at 37° and continuously gassed with the gaseous mixture of 95% O_2/5% CO_2 throughout the subsequent experimental steps.

Agonist stimulation. LiCl (10 μL, final concentration 10 mM) and 20 μL of an appropriate concentration of an antagonist (where applicable) were

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‡ Abbreviations: IHC, inner hair cell; OHC, outer hair cell; 4-DAMP, 4-diphenylacetoxy-*N*-methyl piperidine methiodide; AF-DX 116, 11-((2-((diethylamino)-methyl)-1-piperidinyl)acetyl) - 5,11 - dihydro - 6*H* - pyrido(2,3 - b)-(1,4)-benzodiazepine-6 one.

added to each tube. After 15 min, the stimulatory neuroactive substance or buffer (20 μL) was added. Following an additional 20 min of incubation, the reaction was stopped by the addition of 50 μL perchloric acid (PCA 72%) and, by placing the tubes on ice.

Measurement of [^{3}H]*inositol phosphates.* The labelled IPs were purified and measured according to the method described by Bone *et al.* [14] modified as follows: the cochleas were homogenized using an Ultraturrax homogenizer. The homogenates containing the PCA-extracted [^{3}H]IPs were then centrifuged at 2000 *g* for 20 min. The resulting supernatants were poured off and retained while the pellets were resuspended by homogenization in 1 mL PCA (1%) and recentrifuged at 2000 *g* for 20 min. The two PCA extracts were then pooled and neutralized with a 1.5 M KOH/0.075 M HEPES solution in the presence of universal indicator pH 4–10. The resulting potassium perchlorate salt was then pelleted by centrifugation (5 min at 2000 *g*), resuspended in 2 mL 0.5 mM EDTA/5 mM $Na_2B_4O_7$ and washed by centrifugation (5 min at 2000 *g*). The supernatants from the two centrifugations were then pooled. A solution (10 mL) containing 0.5 mM EDTA/5 mM $Na_2B_4O_7$ was then added to the pooled supernatant. The extracts (containing [^{3}H]inositol and [^{3}H]IPs) thus obtained were then applied to Dowex anion-exchange columns (1X8 formate form; height of resin: 4 cm; diameter: 0.8 cm). Aliquots (1.5 mL) were taken from the flow-through fractions and subjected to liquid scintillation counting. Each column was then washed once with 20 mL water. Glycerophosphoinositides (GPI) were eluted with 20 mL 0.04 M ammonium formate/0.003 M sodium tetraborate. The IPs were eluted with 15 mL 0.8 M ammonium formate/0.1 M formic acid. For both the GPI and IPs fractions, 4-mL aliquots were taken and radioassayed. Columns were regenerated twice with 10 mL 2 M ammonium formate/0.1 M formic acid and twice with 20 mL distilled water prior to re-use. Columns were discarded after five experiments. For liquid scintillation counting, 2.5 mL of Ready Gel scintillation fluid (Beckman Instruments Inc., Ireland) was added to the 1.5-mL aliquots obtained from the flow-through fractions while 6 and 7 mL of Ready Gel were added to the 4-mL aliquots containing the eluted GPI and IPs, respectively.

Data calculation. Dose–response curves for agonists and antagonists were fitted using non-linear regression with experimental values weighted by the reciprocal of the variance, according to the logistic model described by De Lean *et al.* [15, 16]. The computer programme used was the RS/1 release 3.0 program (BBN Software Product Corporation, Cambridge, MA, U.S.A.), run on a VAX 6210 microcomputer.

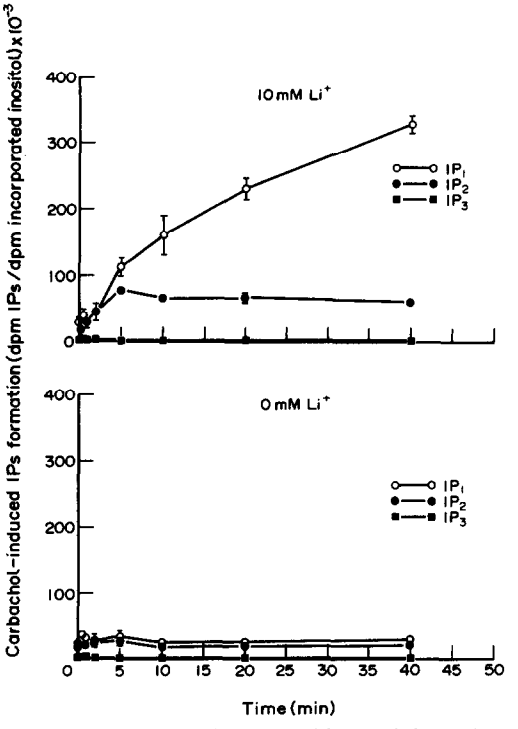

Fig. 1. Time course of carbachol-induced formation of [^{3}H]IP$_1$, [^{3}H]IP$_2$ and [^{3}H]IP$_3$ in rat cochleas. Experimental conditions are as described in Materials and Methods except that the cochleas were prelabelled with 500 μCi [^{3}H]myo-inositol instead of 50 μCi for 75 min, washed five times, and then stimulated for different time periods with 10 mM carbachol in the absence or in the presence of 10 mM LiCl. In addition, the reaction was stopped using 60 μL of a solution containing PCA (72%), EGTA (1 mM) and a nonradioactive mixture of inositol phosphates (2 mg/mL) in order to inhibit the acidic phosphatases which catalyse the dephosphorylation of the various IPs metabolites. The elutions of the various metabolites from the Dowex 1X8 columns were performed as follows: twice with 20 mL of water to remove [^{3}H]myo-inositol, once with 15 mL of 0.04 M ammonium formate/0.003 M sodium tetraborate for GPI, twice with 15 mL of 0.15 M ammonium formate/0.005 M sodium tetraborate for IP$_1$, twice with 15 mL of 0.4 M ammonium formate/0.1 M formic acid for IP$_2$, twice with 15 mL of 0.8 M ammonium formate/0.1 M formic acid for IP$_3$ and once with 15 mL of 2 M ammonium formate/0.1 M formic acid for the other inositol phosphates. Results are means ± SD of experiments done on at least six different cochleas (one IPs determination is performed for each individual cochlea). The basal IP$_1$, IP$_2$ and IP$_3$ accumulation was not significantly affected by the presence of 10 mM Li$^+$.

RESULTS

Time course

Carbachol (carb) enhanced IP$_1$ accumulation in the presence of 10 mM Li$^+$ at 37° in the rat cochlea. This increase was exponential during the first 5 min and linear between 5 and 45 min of incubation (Fig. 1 upper panel). Carb did not induce any IP$_1$ accumulation in the absence of Li$^+$ (Fig. 1 lower panel). Very little radioactivity was found in the IP$_2$ and IP$_3$ fractions and this was not affected by Li$^+$. The basal levels of IP$_1$, IP$_2$ and IP$_3$ did not change with Li$^+$. The results were expressed as the ratios of dpm contained in the various IP fractions to the dpm of incorporated inositol. Similar patterns were obtained when results were expressed as dpm per mg of protein (data not shown). These preliminary experiments prompted us to measure the total IPs (containing about 98% of IP$_1$ and IP$_2$) for a 20 min

Table 1. Effect of neuroactive substances on the IPs formation in the rat cochlea

Agonist	Maximum Concentration Tested (μM)	Effect on IPs accumulation*
Glutamate	1000	100 ± 26
Quisqualate	1000	118 ± 16
N-Methyl-D-aspartate	1000	101 ± 21
Kainate	1000	124 ± 52
Nicotine	10,000	79 ± 29
Carbachol	10,000	617 ± 66‡
Oxtremorine M	1000	727 ± 59‡
Gamma-amino-butyric acid	1000	102 ± 23
Glycine	1000	89 ± 8
Noradrenaline	1000	114 ± 24
5-Hydroxytryptamine	1000	135 ± 23
Dopamine	1000	126 ± 12
Substance P	1	113 ± 26
Neuropeptide Y	1	86 ± 17
Calcitonin gene related peptide	1	96 ± 13
Vasoactive intestinal peptide	1	109 ± 17
Arginine-vasopressin	1	87 ± 12
Cholecystokinin	1	81 ± 10
Met-enkephalin	1	115 ± 29
Leu-enkephalin	1	100 ± 18
Met-enkephalin-Arg^6-Phe^7	1	109 ± 12
Oxytocin	1	99 ± 17
DAGO§	1	82 ± 11
DTLET‖	1	72 ± 3†
DPLPE¶	1	91 ± 34
Potassium	60,000	92 ± 13
A 23187	5	96 ± 23
Monensin	50	145 ± 37
Valinomycin K	1	79 ± 12
Veratridine	10	92 ± 23

* Results are expressed as percentages of control values. The mean control value is $(47 \pm 13) \times 10^{-3}$ dpm IPs/dpm [^{3}H]inositol incorporated and the mean value of [^{3}H]inositol incorporation is 41,130 ± 12,170 dpm per cochlea. Results are expressed as means ± SD of at least six individual determinations. The statistical significance of the agonist-stimulated IPs formation versus the control IPs values was calculated by two-tailed Student's *t*-test. († $P < 0.01$; ‡ $P < 0.001$).

§ DAGO: (D-ala^2, *N* methyl-phe^4, glyol^5) enkephalin.

‖ DTLET: (D-thr^2, thr^6) leu-enkephalin.

¶ DPLPE: (D-pen^2, L-pen^5) enkephalin.

incubation period and to express the results as ratios (dpm IPs/dpm incorporated [^{3}H]myo-inositol) for the following experiments.

Effects of neuroactive substances on the IPs formation in the rat cochlea

A large number of neuroactive substances, some of which are known to be present in the cochlea [5], ions and ionophores were screened for their ability to stimulate IPs formation in the cochlea (see Table 1). With the exception of the muscarinic agonists carbachol and oxotremorine M, none of the substances tested (nicotine included) had any significant stimulatory effect.

Dose–response curve

Carbachol and oxotremorine M induced IPs accumulation in a dose-dependent manner (Fig. 2). From the dose–response relationships, the EC_{50} values were calculated, using a curve fitting programme indicated in Materials and Methods. They were 28 ± 20 μM and 87 ± 19 μM for oxotremorine M and carbachol, respectively. A pre-analysis according to the logistic model proposed by Black *et al.* [17] shows that for the two agonists the slopes are approximately equal to 1. The E_{max} values for carbachol and for oxotremorine M are significantly different. These values expressed as percentages of the basal values are 570 ± 60% and 799 ± 93%, respectively.

Effect of cholinergic antagonists on carbachol-elicited IPs accumulation

A broad range of cholinergic receptor antagonists were assessed for their ability to block the increased accumulation evoked by carbachol. The nicotinic antagonists D-tubocurarine and α-bungarotoxin had no effect. On the contrary, muscarinic antagonists inhibited all the IPs formation induced by 10^{-3} M carbachol (Fig. 3). None of these cholinergic antagonists affected the basal IPs accumulation, except for a high concentration of methoctramine. At

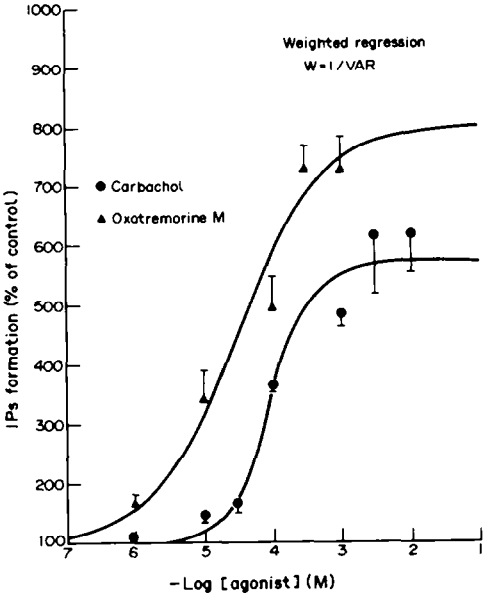

Fig. 2. Concentration–response relationships of carbachol- and oxotremorine M-induced IPs formation in the rat cochlea. Cochleas were labelled for 75 min with 50 μCi [^{3}H]myo-inositol, and then washed four times to remove the excess of radioactive inositol. After preincubation with 10 mM LiCl at 37° for 15 min, increasing concentrations of carbachol or oxotremorine M were added and the cochleas were further incubated for 20 min. The accumulation of [^{3}H]IPs was measured by scintillation counting following Dowex 1X8 column chromatography. Results which are means ± SE of experiments done on at least five different cochleas, show stimulation as a percentage of the basal IPs formation. Basal IPs formation was 54,180 ± 4913 dpm/mg protein (mean ± SE).

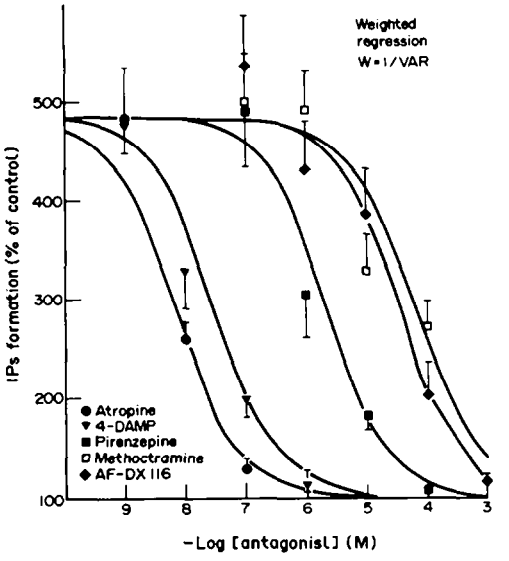

Fig. 3. Effects of muscarinic antagonists on carbachol-induced IPs formation in the cochlea. Experimental conditions are as described in Materials and Methods. Cholinergic antagonists (10^{-7} M–10^{-3} M) were tested for their ability to inhibit IPs formation induced by 1 mM carbachol. Results which are means ± SE of experiments done on at least six different cochleas, show stimulation as a percentage of the basal IPs formation. Antagonists per se (atropine, 4-DAMP, methoctramine, pirenzepine and AF-DX 116) did not affect the basal IPs formation at the concentrations tested (except for high concentrations of methoctramine greater than 10^{-4} M). D-Tubocurarine and α-bungarotoxin were without effect both on the basal and carbachol-stimulated IPs accumulation.

10^{-3} M, methoctramine alone stimulates IPs accumulation (about 400% of basal value). The same level of stimulation is also obtained in the co-presence of 10^{-3} M carbachol. The relative order of potency of the muscarinic antagonists were atropine > 4-DAMP ≫ pirenzepine > methoctramine = AF-DX 116. The IC_{50} values which were calculated from the inhibition curves and were 0.007 ± 0.007, 0.030 ± 0.008, 2.0 ± 0.8, 33 ± 7 and 63 ± 42 μM for atropine, 4-DAMP, pirenzepine, AF-DX 116 and methoctramine respectively. For each antagonist, pseudo-Hill numbers were determined (Table 2). In all cases they are not significantly different from 1. However, for methoctramine, this number is relatively low.

Table 2. Calculated IC_{50} and pseudo-Hill values for cholinergic antagonists for inhibition of 1 mM carbachol-induced IPs formation in the rat cochlea

Antagonist	IC_{50} (μM)	n_H
Atropine	0.007 ± 0.007	0.83 ± 0.47
4-DAMP	0.03 ± 0.008	0.94 ± 0.15
Pirenzepine	2.0 ± 0.8	0.90 ± 0.17
AF-DX 116	33 ± 7	0.91 ± 0.15
Methoctramine	63 ± 42	0.79 ± 0.49

These values were calculated from the results presented in Fig. 3 as described in Materials and Methods. They are expressed as means ± SE of at least six individual determinations.

DISCUSSION

Our results indicate that among the neuroactive substances tested (Table 1), some of which are known to occur in the cochlea [5], only cholinergic agonists are able to stimulate the accumulation of IPs in the cochlea. Although nicotinic receptors seem to occur in the cochlea, (nicotine potentiates the submaximal effects of the stimulation of the crossed olivocochlear bundle [18]) their activation did not lead to IPs stimulation. Conversely, cholinergic agonists of the muscarinic receptor subtype (carbachol and oxotremorine M) increase the metabolism of IPs with a high apparent affinity. The fact that the E_{max} values calculated for these two agonists are significantly different may indicate that carbachol is a partial agonist. The carbachol-stimulated IPs formation is not blocked by nicotinic antagonists such as D-tubocurarine and α-bungarotoxin while it is inhibited by muscarinic antagonists (atropine, pirenzepine, 4-DAMP, methoctramine and AF-DX 116). This clearly demonstrates that a muscarinic

receptor is responsible for the IPs formation in the cochlea. Muscarinic receptors have recently been divided into five subtypes,* named m1, m2, m3, m4 and m5 on the basis of molecular cloning experiments and the expression of this cloned muscarinic receptor in various cells [19–21]. The m1 and m2 receptor subtypes have also been cloned from porcine brain and heart [22–24]. In order to determine which muscarinic receptor is implicated in the cholinergic-induced IPs formation in the cochlea, we have measured the inhibition, by various muscarinic antagonist of the IPs accumulation, induced by 1 mM carbachol. Although it would be better to determine K_i values from Schild plot analyses, we have only measured the IC_{50} value for each antagonist in this paper (Table 2). The reasons for this are technical limitations such as the time taken for dissection, the time-restricted period for cochlear viability and the very small quantities of biological tissue present per cochlea. However, since the same concentration of carbachol was used for studying the antagonist inhibition, IC_{50} values thus determined will provide an approximate rank order of potency of the antagonists. Subsequently, the subtype of receptor involved can be determined. The most widely used classification of muscarinic receptor subtypes was based on the relative affinities of the antagonists, pirenzepine and AF-DX 116 in particular [25–27]. M_1 receptors express a high apparent affinity toward pirenzepine and an intermediate affinity toward AF-DX 116 [26, 28], whereas M_2 receptors "cardiac type" (M_{2alpha}) possess a low apparent affinity for pirenzepine and a high affinity for AF-DX 116 [26–28]. M_3 receptors, previously known as the M_2 "glandular type" (M_{2beta}) present a low apparent affinity toward both pirenzepine and AF-DX 116 and a high affinity toward 4-DAMP [28, 29]. Methoctramine was found to be as effective and selective as AF-DX 116 for the M_2 receptor [30, 31]. No specific antagonists, yet tested, allowed the distinction of m4 or m5 from the rest of the muscarinic receptor subtypes [32]. By using a large variety of antagonists, it is possible to approximate the identification of the specific subtype of muscarinic receptor involved as demonstrated by a recent displacement-binding study carried out in CHO-K1 cells, in which cloned muscarinic receptors were expressed [32]. However, the pharmacological profile determined in this study cannot be easily applied to muscarinic receptor subtype identification in other experimental models. Indeed, artificial receptor gene expression does not necessarily simulate exactly the expression occurring *in vivo*. Another indirect manner of characterization of receptor subtypes is the study of the biochemical responses triggered by their activation. Muscarinic receptors are coupled to a variety of second messenger systems involving adenylate cyclase and phosphatidylinositol metabolism as well as to ion channels [20, 33–41]. In fact, it was reported that M_1 (m1), M_3 (m3) and m5 receptors are coupled with the stimulation of phosphoinositides hydrolysis [20, 26, 29, 35–37, 39, 40] whereas m2 and m4 are mainly linked to the inhibition of adenylate cyclase [36, 38].

Our pharmacological results indicate that the muscarinic receptor subtypes involved in the IPs formation in the rat cochlea is probably a M_3 receptor. In fact, pirenzepine has a low apparent affinity ($IC_{50} = 2 \times 10^{-6}$ M) in inhibiting IPs synthesis which rules out the possibility of an interaction with the M_1 receptor. A similar low apparent affinity of pirenzepine was recently reported in receptor-binding studies to cochlear membranes using [^{3}H]-1-quinuclidinylbenzylate [42]. We found a relatively low efficacy of AF-DX 116 or methoctramine for inhibiting the carbachol-induced IPs accumulation in the rat cochlea ($IC_{50} = 33$ and 63 μM, respectively). This excludes the involvement of a M_2 receptor subtype in this IPs response since these two antagonists present a high affinity for the M_2 receptor subtype [26–30]. The antagonist 4-DAMP inhibits the carbachol-induced IPs response with a high apparent affinity, thus suggesting an action via the M_3 receptor [29]. However, the potency of 4-DAMP has not been tested for its inhibitory action neither on the binding of muscarinic ligands to m4 or m5 receptor subtypes nor on the biochemical responses associated with the activation of these two latter receptor subtypes. Nevertheless, m4 receptors are rather shown to be coupled to adenylate cyclase inhibition than to IPs formation [36], suggesting that the response observed here, is probably not mediated by a m4 muscarinic receptor. On the other hand, the expression of m5 receptors has not yet been observed in any tissue or cell line [32]. The paucity of pharmacological data concerning this receptor subtype, does not allow us to rule out the possibility of the existence of a m5 muscarinic receptor linked to inositol phosphate formation in the cochlea. If this is so, this will be the first indication of the natural expression of the m5 receptor gene in an organ. In fact, the antagonists used present about the same rank order of potencies in the binding studies on both the m3 and m5 receptors expressed in CHO-K1 cells: atropine $\gg$ methoctramine $>$ pirenzepine $\gg$ AF-DX 116 [32]. In our experiments, we found the following order of inhibitory action on carbachol-stimulated IPs formation: atropine $\gg$ pirenzepine $\gg$ AF-DX 116 = methoctramine. The apparent discrepancy concerning the inhibitory effect of methoctramine between the two sets of results may originate from the fact that methoctramine at concentrations greater than 10^{-4} M becomes an agonist in the cochlea. This finding, which has also been reported in the rat cerebral cortex [43], may explain why the Hill number for methoctramine is not equal to 1. A low Hill number for methoctramine has also been obtained in [^{3}H]-*N*-methylscopolamine displacement experiments on rat submaxillary gland membranes, thought to possess only M_3 receptors [32]. Taken

* The nomenclature of the muscarinic receptor subtypes used in this paper is that recommended recently in the supplement of Trends in Pharmacological Sciences, December 1989, p. VII by the nomenclature committee of the fourth symposium on muscarinic subtypes. According to this nomenclature, the pharmacologically characterized receptor subtypes are known as M_1, M_2 and M_3, while those characterized by molecular cloning techniques are named m1, m2, m3, m4 and m5. It is likely that the m1 sequence corresponds to that of the M_1 receptor, m2 to the M_2 receptor and m3 to the M_3 receptor.

together our results seem to indicate that cholinergic-induced IPs formation in the cochlea is probably mediated by the activation of a M_3 muscarinic receptor.

Our results also show that neither glutamate nor quisqualate stimulate IPs formation in the rat cochlea. Nevertheless several reports have shown that an excitatory amino acid is probably involved in the transmission between the IHCs and the primary auditory neurons [44] by activating a quisqualate receptor [1, 45]. If so, the quisqualate receptor involved did not correspond to that linked to the phosphoinositide metabolism [46]. These facts reinforce the conclusion of our previous work [46] which indicate that two subtypes of quisqualate receptors do indeed exist in the central nervous system, one linked to ion channels, the other one to phosphoinositides metabolism.

In conclusion, our data strongly suggest that among the known neuroactive substances found in the rat cochlea only muscarinic agonists mediate the stimulation of IPs turnover likely via a M_3 receptor subtype. Acetylcholine is thought to be the main neurotransmitter between the medial efferent system and the OHCs [6], and inositol triphosphate causes the contraction of permeabilized OHCs *in vitro* [11, 12]. Thus, the activation of this second messenger pathway in the cochlea by acetylcholine via a M_3 receptor may play a key role in the triggering or the control of slow OHCs motility, although the accurate cellular location of this second messenger remains to be elucidated.

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