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Localisation of functional muscarinic receptors in the rat cochlea: evidence for efferent presynaptic autoreceptors

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In the rat cochlea, the activation of muscarinic receptors stimulates the hydrolysis of phosphoinositides but the importance of this muscarinic effect is still unknown. In order to find out about the role of the muscarinic receptors in the cochlea, we examined their functional distribution within this organ. This was achieved by measuring the formation of [^3H]inositol phosphates induced by carbachol (1 mM) in two regions of the cochlea: the modiolus and the organ of Corti. At both sites, carbachol enhanced the accumulation of inositol phosphates in an atropine-sensitive way. These stimulations were completely antagonised by 4-diphenylacetoxy-*N*-methyl piperidine methiodide (1 μM) but unchanged by pirenzepine (1 μM). In cochleas depleted of outer hair cells by a treatment with amikacin, the carbachol-induced formation of inositol phosphates is not altered with respect to control, undamaged cochleas. Conversely, when the medial cholinergic axons which form synapses with the outer hair cells are destroyed by the section of the crossed olivocochlear bundle the carbachol-stimulated inositol phosphates response is reduced by 35% in the organ of Corti. This section has no effect in the modiolus, despite the degeneration of some modiolar fibers. Our results show that functional muscarinic receptors are distributed both in the organ of Corti and in the modiolus. These two structures contain presumably the same class of cholinergic receptor. The effects of selective destruction clearly demonstrate that a population of muscarinic receptors is located on presynaptic membranes at the level of the medial axon-outer hair cell contacts. They also point to spiral ganglion neurons and/or the Schwann cells as sites for the functional cholinergic receptors in the modiolus.

INTRODUCTION

The organ of Corti, the neuro-sensory epithelium of the cochlea, is innervated by efferent axons from the brainstem. The axonal population is dichotomised between the medial and lateral efferent systems. The former innervates the outer hair cells, the latter forms axo-dendritic synapses with the radial afferent fibers innervating the inner hair cells^{53,57}. An interesting anatomical feature of the medial system is that most of its axons project predominantly to the contralateral cochlea and cross the brain midline on the floor of the fourth ventricle. Such a circuit provides the possibility to alter and study specifically the function of the efferent medial system. Concerning its physiology, it is now accepted that the medial efferent system modulates the active mechanisms in the cochlea^{32,38,45,51} by means of acetylcholine^{9,10,32,51}. Support for identifying acetylcholine as the principal medial transmitter also comes

from immunohistologic surveys which have repeatedly demonstrated that most of the medial efferent neurons express the choline acetyltransferase activity^{1,13,20,47,48}. Concerning the lateral system, its function is rather complicated since recent data showed that a single lateral neuron possesses several neurotransmitters, both excitatory and inhibitory, in order to modify the sensory-neural transduction⁴⁸.

In the cochlea, acetylcholine alters the behaviour of its target cells by acting through both nicotinic^{11,25,43,44} and muscarinic cholinergic receptors^{3,4,6,19,24,27,28,41}. Both cholinergic receptors are present on the outer hair cell and their activation results in the hyperpolarization of the outer hair cells^{25,29}. But the distribution of the muscarinic cholinergic receptor is not restricted to these cells because binding studies report the presence of muscarinic sites in the cochlear nerve^{27,58}, in the modiolus²⁷ and in the organ of Corti²⁷. However, such a distribution may not match accurately the localisation of functional mus-

carinic proteins, which are cell surface receptors⁵⁵. Indeed, the ligand used in these studies, quinuclidinyl benzylate may label internalised receptors in addition to the plasma membrane bound receptors²³.

Functional muscarinic receptors are linked to the inositol phosphate (IP) signalling pathway in the rat cochlea^{3,4,24} and in the guinea pig organ of Corti⁴¹. Pharmacological characterisation indicates that the cholinceptors include the M₃ muscarinic subtype in the rat cochlea^{4,6,19,24}. Besides the muscarinic activation of potassium currents in outer hair cells²⁸, it is possible that muscarinic receptors are involved in the modulation of the motility of these cells. Indeed motile responses occur in permeabilized isolated outer hair cells exposed to inositol 1,4,5-trisphosphate^{49,50}. Furthermore the suppressive effects of olivocochlear efferent activation on distortion product otacoustic emissions are mimicked by acetylcholine³² and blocked by muscarinic antagonists³³. However, a direct muscarinic stimulation of the IP turnover has not been evidenced in outer hair cells yet and, more controversially, Plinkert and co-workers⁴⁴ did not detect any muscarinic binding site in isolated outer hair cells using tritiated quinuclidinyl benzylate as a ligand.

Therefore considering published data, the role of muscarinic receptors in the peripheral auditory system is still an open question. The determination of the cochlear structures bearing functional muscarinic receptors will provide useful information in this respect. The aim of the present work is therefore to identify some of these structures. Since the function of the medial system is better understood than that of the lateral system and since the trajectory of the medial fibers in the brainstem facilitates their experimental manipulation, this study mainly focuses on the outer side of the organ of Corti. Furthermore, indirect evidence associating muscarinic receptors to the outer region of this organ is found in the following spatio-temporal correlation: the muscarinic stimulation of the IP turnover is particularly enhanced during the period when efferents are innervating the outer hair cells^{3,4}, and the muscarinic stimulation of the IP turnover is higher at the base than at the apex of the organ of Corti⁴¹, paralleling the distribution of the medial efferent innervation which is more abundant at the base than at the apex of the cochlea⁵³.

The strategy used was to study the effect of selectively destroying either the medial efferents or the outer hair cells on the metabolism of IPs activated via muscarinic receptors in the rat cochlea. Chronic treatment with aminoglycoside antibiotic was carried out in order to destroy the outer hair cells^{22,34,46} and surgical transections of the crossed olivocochlear bundle were

performed in order to eliminate medial efferent terminals^{26,59}. Partial results have been presented in a preliminary form⁵.

MATERIALS AND METHODS

Materials

Myo-[2-(³H)]inositol (spec. act. 17.02 Ci/mmol) was purchased from Dositek (France) and 4-diphenylacetoxy-N-methyl piperidine methiodide (4-DAMP) from Research Biochemical Inc. Carbachol, amikacin sulphate, atropine and pirenzepine were obtained from Sigma. All other compounds were of analytical grade.

Dissection of the cochleas for biochemical studies

Wistar rats which completed cochlear maturation (26-day-old and adult) were stunned and killed by cervical transection. The cochleas were rapidly dissected and collected in Krebs-Ringer buffer (concentration in mM: 125 NaCl, 3.5 KCl, 1.25 KH₂PO₄, 1.2 MgSO₄, 1.5 CaCl₂, 25 NaHCO₃ and 10 glucose). Throughout the experiment, the dissected cochleas (consisting of the organ of Corti, the spiral ganglion and peripheral part of the cochlear nerve contained in the modiolus) were maintained in the Krebs-Ringer buffer, which was equilibrated to pH 7.4 by saturation with a 95% O₂/5% CO₂ mixture. In some sets of experiment, the cochleas were further dissected into two parts: the organ of Corti and modiolus. This latter part contains the spiral ganglion and the nerve fibers of both the efferent and afferent systems.

Labelling of the cochlear tissues

[³H]Inositol incorporation into cochleas or cochlear parts (organ of Corti and modiolus) was carried out at 37°C, for 75 min in Krebs-Ringer buffer containing 1 mM cytidine and 10.8 µCi/ml of myo-[2-(³H)]inositol. Labelling of the cochlear tissues was achieved by using 90 µl of radioactive Krebs-Ringer buffer per cochlea, or 45 µl per cochlear part. The cochlear samples were washed four times with Krebs-Ringer buffer and then individually distributed in plastic test

Fig. 1. Formation of IPs in the organ of Corti and in the modiolus. The data are means $\pm$ S.E.M. of 6 experiments, at least, and are expressed as percentage of their respective basal levels of IPs accumulation. These basal levels are 0.125 ± 0.014 dpm [³H]IPs/dpm [³H]inositol in the organ of Corti and 0.038 ± 0.002 dpm [³H]IPs/dpm [³H]inositol in the modiolus. Carbachol was used at 1 mM while atropine, 4-DAMP and pirenzepine were applied at 1 µM. C, carbachol; ATR, atropine; PZ, pirenzepine. * Different from control with $P < 0.05$; ** different from control with $P < 0.01$; *** different from control with $P < 0.001$.

tubes containing 500 μ l Krebs-Ringer buffer. The tubes were quickly transferred to a water bath thermostatically maintained at 37°C and continuously bubbled with the 95% O₂/5% CO₂ mixture.

Stimulation of tritiated IP formation

LiCl (10 mM) and the muscarinic antagonists 4-DAMP and pirenzepine (where applicable) were added to each tube 15 min prior

to stimulation by carbachol (1 mM). Carbachol was allowed to react for 20 min. The reaction was stopped by the application of 50 μ l of perchloric acid (72%) per tube and by placing the tubes on ice. The cochlear tissues were homogenised by sonication and the homogenates were centrifuged at 2000 $\times$ g for 5 min. The supernatants containing the [³H]IPs were neutralised with 1.5 M KOH/0.075 M HEPES. After separation from [³H]inositol and glycerophosphoinos-

Fig. 2. Loss of the outer hair cells following the chronic treatment with amikacin. Ten-day-old rats received a daily injection of amikacin sulphate (20 μ l, 479 mM) for 6 days. Following a 10-day recovery, the animals were sacrificed and their cochleas processed for scanning and transmission electron microscopy. a, b and c are scanning electron micrographs of the surface of the organ of Corti. The inner hair cells (I), localised by their stereocilia bundles are present throughout the organ of Corti, while the outer hair cells (O) are still present at the apex (a) but have been destroyed in the medial (b) and basal (c) turns. A semi-thin radial section of an amikacin-treated organ of Corti in the lower medial turn is shown in d. Arrows indicate the inner hair cell (I) and the Deiters' cells (D) that took the place of the destroyed outer hair cells. Panel e depicts the apical pole of the Deiters' cells (D) that extended towards the surface of the organ of Corti, numerous nerve fibers (arrows) are present in the space between the Deiters' cells and the pillar cells (P). f: high magnification of the fibers that allows the recognition of efferents (e). Bars = 10 μ m (a–d), 2 μ m (e), and 1 μ m (f).

itols by anion-exchange chromatography, the [^3H]IPs formed were measured in each cochlear sample as previously described²⁴.

Chronic treatment with the aminoglycoside amikacin

Ten-day-old Wistar rats from several litters received a daily subcutaneous injection of amikacin sulphate for 6 days. The injected volume was 20 μl of a sterile saline solution of amikacin sulphate concentrated at 479 mM. Following injections, the baby rats were returned to their mothers. This treatment represents a dosage of 300 mg of amikacin/kg body weight/day for a 12-day-old rat. At the end of the treatment, the rats were allowed to recover for 10 days. They reached then the age of 26 days, an age where morphological and physiological studies have demonstrated that the cochlea is mature. At this time, the animals were anaesthetised and killed and the cochleas were dissected, as described above, and processed for histology and measurement of the metabolism of IPs.

Section of the crossed olivocochlear bundle

Mature Wistar rats (about 3-month-old) were deeply anaesthetised by an intraperitoneal injection of Equithesin (300 μl /100 g body weight) and placed in a stereotaxic instrument. The scalp was incised and the skull was opened along a 2.5-mm-wide midline, from the base of the occipital bone to the middle of the vertex in order to visualise the venous sinus. Without damaging the sinus, the brain was split by a rostro-caudal section from the vertex to the base of the occipital bone. The cut goes through the cerebellum and the brain stem to the bottom of the skull. The opening on the top of the occipital bone was closed with gel-foam and the skin was sewed. After an 11 day survival period, the animals were anaesthetised and killed, the cochleas were dissected, as described above, and processed for histology and measurement of the metabolism of IPs. The duration of the survival was determined by a preliminary time-course study (unpublished data).

Transmission electron microscopy

Following a deep anaesthesia with sodium pentobarbital and decapitation, the cochleas were dissected from the bulla and fixed in 2.5% glutaraldehyde buffered with sodium cacodylate (0.1 M, pH 7.4). The cochleas were quickly perfused with the same fixative through the windows and then kept in the fixative for 2 h. Following a buffer wash, the cochleas were further dissected and then postfixed in an aqueous solution of 1% osmium tetroxide for 1 h. Samples were dehydrated in graded series of ethanol (35–100%), embedded in Spurr resin and ultrathin sections were cut at different base-to-apex levels, in the transverse plane. The sections were observed with a Philips EM 301 electron microscope. Semithin sections were also cut for observation with Nomarski microscopy.

Scanning electron microscopy

The cochleas were processed as for the transmission electron microscopy, except that fixation time with osmium tetroxide was reduced to 10 min. During the dissection, special care was taken in the removal of the tectorial membrane. After dehydration, the specimens were critical point dried in liquid CO_2 and coated with gold, samples were observed with a Hitachi S4000 electron microscope examining on the outer hair cells from the base to the apex of the organ of Corti.

Data expression and statistical analysis

The data are expressed as ratios of the amount [^3H]IPs (in dpm) formed to the amount of dpm of [^3H]inositol taken up per cochlea (dpm [^3H]IPs/dpm [^3H]inositol). The total IPs are composed essentially of inositol trisphosphate (2%), inositol bisphosphate (23%) and inositol monophosphate (75%)²⁴. The amount of inositol taken up per cochlea is measured in the 'flow through' fraction of the anion-exchange chromatography used to purify the IPs. The data were not normalised to the amount of protein due to difficulty to measure very accurately the protein content of a single, dissected cochlea or cochlear region. The data are also given as percentage of the basal level of IP synthesis measured in the absence of neuroactive mole-

cules. All the data points are means $\pm$ S.E.M. of 6 experiments, at least. The statistical significance of the data was determined using one-way ANOVA.

RESULTS

Whatever the experimental destruction, the amounts of tritiated inositol taken up per cochlea were not different from controls.

Metabolism of IPs in the organ of Corti and the modiolus of the mature cochlea

The basal accumulation of IPs is higher in the organ of Corti, with a level of 0.125 ± 0.014 dpm [^3H]IPs/dpm [^3H]inositol, than in the modiolus which the level of formation of IPs is 0.038 ± 0.002 dpm [^3H]IPs/dpm [^3H]inositol ($P < 0.001$).

Following the application of carbachol (1 mM), the metabolism of IPs is stimulated in both cochlear compartments (Fig. 1). In the organ of Corti, carbachol raises the IP response to 0.228 ± 0.024 dpm [^3H]IPs/dpm [^3H]inositol, corresponding to $182 \pm 19\%$ of the basal level. In the modiolus, the formation of IPs is increased to 0.066 ± 0.005 dpm [^3H]IPs/dpm [^3H]inositol ($174 \pm 13\%$ of control).

In the organ of Corti, the addition of the muscarinic antagonists atropine and 4-DAMP (both used at 1 μM) completely blocks the stimulation induced by carbachol (1 mM), but they do not significantly change the basal

Fig. 3. Carbachol-induced accumulation of IPs in the amikacin-damaged cochleas. The accumulation of [^3H]IPs was stimulated by 1 mM of carbachol in amikacin-damaged and control cochleas (top two bars). The data are means $\pm$ S.E.M. of 6 experiments, at least and are expressed as percentage of their respective basal levels of IPs accumulation. These basal levels are 0.020 ± 0.003 dpm [^3H]IPs/dpm [^3H]inositol in control and 0.036 ± 0.008 dpm [^3H]IPs/dpm [^3H]inositol in amikacin-treated cochleas. The carbachol-induced synthesis of IPs was subjected to antagonism by atropine, 4-DAMP and pirenzepine (bottom three bars) that were applied at 1 μM . C, carbachol; ATR, atropine; PZ, pirenzepine.

accumulation of IPs (Fig. 1). Unlike these compounds, pirenzepine, at $1\ \mu\text{M}$, reduces the carbachol-activated synthesis of IPs by $63 \pm 26\%$ and the basal level by $42 \pm 1\%$ (Fig. 1).

In the modiolus, atropine, 4-DAMP and pirenzepine, all of them applied at $1\ \mu\text{M}$, do not affect the basal synthesis of IPs. However, the former two antagonists completely block the stimulatory effect of carbachol, while pirenzepine induces a $29 \pm 1\%$ inhibition of the carbachol-enhanced formation of IPs (Fig. 1).

Effects of the chronic treatment with amikacin

Observation of the surface of the organ of Corti using the scanning electron microscope, along the

cochlear coil, reveals that the apical surfaces of the outer hair cells have almost completely disappeared. At the apex, some outer hair cells may have survived since their apical membranes are still present (Fig. 2a). No outer hair cells are left in the medial and basal turns of the cochlea (Fig. 2b,c). Occasionally, some inner hair cells are missing in the basal turn (Fig. 2c). Based on scanning electron microscopy data, an assessment of the amikacin-induced outer hair cell loss indicates that about 90% of the outer hair cells have been destroyed. However, this assessment may underestimate the extent of the hair cell loss because it has been shown that the apical surface of an outer hair cell can remain in the organ of Corti long after the degenera-

Fig. 4. Effects of crossed olivocochlear bundle section. Transmission electron micrographs a, b, c and d depict the synaptic poles of outer hair cells (O). In most of the cases the efferent terminals have degenerated (a), leaving a space (stars) between the top of the Deiters' cells and the postsynaptic membrane (arrowheads). Some relics of the terminals are also found in the spaces (b,c), together with expansions of the Deiters' cells (c, double arrow). Few large efferent terminals (e) are still connected with the outer hair cell (d) but irregularities are visible at the cytoplasmic membrane (small arrows). Panel e shows abnormal efferent fibers (large arrows) within the intraganglionic bundle. Bars = $1\ \mu\text{m}$ (a-e).

tion of the cell body^{16,22,35}. Light microscope observations of transverse sections of the organ of Corti of treated rats show that the outer hair cells are missing (Fig. 2d). The use of electron microscopy confirms this fact and also shows that Deiters' cells have expanded and taken the place of the outer hair cells (Fig. 2e). The treatment with amikacin does not affect the nerve fibers since many of them are seen in every section (Fig. 2e,f). Amongst these fibers, some large efferent terminals that contain a great amount of synaptic vesicles are often found (Fig. 2f).

Metabolism of IPs in the amikacin-exposed cochlea

The basal synthesis of IPs is statistically similar in both control and treated cochleas, with the level of formation of IPs being 0.020 ± 0.003 dpm [^3H]IPs/dpm [^3H]inositol in control cochleas and 0.036 ± 0.008 dpm [^3H]IPs/dpm [^3H]inositol in amikacin-treated cochleas.

The activation of the IP metabolism by carbachol at 1 mM, elicits identical responses in both groups of cochleas. The carbachol-induced accumulation of IPs reaches 0.065 ± 0.004 dpm [^3H]IPs/dpm [^3H]inositol ($326 \pm 19\%$ of basal control level) in untreated cochleas and 0.113 ± 0.007 dpm [^3H]IPs/dpm [^3H]inositol ($313 \pm 21\%$ of basal 'treated' level) in amikacin-damaged cochleas (Fig. 3). This latter stimulation is completely abolished by the addition of $1 \mu\text{M}$ of either atropine or 4-DAMP (Fig. 3) but it is not affected by pirenzepine (Fig. 3).

Effects of the section of the crossed olivo-cochlear bundle

Interrupting the efferent axons on the floor of the fourth ventricle results in a drastic degeneration of the efferent terminals that formed synapses with basal membranes of the outer hair cells 11 days postoperatively (Fig. 4). Large spaces appear between the Deiters' cells and outer hair cells in place of the efferent terminals (Fig. 4a). Remnants of degenerated terminals are sometime found in these spaces (Fig. 4b,c), as well as expansions of Deiters' cells (Fig. 4c). Observation of cochleas from seven operated animals leads to the conclusion that about 50% of the outer hair cells have lost their synaptic contacts with the medial efferent fibers. In a quarter of cases, remains of terminals are seen associated with post-synaptic specializations of the membrane of the outer hair cells. Lastly, about 25% of the outer hair cells are still contacted by efferent synaptic boutons (Fig. 4d). At the level of the spiral ganglion, some degenerating efferent fibers are seen in the intraganglionic bundle (Fig. 4e).

Metabolism of IPs in the organ of Corti and the modiolus following the section of the crossed olivocochlear bundle

The unstimulated, basal level of IP synthesis is statistically similar in control organs and in organs of Corti from operated rats since in the latter the basal accumulation of IPs is 0.101 ± 0.030 dpm [^3H]IPs/dpm [^3H]inositol (control level being 0.125 ± 0.014 dpm [^3H]IPs/dpm [^3H]inositol). In modiolar parts, the unstimulated IP formation reach 0.029 ± 0.003 dpm [^3H]IPs/dpm [^3H]inositol following the section of the crossed olivocochlear bundle. This value is not statistically different from the basal production of IP in control modiolar tissue (0.038 ± 0.002 dpm [^3H]IPs/dpm [^3H]inositol).

The stimulation of cochlear regions of operated rats with carbachol (1 mM) enhances the synthesis of IPs to 0.149 ± 0.014 and 0.062 ± 0.008 dpm [^3H]IPs/dpm [^3H]inositol in the organ of Corti and the modiolus, respectively (Fig. 5). The stimulatory effects of carba-

Fig. 5. IP metabolism in the organ of Corti and in the modiolus following the section of the crossed olivocochlear bundle. Data are means $\pm$ S.E.M. of 6 experiments, at least. Black bars show the basal and carbachol- (1 mM) evoked accumulations of IPs in control, unoperated cochlear regions. White bars depict the basal and carbachol- (1 mM) evoked accumulations of IPs, as well as the block of the carbachol stimulation by atropine ($1 \mu\text{M}$) in operated organs of Corti (top panel) and operated modiolus (bottom panel). UNSTIM, unstimulated basal level; CARB, carbachol; ATR, atropine.

chol are abolished completely by the addition of atropine (1 μ M) in both regions of the cochlea (Fig. 5). In the presence of the muscarinic antagonist, the formation of IPs amounts to 0.080 ± 0.022 and 0.037 ± 0.011 dpm [3 H]IPs/dpm [3 H]inositol in the organ of Corti and the modiolus, respectively.

When comparing the stimulation of the IP metabolism produced by carbachol in the cochlear compartments of control and operated rats, a difference becomes evident in the case of the organ of Corti. A carbachol-induced rise of $47 \pm 14\%$ above basal formation of IPs is obtained following the section of the crossed efferent bundle, while the carbachol-evoked increase of the IP response represents a $82 \pm 19\%$ rise above basal level in normal animals. The stimulated IP response is significantly smaller in organs of Corti of operated rats than in controls ($P < 0.01$). No difference was noticed between modiolar fractions from both groups of rats.

DISCUSSION

Stimulation of muscarinic receptors enhance the hydrolysis of phosphatidylinositol 4,5-bisphosphate in the organ of Corti and in the modiolus. In both cochlear parts, the stimulatory effect of carbachol (1 mM) is antagonised by atropine (1 μ M), showing that carbachol elicits the synthesis of IPs via an interaction with muscarinic receptors. The application of 4-DAMP (1 μ M) results in the complete blockage of the carbachol stimulation while pirenzepine (1 μ M) lacks significant effects. Actually, a partial inhibition of the carbachol-induced formation of IPs is obtained with pirenzepine in the modiolus, but in fact this inhibition is due to a reduction of the basal metabolism of IPs, the reason for which remains unclear; one may speculate that some endogenous ligands for pirenzepine-sensitive sites are present in our *in vitro* model.

Recently, in the mice cochlea, Drescher and co-workers¹⁶ identified mRNAs encoding three muscarinic receptors coupled to IP metabolism, namely m1, m3 and m5 mRNAs. This result suggests the presence of the M_1 , M_3 and M_5 receptors in the cochlea. While the identification of M_5 receptors is prevented by the lack of selective ligand, previous electrophysiological and biochemical observations have shown that cochlear muscarinic receptors have a very low affinity for pirenzepine (M_1 selective) and a high affinity for 4-DAMP (M_3 selective)^{4,6,24,33}. For instance, previously-reported dose-response curves for the effects of specific antagonists on carbachol-induced IP synthesis showed that 1 μ M of 4-DAMP abolishes completely the effect of carbachol (1 mM) while piren-

zepine at 1 μ M does not affect the carbachol-activated transduction system^{4,24}. These data indicate that muscarinic receptors are mainly of the M_3 type in the cochlea. In addition, binding surveys demonstrated the existence of only one population of muscarinic sites in the cochlea^{6,27,56,58}. Together with mRNA analysis¹⁶, the whole body of data suggests that the synthesis of M_1 and M_5 proteins may be regulated posttranscriptionally. Consequently and since, in the present study, 4-DAMP (1 μ M) is more potent than pirenzepine (1 μ M) in both the organ of Corti and the modiolus, it appears that both cochlear parts contain the same type of functional muscarinic receptor which is probably the M_3 type.

Muscarinic receptors in the modiolus

Although binding experiments that detected muscarinic sites in both cochlear nerves and spiral ganglions^{27,58} suggest the transport of muscarinic receptors, the fact that carbachol activates the synthesis of IPs via an atropine-sensitive mechanism in the modiolus, demonstrates the fairly unexpected presence of functional muscarinic receptors in this cochlear compartment. It appears, therefore, that the binding sites may be related to both transported and cell surface functional cholinceptors⁵⁵.

The carbachol-induced release of IPs in the modiolus suggests that muscarinic receptors could be located at the level of the cell bodies of the spiral ganglion neurons and/or nerve fibers and/or Schwann cells. Although there is no previous data providing support for the presence of functional receptors on the neuronal cell bodies, this possibility cannot be excluded yet. The presence of functional muscarinic receptors in the fibers is questionable because the carbachol-activated IP turnover is unchanged in the modiolus despite the degeneration of some fibers, following the section of the crossed olivocochlear bundle. However, functional receptors may be located in the fibers, as this has been shown in the sciatic and vagus nerves^{14,52} where phosphatidylinositol 4,5-bisphosphate is hydrolysed following the activation of M_1 and M_3 cholinceptors^{14,52}, which are likely to be coupled to a calcium-sensitive phospholipases C^{37,40} via specific G proteins Gq/G₁₁⁷. Muscarinic receptors could be involved in the regulation of the axoplasmic transport because the stimulation of these receptors suppresses the fast axonal transport in cultured superior cervical neurons⁵⁴.

Despite the lack of direct evidence for the expression of muscarinic receptors coupled to the IP cycle in Schwann cells, there are reports suggesting indirectly that Schwann cells may possess such receptors within their myelin sheath. Indeed, in the peripheral nervous

system, myelin sheaths are particularly rich in phosphatidylinositol 4,5-bisphosphate³⁶ and contain both phospholipase C enzymes and their stimulatory G proteins^{7,37}. Moreover, the carbachol-stimulation of muscarinic receptors enhances the IP turnover in brain myelin^{28,34}. Then, if cochlear Schwann cells do bear muscarinic receptors, these receptors may regulate the turnover of phospholipid within the myelin sheath.

Muscarinic receptors in the organ of Corti

The responses obtained in the rat organ of Corti show the expression of functional muscarinic receptors and support the previous demonstration of the presence of muscarinic receptors linked to the activation of phospholipase C in the guinea pig organ of Corti⁴¹. At the cellular level, the most obvious sites of muscarinic receptors are at the cholinergic synapses^{1,13,20,47,48}. In order to shed more light on the synaptic position of the metabotropic cholinergic receptor, we investigated, in the outer region of the organ of Corti, the effects of the loss of presynaptic and postsynaptic elements on the carbachol-evoked IP response.

Amikacin treatment leads to the degeneration of almost all outer hair cells without changing the magnitude of the carbachol-induced formation of IPs. At first sight, this would indicate that outer hair cells do not possess muscarinic receptors, as first suggested by Plinkert and co-workers⁴⁴. However, a reservation must be brought to this straightforward conclusion since outer hair cells represent a small proportion of the total cells in the whole cochlea, and because muscarinic, electrophysiological responses have been recorded in isolated outer hair cells^{19,29}. It is possible that our assay is not sensitive enough to detect changes in the formation of IPs caused by outer hair cell loss, because the synthesis of IPs is also enhanced by carbachol in other cochlear cells, as indicated for instance by the current results obtained in the modiolus. Thus, a more careful conclusion would be to state that the localisation of muscarinic cholinergic receptors is not restricted to outer hair cells. The muscarinic receptors of these latter cells may be involved in changes in the cytosolic concentration of calcium ions which have been proposed to modulate the slow motility of the outer hair cells¹⁸.

The interruption of the crossed olivocochlear bundle causes a significant reduction of the level of carbachol-enhanced release of IPs. Since the medial terminals which form synapses with the outer hair cells, are selectively lost following the surgery, this demonstrates that many muscarinic receptors are associated with these presynaptic terminals. Although no clue is available to point to a role for these presynaptic cholinergic

receptors, a tempting hypothesis is that muscarinic receptors are autoreceptors which modulate the release of acetylcholine³⁰. Support for this hypothesis is found in the ability of M₃ prejunctional autoreceptors to inhibit the release of acetylcholine in the guinea pig ileum^{12,31}, the rat striatum¹⁵ and the bovine cerebral arteries²¹. Besides regulating acetylcholine release, these muscarinic receptors may control the release of ATP which increases the calcium permeability in isolated outer hair cell^{2,39} as well as the IP turnover in the organ of Corti⁴². Such a control of purinergic transmission has been reported in adrenal chromaffin cells⁶⁰.

Conclusion

We demonstrate that muscarinic receptors functionally coupled to phospholipase C are distributed in both the organ of Corti and the modiolus. The pharmacological features of these receptors are similar in both structures and seem indicative of M₃ muscarinic receptors. While the cellular localisation of the cholinergic receptors in the modiolus is still to be determined, the current results clearly show that a large number of muscarinic receptors are present on presynaptic terminals of the medial efferent system in the organ of Corti. Such autoreceptors may regulate the release of neurotransmitter at the synapses between the medial cholinergic axons and the outer hair cells.

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