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## Carbachol-induced inositol phosphate formation during rat cochlea development

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The age related-intensity developmental pattern of the phosphoinositide breakdown, which leads to the formation of intracellular second messengers, was investigated in rat cochleas by measuring the accumulation of inositol phosphates induced by carbachol in the presence of LiCl. The accumulation of the phosphoinositide metabolites elicited by this muscarinic agonist is very low at post-natal day 1 and particularly large during the period between post-natal days 8 and 14 with a peak around day 12. In the 25-day-old rat cochlea, carbachol induced a 2-fold increase in inositol phosphates (IPs) accumulation, with respect to the basal control level. The apparent affinities of the carbachol-induced IPs responses are 49.6, 31.6 and 36.7  $\mu$ M in cochleas of 12-, 16- and 25-day-old rats, respectively, thus suggesting that the specific developmental changes are rather due to a modification in the number of muscarinic cholinergic receptors than to alterations of the apparent affinity of carbachol for its receptors. This developmental pattern of carbachol-elicited IPs accumulation reveals a striking time coincidence with both the efferent synaptogenesis at the outer hair cells (OHCs) level and the period of increased sensitivity of OHCs to aminoglycoside toxicity. Phosphoinositide breakdown may, consequently, play a role in the maturation of OHCs and their efferent supply. In addition, the remaining IPs response measured at 25 post-natal days indicates that muscarinic agonist-mediated IPs metabolism also occurs in mature cochlea, and might be involved in the regulation of OHCs motility.

### Introduction

The degradation of phosphatidylinositol 4,5 bisphosphate, a membrane polyphosphoinositide, catalysed by phospholipase C, leads to the formation of diacylglycerol and inositol phosphates. Some of these metabolites, diacylglycerol and inositol 1,4,5-trisphosphate are considered as second messengers (for reviews, see Berridge, 1984; Berridge and Irvine, 1989; Nishizuka, 1984). The former directly activates the protein kinase C enzymes, which catalyse protein phosphorylation; the latter elicits a massive release of calcium from intracellular stores. This transduction system has been found to be driven by specific agonist-activated receptors such as muscarinic cholinergic

and glutamatergic receptors (Fisher and Agranoff, 1987 and Sladeczek et al., 1988).

We recently found that among several neuroactive substances tested, only muscarinic cholinergic agonists are able to stimulate the accumulation of the various inositol phosphate metabolites (IPs) in the cochlea of 12-day-old rats via, probably, a  $M_3$  receptor subtype (Guiramand et al., 1990). At this age, the development of the rat cochlea is not yet complete (Lenoir et al., 1980, 1987). In particular, an entire reorganization of the innervation of the outer hair cells (OHCs) is occurring with the formation of synapses between the medial efferent system, which is thought to be cholinergic (Eybalin et al., 1987), and OHCs (Lenoir et al., 1980). Moreover, at this stage of development, a period of increased susceptibility of the cochlea to aminoglycoside antibiotics has been demonstrated in the rat (Osako et al., 1979; Carlier and Pujol, 1980) and in different species (Pujol, 1986a). With respect to other cochlear cells, the OHCs are more sensitive to aminoglycoside toxicity (Hawkins,

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1976). Aminoglycosides such as neomycin have been shown to bind to polyphosphoinositide substrates for phospholipase C (Schacht, 1974) in several tissues (Prentki et al., 1986; Siess and Lapetina, 1986; Tysnes et al., 1988; Gabev et al., 1989), including the inner ear (Schacht, 1979; Williams et al., 1987). This suggests that the period of development of the OHCs-cholinergic efferent system may be related to the period of increased sensitivity of the OHCs to aminoglycosides ototoxicity, which may, in turn, result from the inhibition of the enhanced phosphoinositide turnover mediated by cholinergic agonists.

In the present paper, we thus measured the accumulation of the various IPs metabolites elicited by carbachol, a muscarinic agonist, during rat cochlear development, in order to investigate whether developmental changes in IPs metabolism correlate with (1) the formation of the OHCs-efferent fibers synapses and (2) the critical period of cochlear supranormal susceptibility to aminoglycosides.

## Methods

Wistar rats of increasing ages (1-, 4-, 8-, 10-, 12-, 14-, 16-, 20- and 25-day-old) were sacrificed by decapitation. For each experiment both cochleas of 36 to 40 rats were used (6 to 8 per each age). The bony capsules, surrounding the cochleas, and the stria vascularis were dissected out under a light microscope. Intact cochleas, made up of the modiolus, the spiral ganglion and the organ of Corti, were rapidly placed in Krebs-Ringer buffer, pH 7.4. The Krebs-Ringer buffer contained in mM: 125 NaCl; 3.5 KCl; 1.25  $\text{KH}_2\text{PO}_4$ ; 1.2  $\text{MgSO}_4$ ; 1.5  $\text{CaCl}_2$ ; 25  $\text{NaHCO}_3$  and 10 glucose. The cochleas were maintained throughout the experiment in this buffer, which was equilibrated to pH 7.4 before use and maintained at this pH during the whole duration of the experiment by saturation with a gaseous mixture of  $\text{O}_2$  95% and  $\text{CO}_2$  5% (vol/vol).

The radioactive inositol incorporation in cochleas was carried out at 37°C, for 75 min in Krebs-Ringer buffer containing 1 mM cytidine and 50  $\mu\text{Ci}$  of Myo-[2-( $^3\text{H}$ )]-inositol (specific activity: 17 Ci per mmol, obtained from CEA Saclay, France). Labelling of cochleas, pooled into groups

of cochleas of the same age, was carried out by using 67  $\mu\text{l}$  of radioactive Krebs-Ringer buffer per cochlea. Each pool of cochleas was then washed four times using Krebs-Ringer buffer, pH 7.4, at 37°C, before the cochleas were individually distributed in plastic test tubes containing 500  $\mu\text{l}$  Krebs-Ringer buffer. The tubes were immediately transferred to a water bath maintained at 37°C and continuously gassed with the mixture of  $\text{O}_2$  95% and  $\text{CO}_2$  5%.

LiCl (10 mM) was added to each tube, 15 min prior to carbachol (Sigma, France) stimulation. Carbachol (20  $\mu\text{l}$ ), at various concentrations, was allowed to react for 20 min. The reaction was then stopped by the application of 50  $\mu\text{l}$  of perchloric acid (72%) and by placing the tubes on ice. The cochleas were homogenized by sonication, homogenates were then centrifugated at  $2000 \times g$  for 20 min. The resulting supernatants, containing the perchloric acid-extracted ( $^3\text{H}$ )-IPs, were taken and neutralized with 1.5 M KOH/0.075 M HEPES. After separation on anion-exchange columns according to Bone et al., 1984, the ( $^3\text{H}$ )-IPs formed in the absence or the presence of carbachol were measured in each individual cochlea, as described elsewhere (Guiramand et al., 1990).

The results were expressed as the ratios of dpm of ( $^3\text{H}$ )-IPs accumulated to dpm of ( $^3\text{H}$ )-inositol taken up by the cochlea. The statistical significance of the carbachol-stimulated IPs formation versus the control IPs values was calculated by the 2-tailed Student's *t*-test.

## Results

### *Age-dependent stimulation of ( $^3\text{H}$ )-IPs formation by carbachol*

The developmental changes of basal and  $10^{-3}$  M carbachol-induced ( $^3\text{H}$ )-IPs formation in rat cochlea is shown in Fig. 1. The ( $^3\text{H}$ )-inositol uptake into cochlear cells is independent of the age of the rat (data not shown). The basal IPs accumulation is very low in 1-day-old rats, slightly increases in the cochlea up to 12-day-old rats, then decreases regularly after this age. The carbachol elicited IPs synthesis is very low in the cochlea of 1-day-old animals, but it increases sharply between 1 and 12 days where it reaches its maximal value (about 450% of the corresponding basal

value which is  $0.067 \pm 0.005$  expressed as dpm of ( $^3\text{H}$ )-IPs/dpm of ( $^3\text{H}$ )-inositol taken up). Thereafter, the carbachol evoked IPs response decreases to a value which is twice that of the corresponding control at day 25. The control value at this age is  $0.023 \pm 0.004$  dpm of ( $^3\text{H}$ )-IPs/dpm of ( $^3\text{H}$ )-inositol taken up.

*Concentration-response curve of carbachol induced ( $^3\text{H}$ )-IPs formation in cochlea of various ages*

The potency of carbachol in stimulating IPs accumulation was examined in the cochleas of 12-, 16-, and 25-day-old rats (Fig. 2).  $\text{EC}_{50}$  values (concentrations required to obtain an IPs accumulation of 50% of the maximal response) were graphically determined and were 49.6, 31.6 and 36.7  $\mu\text{M}$  at 12, 16 and 25 days, respectively. No significant changes in the  $\text{EC}_{50}$  values are observed. On the other hand, the maximal values of stimulated IPs accumulations clearly decrease from 12 days to 25 days. These maximal values represent 512 %, 292% and 206% of their respective control values obtained using cochleas from 12-, 16- and 25-day-old rats. The corresponding mean

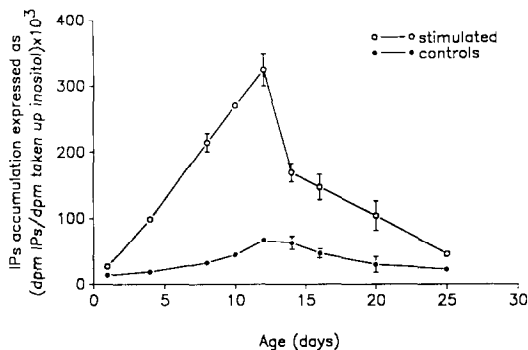


Fig. 1. Ontogenic development of the basal- and carbachol-stimulated IPs formation in the rat cochlea. Following the incorporation of ( $^3\text{H}$ )-myoinositol for 75 min at  $37^\circ\text{C}$ , the cochleas were washed and incubated for 15 min in 10 mM LiCl and for a further 20 min in the absence or the presence of carbachol (1 mM). The data are expressed as the ratio of the quantity of dpm corresponding to ( $^3\text{H}$ )-IPs formed to the amount of dpm of ( $^3\text{H}$ )-myoinositol taken up in the cochlea. The IPs accumulation was measured for each cochlea. For each experiment, three independent cochleas were used to determine one value. The values presented in this figure are means  $\pm$  SEM of at least three independent experiments. When error bars are not indicated in the figure, the width of the error bars are smaller than the thickness of the symbols.

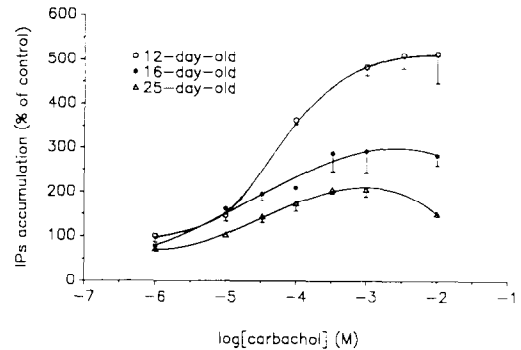


Fig. 2. Evolution of the dose-dependent carbachol-elicited IPs accumulation in the rat cochlea during postnatal development. The cochleas were dissected out from 12 day-, 16 day- and 25 day- old rats. The values are the means  $\pm$  SEM of at least three independent experiments, each performed in triplicate. The results are expressed as percentages of the corresponding control values.  $\text{EC}_{50}$  values, graphically determined, were 49.6, 31.6 and 36.7  $\mu\text{M}$  for cochleas taken from rats of ages 12, 16 and 25 days respectively.

control values ( $\pm$  SEM), for 12-, 16- and 25-day-old rat cochlea, are  $0.067 \pm 0.005$ ,  $0.047 \pm 0.004$  of ( $^3\text{H}$ )-IPs/dpm of ( $^3\text{H}$ )-inositol taken up by the cochlea.

## Discussion

In the mammalian cochlea, cholinergic transmission is thought to take place at both the level of the OHCs and the level of the contacts, between lateral efferent terminals and the afferent dendrites innervating the inner hair cells (IHCs). This suggests two putative locations of the carbachol-elicited IPs formation within the cochlea.

The developmental pattern of the carbachol-induced IPs formation in the rat cochlea shows a very low IPs formation in 1-day-old animals and a high activation of this response between 8 and 14 days of age, with a peak in between at 12 days (Fig. 1). This period of enhanced activation of this second messenger system is apparently concomitant with the reorganization of the innervation of the OHCs in the rat (Lenoir et al., 1980). In fact, during this period, arriving efferent endings from the medial cholinergic system, are competing with the initial afferent synapses to contact the basal membranes of the OHCs. Electron microscopic studies show that at 12 days, the first efferent

synapses are recognizable while adult-like efferent synapses are formed at 14–16 days (Lenoir et al., 1980). This may indicate that the increased phosphoinositide turnover, stimulated by carbachol, plays a key role in the plastic phenomena occurring at the level of OHCs, during this period. The involvement of IPs in neuronal plasticity was indeed suggested in the central nervous system where kindling or lesions of some glutamatergic pathways result in an increase in IPs formation elicited by glutamate. In addition, protein kinase C, a target enzyme for diacylglycerol, another second messenger resulting from the phosphoinositides breakdown was shown to be involved in long-term potentiation which is also a neuronal, plastic phenomenon (Linden et al., 1987; Lovinger et al., 1987). Long-term potentiation is a plastic event thought to take part in memory storage mechanisms; it is caused by very brief periods of intense stimulation of central nervous pathways, such as the hippocampal pathways. This results in the persistent increase of synaptic efficacy.

However, the possibility that the IPs are synthesized at the level of the synaptic contacts between the afferent dendrites, innervating the IHCs, and the terminals of the lateral efferent system which is also partly cholinergic, cannot be excluded despite the lack of data supporting this hypothesis. On the other hand, another body of evidence based on the study of aminoglycosidic ototoxicity favours the involvement of IPs turnover at the level of the OHCs-medial efferent system. Actually, it has been demonstrated that the rat cochlea presents a higher sensitivity to aminoglycoside toxicity during its period of development around 12 days (Carlier and Pujol, 1980). This increased sensitivity is mainly expressed at the level of the OHCs (Lenoir et al., 1983). Interestingly, it is known that aminoglycoside antibiotics bind to the phosphatidylinositol 4,5-bisphosphate, precursor of the inositol phosphates, thus resulting in aminoglycoside toxicity due to the inhibition of IPs turnover (Schacht, 1986). Consequently, there is, in the cochlea, a striking time coincidence (around day 12) between three events: the setting up of the efferent synapses at the base of OHCs, the increased sensitivity of OHCs for aminoglycoside antibiotics and the maximal increase of the intracellular IPs metabolism elicited by the

muscarinic agonist. Although we have not yet demonstrated that the IPs formation occurs at the OHC level, the body of evidence described above strongly suggests this possibility.

The IPs formation elicited by carbachol shows a specific developmental pattern with a peak at day 12. Among the numerous possibilities that could be proposed to explain this pattern, we have examined the two simplest ones: a modification of the apparent affinity of muscarinic agonists for their receptors or a change in the number of receptor sites. The carbachol dose-response curve indicates that the apparent  $EC_{50}$  values obtained are similar whatever the age of the rat used (Fig. 2). This result rules out a change in the apparent receptor affinity during development and favours the possibility of a variation in the number of muscarinic receptors. This is reinforced by the fact that the last stage of cochlear development is characterized both by an active phase where the cholinergic efferent medial fibers set up direct synaptic contacts with the OHCs and by regressive phenomena which involved synapse elimination between the OHCs and the nerve fibers of the afferent system (Pujol, 1986b). One could speculate that during this phase which occurs between 8 and 16 days in the rat (Lenoir et al., 1980), the OHCs possess a high density of muscarinic receptors to attract the cholinergic fibers coming from the brain. As soon as these synapses are almost mature (around 14–16 days) (Lenoir et al., 1980) the need for a high receptor density is no longer necessary. This could in some way be compared to the cholinergic fibers innervating muscle at the motor end plate, where a rearrangement of the cholinergic receptors and a decrease in their density occur (Changeux and Danchin, 1976).

The developmental features of carbachol-induced IPs formation cannot, in any case, eliminate the possibility that IPs turnover may play a role in the adult cochlea. At 25 days, when the rat cochlea is considered to be mature, carbachol induces a statistically significant 2-fold increase in IPs turnover, as it does in adult cochlea (data not shown). Such a stimulation could be large enough to provoke an intracellular calcium mobilization that subsequently could modulate cell motility, since this second messenger pathway is an amplifying mechanism. Indeed, the possibility that IPs

metabolites control OHCs motility is very likely to occur in the organ of Corti is supported by the observation that inositol 1,4,5-trisphosphate induces slow contractions in isolated OHCs (Schacht and Zenner 1986, 1987). Such a control of motility has been shown to exist in platelets and in several other tissues (Lassing and Lindberg, 1985, 1988).

In conclusion, the whole body of data suggests that the IPs transduction system may actively participate in the maturation phase of synapses that occurs at the OHCs-efferent fibers level. This transduction mechanism may also serve the function of modulating OHCs motility in the fully-grown stage.

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