Receptor-mediated release of inositol phosphates in the cochlear and vestibular sensory epithelia of the rat

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Various neurotransmitters, hormones and other modulators involved in intercellular communication exert their biological action at receptors coupled to phospholipase C (PLC). This enzyme catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) to inositol 1,4,5-trisphosphate (InsP$_3$) and 1,2-diacylglycerol (DG) which act as second messengers. In the organ of Corti of the guinea pig, the InsP$_3$ second messenger system is linked to muscarinic cholinergic and P$_2$y purinergic receptors. However, nothing is known about the the InsP$_3$ second messenger system in the vestibule. In this study, the receptor-mediated release of inositol phosphates (InsPs) in the vestibular sensory epithelia was compared to that in the cochlear sensory epithelia of Fischer-344 rats. After preincubation of the isolated intact tissues with *myo*-[^{3}H]inositol, stimulation with the cholinergic agonist carbamylcholine or the P$_2$ purinergic agonist ATP-γ-S resulted in a concentration-dependent increase in the formation of [^{3}H]InsPs in both epithelia. Similarly, the muscarinic cholinergic agonist muscarine enhanced InsPs release in both organs, while the nicotinic cholinergic agonist dimethylphenylpiperadinium (DMPP) was ineffective. The muscarinic cholinergic antagonist atropine completely suppressed the InsPs release induced by carbamylcholine, while the nicotinic cholinergic antagonist mecamylamine was ineffective. Potassium depolarization did not alter unstimulated or carbamylcholine-stimulated release of InsPs in either organ. In both tissues, the P$_2$ purinergic agonist α,β-methylene ATP also increased InsPs release, but the P$_1$ purinergic agonist adenosine did not. These results extend our previous observations in the organ of Corti of the guinea pig to the rat and suggest a similar control of the InsP$_3$ second messenger system in the vestibular sensory epithelia. In contrast to the cochlear sensory epithelia, atropine also significantly suppressed unstimulated InsPs release in the vestibular sensory epithelia. This suggests that the physiological mechanisms of the efferent nervous systems involving InsP$_3$ second messenger system might be different in vestibular versus cochlear sensory epithelia.

Inositol phosphates; Second messenger; Cochlea; Vestibule; Muscarinic cholinergic receptor; Purinergic receptor; Rat

Introduction

The activation of phospholipase C (PLC) is the metabolic step regulated by many neurotransmitters, hormones and other modulators involved in intercellular communication. The plasma membrane receptors of these agents are coupled to this enzyme which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) into inositol 1,4,5-trisphosphate (InsP$_3$) and 1,2-diacylglycerol (DG). Both InsP$_3$ and DG then act as intracellular second messengers. InsP$_3$ raises calcium levels by releasing calcium ions from intracellular stores such as the endoplasmic reticulum, while DG activates calcium-dependent protein kinase C. These two events result in protein phosphorylation which regulates enzyme activities and a variety of physiological cellular responses including intermediary metabolism, cell motility, ion channels and neurotrans-

mitter synthesis and release (Nishizuka, 1984; Berridge and Irvine, 1984).

The InsP$_3$ second messenger system is present in the cochlea and coupled to muscarinic cholinergic and P$_2$y purinergic receptors as determined from studies of phosphoinositide metabolism and release of InsP$_3$ (Orsulakova et al., 1976; Schacht and Zenner, 1986, 1987; Ono and Schacht, 1989; Bartolami et al., 1990; Guiramand et al., 1990; Wang and Schacht, 1990; Niedzielski and Schacht, 1991, 1992; Niedzielski et al., 1992). The expression of the appropriate muscarinic receptor subtypes m1, m3 and m5 was also recently documented in cochlear tissues (Drescher et al., 1992). However, the precise localization and function of the phosphoinositide second messenger system in the cochlea remains largely speculative. Given its involvement in diverse aspects of cellular physiology, multiple sites and actions can be expected. Indeed, components of this second messenger system have so far been found in the lateral wall tissues (Orsulakova et al., 1976), hair cells (Tachibana et al., 1985; Schacht and Zenner, 1987) and Deiters' cells (Dulon et al., 1993). Because of the participation of calcium in slow shape

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changes of isolated outer hair cells (Zenner, 1986; Flock et al., 1986; Dulon et al., 1990), it has been speculated that phosphoinosites may regulate 'slow motility' via release of $InsP_3$ and elevation of intracellular calcium (Schacht and Zenner, 1987; Dulon and Schacht, 1992). Application of $InsP_3$ to permeabilized cells indeed elicited a slow motile response of outer hair cells (Schacht and Zenner, 1986, 1987). Whether or not acetylcholine, the presumed cochlear efferent neurotransmitter, can elicit motile responses (via activation of muscarinic receptors and the $InsP_3$ cascade) remains controversial (see Bobbin et al., 1990; Dulon and Schacht, 1992).

The vestibular sensory epithelia consist of the macula utriculi, macula sacculi and cristae ampullares of semicircular canals. Two types of hair cells are present in these epithelia. Type I hair cells are flask-shaped and surrounded by an afferent nerve calice that receives efferent innervations, while type II hair cells are cylindrical and innervated directly by the afferent and efferent nerves. Several morphological and physiological observations have led to the speculation that the vestibular hair cells might also possess an active motility (Sans et al., 1989; Zenner et al., 1990; Valat et al., 1991, Lepyre and Cazals, 1991). The functional similarities of cochlear and vestibular hair cells as mechanoreceptors and their motile properties suggest that the $InsP_3$ second messenger system might also exist in the vestibule. However, nothing is known about the $InsP_3$ second messenger system in these tissues. In the present paper, we compare receptor-mediated release of InsPs in the vestibular sensory epithelia to that in the cochlear sensory epithelia. These results have been presented in a preliminary report (Ogawa and Schacht, 1993).

Materials and Methods

Materials

Fischer-344 rats (3 month-old, male; from Charles River, Kingston, NY) with a positive Preyer reflex were used in this study. Myo-[^{3}H]inositol (specific activity, 82 Ci/mmol) was obtained from Amersham Corporation (Arlington Heights, IL) and Hanks' balanced salt solution from Gibco BRL Life Technologies Inc. (Gaithersburg, MD). All other reagents were purchased from Sigma (St. Louis, MO).

Tissue preparation

The otic capsules were quickly removed following decapitation and placed in incubation buffer at room temperature. The incubation buffer was a Hanks' balanced salt solution (137.9 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 0.3 mM KH_2PO_4, 5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5.6 mM D-glucose) with 5

mM sodium N-2 hydroxyethylpiperazine-N'-2 ethane sulfonic acid (HEPES). The buffer was gassed with 95% O_2 and 5% CO_2 for 30 min prior to use, and then its pH was adjusted to 7.4 with NaOH and its osmolality to 300 ± 2 mOsm with NaCl. The cochlear sensory epithelia (including inner and outer hair cells, supporting cells, basilar membrane and lateral part of the spiral limbus) and the vestibular sensory epithelia (including macula utriculi, macula sacculi and cristae ampullares of semicircular canals) were isolated by microdissection and transferred into the incubation buffer.

Assay of phosphoinositide hydrolysis

In order to label the inositol lipid pool, tissues were incubated for 2 hr at 37°C in 50 μl of buffer with 1 mM cytidine and 16 μCi myo-[^{3}H]inositol (specific activity, 82 Ci/mmol) as described previously (Niedzielski and Schacht, 1991). After removing the myo-[^{3}H]inositol-containing incubation medium and washing twice with 0.5 ml of radioactivity-free buffer, 90 μl of buffer with 10 mM LiCl was added and incubation continued for 10 min. Then, 10 μl of buffer with cholinergic or purinergic agonists, antagonists or both of them were added. For potassium depolarization, a potassium chloride/Hanks' balanced salt solution (sodium replaced by potassium) with 5 mM HEPES was added (50 mM KCl final concentration). After 30 min at 37°C, phosphoinositide hydrolysis was terminated by the addition of 300 μl of chloroform/methanol (1:2, vol/vol). In order to separate aqueous and chloroform phases, 100 μl of distilled water with a phytic acid hydrolysate and 200 μl of chloroform with bovine brain extract (Sigma type I) were added. Phytic acid hydrolysate and bovine brain extract served as a carriers to minimize the loss of radiolabelled inositol phosphates and lipids during the isolation procedures. The aqueous phase was processed for inositol phosphates and the interface for protein (see below). Radioactivity of the chloroform phase was measured by liquid scintillation counting to determine the radioactivity of [^{3}H]inositol incorporation into phosphoinositides (PtdInsPs) (Brown et al., 1984).

Separation of labelled inositol phosphates

Inositol phosphates in the aqueous phase were separated from inositol on a Dowex-1 formate column as described (Berridge et al., 1983; Dean and Beaven, 1989) with minor modifications. The column was a Pasteur capillary pipet filled with Dowex-1 formate (cross-linkage 8%, mesh size 200–400) to a height of 25 mm. Two hundred μl of aqueous phase diluted to a final volume of 1.5 ml with distilled water was passed over the column which was then washed with 8 ml of 5 mM myoinositol. Combined inositol phosphates were eluted with 5 ml of 1 M ammonium formate in 0.1 M

formic acid. For the separation of individual inositol phosphates, 5 ml of 5 mM sodium tetraborate in 60 mM sodium formate, 0.2 M ammonium formate in 0.1 mM formic acid, 0.4 M ammonium formate in 0.1 mM formic acid, 1 M ammonium formate in 0.1 mM formic acid were applied sequentially to elute glycerophosphoinositol (GPI), inositol monophosphate (InsP), inositol bisphosphate (InsP$_2$) and inositol trisphosphate (InsP$_3$), respectively. Radioactivity of each fraction was determined by liquid scintillation counting. Columns were regenerated with 10 ml of 1 M sodium formate in 0.1 M formic acid and then rinsed with 10 ml of distilled water; they were discarded after 5 experiments.

Other procedures

Protein content in the interface was determined by the method of Bradford (1976) against bovine serum albumin as a standard. After removing both aqueous and chloroform phases, 50 μl of 1N NaOH was added to the remaining pellet. Following 5 days of incubation at room temperature, an aliquot was assayed.

The amount of radioactive inositol phosphates was expressed as dpm of labelled InsPs per μg protein. Similar results were obtained when InsPs were expressed as % of total radioactive label (PtdInsPs plus InsPs) (data not shown). Results are reported as means ± SD. Statistical significance was tested using ANOVA.

The care and use of animals reported on in this study were reviewed under grants DC-00078 and AG-08885, and approved and supervised by the University of Michigan Unit on Laboratory Animal Medicine.

Results

Release of InsPs after the preincubation of the isolated intact organs with myo-[^{3}H]inositol was 3703 ± 950 dpm/μg protein in the cochlear sensory epithelia and 4250 ± 2076 dpm/μg protein in the vestibular sensory epithelia. Addition of the cholinergic agonist carbamylcholine or the P$_2$ purinergic agonist adenosine 5'-O-(3-thiophosphate) (ATP-γ-S) resulted in a concentration-dependent increase in the formation of [^{3}H]InsPs (Fig. 1). In the cochlear sensory epithelia, increases of InsPs over controls were significant at and above 100 μM carbamylcholine and at and above 20 μM ATP-γ-S. In the vestibular sensory epithelia, 1 mM or more carbamylcholine and 200 μM or more ATP-γ-S significantly increased InsPs release. Maximal stimulation was 2.1-fold and 1.9-fold for carbamylcholine in the cochlear and vestibular sensory epithelia, respectively. ATP-γ-S (200 μM) caused a 8.7-fold and 4.1-fold increase in InsPs release in the cochlear and vestibular sensory epithelia, respectively.

The combined InsPs fraction included GPI, InsP, InsP$_2$ and InsP$_3$. After separation, the relative amounts
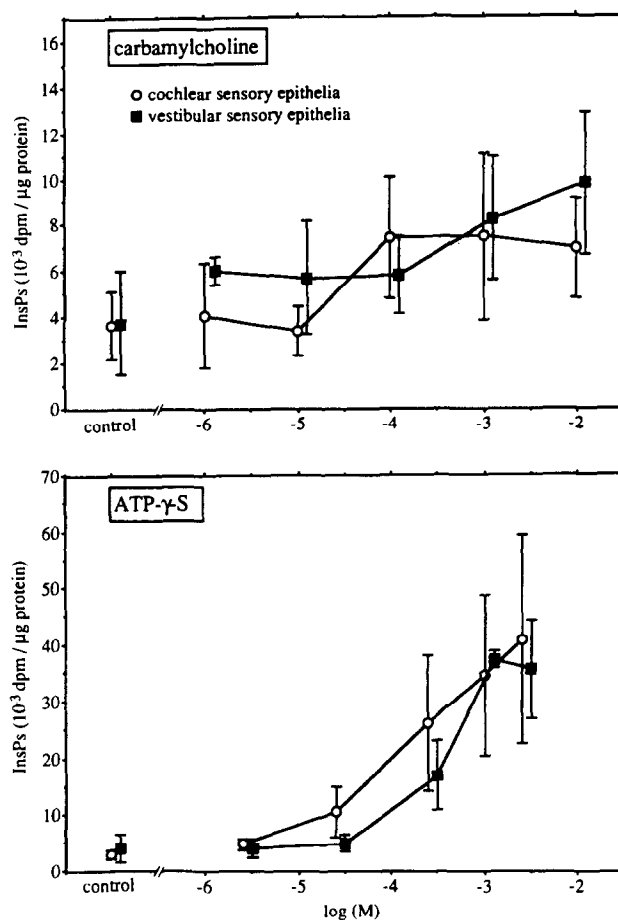

Fig. 1. Dose-response curves of InsPs release stimulated by carbamylcholine and ATP-γ-S. The cochlear and vestibular sensory epithelia were prelabelled with 16 μCi myo-[^{3}H] inositol and incubated with 0.01 ~ 10 mM carbamylcholine chloride or 0.002 ~ 2 mM ATP-γ-S for 30 min as described in Materials and Methods. Open circles represent InsPs release in the cochlear sensory epithelia and closed squares represent those in the vestibular sensory epithelia. Each point is the mean ± SD of 4 ~ 7 independent experiments. In the cochlear sensory epithelia, increases of InsPs over controls were significant (p < 0.05) at and above 100 μM carbamylcholine and at and above 20 μM ATP-γ-S. In the vestibular sensory epithelia, 1 mM or more carbamylcholine and 200 μM or more ATP-γ-S significantly (p < 0.05) increased InsPs release.

of GPI, InsP, InsP$_2$ and InsP$_3$ in controls were determined to be 36.0 ± 15.0%, 37.6 ± 18.3%, 10.5 ± 4.7% and 16.0 ± 6.1% in the cochlear sensory epithelia and 22.8 ± 8.0%, 48.0 ± 5.9%, 13.8 ± 5.9% and 15.3 ± 6.9% in the vestibular sensory epithelia, respectively. Carbamylcholine (1 mM) significantly increased InsP and InsP$_3$ 2.7-fold and 2.3-fold in the cochlear and 2.5-fold and 2.0-fold in the vestibular sensory epithelia, respectively. On the other hand, ATP-γ-S (200 μM) significantly increased InsP, InsP$_2$ and InsP$_3$ release 13.0-fold, 5.1-fold and 4.2-fold in the cochlear and 8.3-fold, 3.6-fold and 4.9-fold in the vestibular sensory epithelia, respectively (Fig. 2).
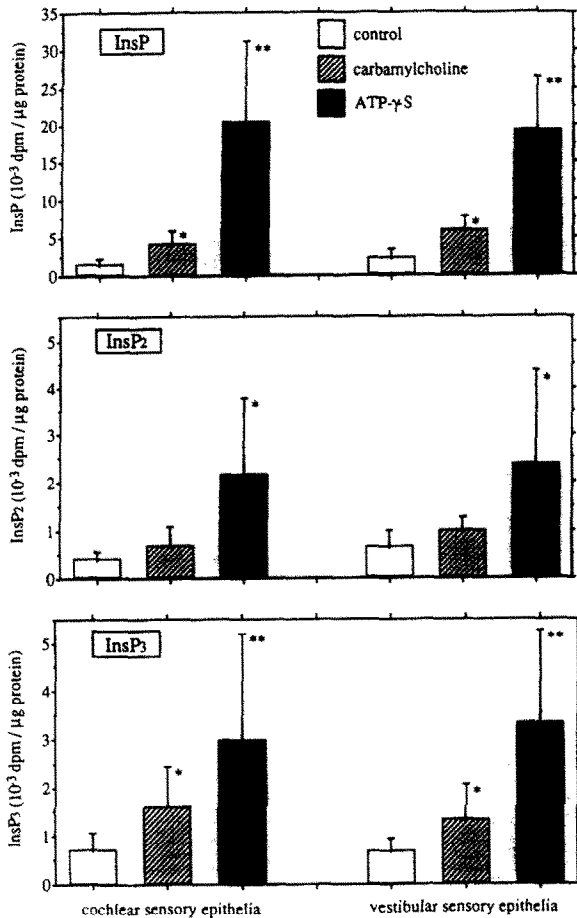
Fig. 2. Effect of carbamylcholine and ATP-γ-S on InsP, InsP$_2$ and InsP$_3$. The cochlear and vestibular sensory epithelia were prelabelled and incubated with 1 mM carbamylcholine chloride or 200 μM ATP-γ-S for 30 min as described in Materials and Methods. Each figure is the mean ± SD of 6 independent experiments. Statistical significance of differences from controls were determined by ANOVA (*, $0.01 < P < 0.05$; **, $P < 0.01$).

Fig. 4. Effect of the muscarinic cholinergic antagonist atropine and the nicotinic cholinergic antagonist mecamylamine on InsPs release. The cochlear and vestibular sensory epithelia were prelabelled and incubated with 10 μM atropine sulfate or 10 μM mecamylamine with or without 1 mM carbamylcholine chloride for 30 min as described in Materials and Methods. Each figure is the mean ± SD of 4 ~ 5 independent experiments.

The subtype of cholinergic receptor coupled to InsPs release was determined by the use of selected muscarinic or nicotinic cholinergic agonists and antagonists. The muscarinic cholinergic agonist muscarine (1 mM) acted similarly to 1 mM carbamylcholine, causing a 1.9-fold and 1.4-fold increase of InsPs in the cochlear and vestibular sensory epithelia, respectively. In contrast, the nicotinic cholinergic agonist 1,1-dimethyl-4-phenylpiperadinium (DMPP, 30 μM) did not significantly affect InsPs release in these tissues (Fig. 3).

The muscarinic cholinergic antagonist atropine (10 μM atropine sulfate) completely suppressed stimulated InsPs release induced by 1 mM carbamylcholine in both tissues, while the nicotinic cholinergic antagonist mecamylamine (10 μM) did not. Atropine and mecamylamine alone had no effect on unstimulated InsPs accumulation in the cochlear sensory epithelia.
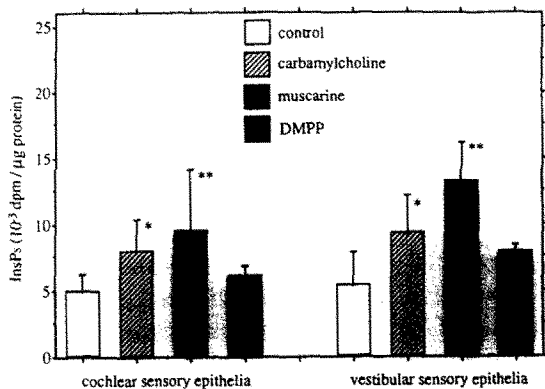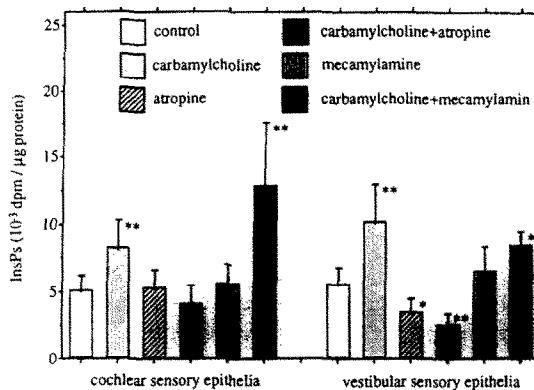

Fig. 3. Effect of the muscarinic cholinergic agonist muscarine and the nicotinic cholinergic agonist DMPP on InsPs release. The cochlear and vestibular sensory epithelia were prelabelled and incubated with 1 mM muscarine or 30 μM DMPP for 30 min as described in Materials and Methods. Each figure is the mean ± SD of 4 ~ 5 independent experiments.
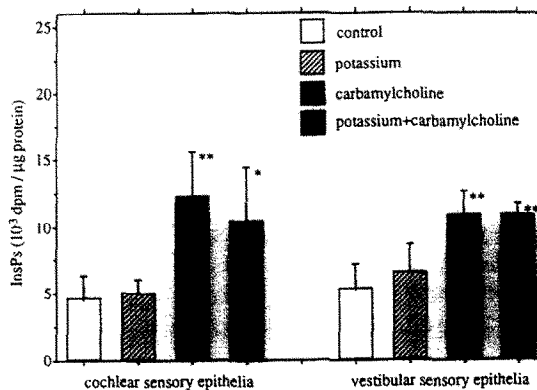

Fig. 5. Effect of potassium-induced depolarization on InsPs release. The cochlear and vestibular sensory epithelia were prelabelled and incubated with 50 mM KCl with or without 1 mM carbamylcholine chloride for 30 min as described in Materials and Methods. Each figure is the mean ± SD of 4 independent experiments.
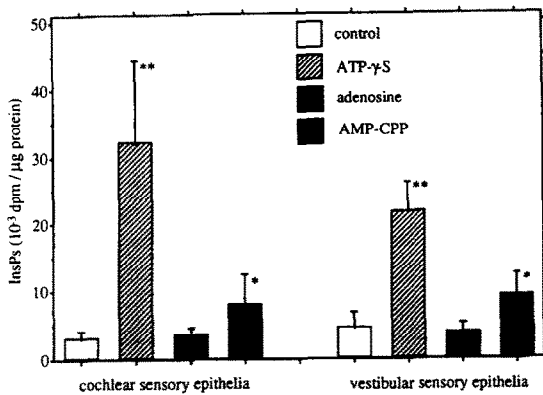
Fig. 6. Effect of the P_2 purinergic agonists ATP-γ-S and AMP-CPP and the P_1 purinergic agonist adenosine on InsPs release. The cochlear and vestibular sensory epithelia were prelabelled and incubated with 200 μM ATP-γ-S, 200 μM AMP-CPP or 200 μM adenosine for 30 min as described in Materials and Methods. Each figure is the mean ± SD of 4 independent experiments.

In the vestibular sensory epithelia, however, atropine significantly suppressed both unstimulated and carbamylcholine-stimulated InsPs release (Fig. 4).

The effect of potassium depolarization on carbamylcholine-stimulated InsPs release was determined by incubating cochlear and vestibular tissues with 50 mM [K^+] for 30 min with or without carbamylcholine. Neither unstimulated nor carbamylcholine-stimulated InsPs release was affected by potassium depolarization (Fig. 5).

Purinergic receptor agonists were used in an effort to determine the subtype of purinergic receptor coupled to InsPs release. Although the P_2 purinergic agonist a,b-methyladenosine 5'-diphosphate (AMP-CPP, 200 μM) increased InsPs release 2.6-fold in the cochlear sensory epithelia and 2.0-fold in the vestibular sensory epithelia, its effect was significantly smaller than that of ATP-γ-S (10.2-fold and 4.7-fold). In addition, InsPs release was not stimulated by the P_1 purinergic agonist adenosine (200 μM) (Fig. 6).

Discussion

In both the cochlear and vestibular sensory epithelia, the $InsP_3$ second messenger system appears to be linked to muscarinic cholinergic and P_2y purinergic receptors. In both organs, the cholinergic agonist carbamylcholine and the P_2 purinergic agonist ATP-γ-S elicited a concentration-dependent increase in InsPs release with a much higher effect induced by ATP-γ-S. These findings extend our previous results in the organ of Corti of the guinea pig (Niedzielski and Schacht, 1991, 1992; Niedzielski et al., 1992) to the rat and suggest the existence of a similar $InsP_3$ second messenger system in the rat vestibule. While the magnitude of

stimulation by both agonists is similar in rat and guinea pig cochlea, the amount of [^3H]inositol radioactivity in the IPs fraction is considerably higher in the rat. This may reflect differences in assay conditions (aeration of the incubation buffer in the present study) or species differences in intrinsic inositol levels or lipid metabolism.

The accumulation of InsP was increased more than that of $InsP_2$ and $InsP_3$ by both agonists. $InsP_3$, the immediate second messenger produced from $PtdInsP_2$, is rapidly metabolized primarily yielding $InsP_2$ and InsP as intermediates and, finally, free inositol. However, InsPs can be trapped for analysis at the level of InsP by the use of LiCl, an inhibitor of inositol monophosphatase. InsP and $InsP_2$ can also be derived directly from hydrolysis of phosphatidylinositol and phosphatidylinositol monophosphate (Majerus et al., 1988). A previous study of the whole cochlea (Guiramand et al., 1990) had failed to identify any increase of $InsP_3$ in response to carbamylcholine leaving the possibility open that InsPs were not derived from $PtdInsP_2$. The significant increase of $InsP_3$ seen in the present investigation suggests that the primary pathway of $InsP_3$ generation from $PtdInsP_2$ is present in the cochlear and vestibular sensory epithelia.

Potassium depolarization is known to potentiate carbamylcholine-stimulated InsPs release in the brain (Eva and Costa, 1986; Challis and Nahorski, 1991). It has been suggested that depolarization enhances the coupling of muscarinic receptors and PLC by activating the GTP-binding protein (G-protein) (Eva and Costa, 1986). In our study, however, potassium depolarization neither affected unstimulated nor carbamylcholine-stimulated InsPs release in the cochlear and vestibular sensory epithelia. This difference may be related to the type of G-protein associated with the receptor. Although several classes of G-protein have been identified in the organ of Corti (Canlon et al., 1991; Tachibana et al., 1992), nothing is known about the type of G-protein coupled to the $InsP_3$ second messenger system in the inner ear.

The function of the vestibular efferent system remains speculative. It may regulate the sensitivity of the hair cells during spontaneous or reflex-induced movement like the muscle spindle under γ-neuron control. In addition, this system may be related to vestibular habituation or compensation (Goldberg and Fernandez, 1975. for review). Although acetylcholine is assumed to be a neurotransmitter in the vestibular efferent system as in the cochlea (Klinke, 1986; Schwartz et al., 1986; Anniko and Arnold, 1991), little is known about the receptors linked to the vestibular efferent system. Electrical stimulation of vestibular efferent fibers produces both inhibition and facilitation of the afferent activity (Dechesne and Sans, 1980; Goldberg and Fernandez, 1980). It is not clear whether these two

types of actions use different neurotransmitter mechanisms. However, the stimulation by cholinergic agonists also produces both inhibition and facilitation of the afferent activity, suggesting that acetylcholine is a putative neurotransmitter for both types of efferent effects (Housley et al., 1990). Recently, a study using α-bungarotoxin binding suggested the presence of nicotinic cholinergic receptors in the frog vestibular sensory epithelia (Thornhill, 1991), and mRNA of the nicotinic cholinergic receptor subunit was shown to be expressed in vestibular hair cells (Wachym et al., 1991). However, the inhibitory effect of atropine on IPSPs elicited by electrical stimulation of the frog sacculus suggests the additional presence of muscarinic cholinergic receptors (Sugai et al., 1992). Our results support the existence of muscarinic cholinergic receptors in the vestibular sensory epithelia.

The differential response of InsPs release to atropine in the vestibular sensory epithelia is interesting. In the cochlear sensory epithelia, carbamylcholine-induced InsPs release was completely blocked by atropine, but atropine had no effect on unstimulated InsPs release. These results agree with those in the whole rat cochlea (Guiramand et al., 1989) and in the organ of Corti of guinea pig (Niedzielski and Schacht, 1990). In contrast, atropine lowered both the unstimulated and stimulated InsPs release in the vestibular sensory epithelia. This suggests that the muscarinic cholinergic receptor in the vestibular sensory epithelia could be stimulated by endogenous acetylcholine during the experiment. Even in the resting state, endogenous acetylcholine might be released constantly to regulate the spontaneous activity of vestibular afferent system.

ATP may serve as a neuromodulator in the central and peripheral nervous system by increasing or decreasing the release of other neurotransmitters, or by modulating their action (Gordon, 1986). Purinergic receptors are classified into P_1 and P_2 receptors which are further divided into P_2x and P_2y receptor subtypes based on their affinity for different purinergic agonists (Burnstock, 1990, for review). The order of potency of agonists for InsPs release obtained in this study, ATP-γ-S > α,β-methylene-ATP > adenosine, corresponds to that of a P_2y purinergic receptor. This result is in agreement with the studies that demonstrate P_2y receptors coupled to PLC in nerve cells (Ehrlich et al., 1988; Fisher and Landon, 1991; Kastritsis et al., 1992). While ATP is known to coexist with acetylcholine in the autonomic nervous system (Burnstock, 1981), there is no anatomical evidence for purinergic receptors in the cochlear and vestibular sensory epithelia. Thus, a role for ATP and ATP-coupled second messengers remains to be established.

Finally, the question arises as to the physiological role of the $InsP_3$ second messenger system in the inner ear. The cholinergic control points to the efferents where both the lateral and the medial system may utilize this second messenger for an as yet unknown aspect of their function. Both muscarinic cholinergic and purinergic receptor agonists increase intracellular calcium concentrations in isolated outer hair cells (Shigemoto and Ohmori, 1990; Nakagawa et al., 1990). Such an effect might link the phosphoinositides with the regulation of calcium levels and therefore, possibly, to some aspects of the control and modulation of 'slow motility' (Dulon and Schacht, 1992). Acetylcholine has indeed been linked to inositol phosphates in outer hair cells of the guinea pig (Kakehata et al., 1992). On the other hand, some hair cell responses to this neurotransmitter, for example, hyperpolarization, may be mediated by a nicotinic-like receptor (Housley and Ashmore, 1991).

Several anatomical and physiological observations have led to the speculation that the vestibular hair cells also possess active motile properties regulated by intracellular calcium mobilization (Sans et al., 1989; Zenner et al., 1990; Valat et al., 1991, Lepyre and Cazals, 1991). Calcium in vestibular hair cells may, then, likewise be under regulation by phoshoinositides and $InsP_3$. However, such an action may at best be one of many potential physiological functions of this second messenger system in the cochlea and the vestibule. The precise role of phosphoinositides, calcium and protein phosphorylation in inner ear physiology remains to be established.

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