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Evidence that phosphoinositides mediate motility in cochlear outer hair cells

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Cochlear outer hair cells are postulated to modulate auditory transduction by their mechanical properties which presumably are controlled by efferent neurons and regulated by the levels of intracellular calcium. In a number of biological systems these calcium levels are controlled by inositol trisphosphate ($InsP_3$), the second messenger of the phosphoinositide cascade. We have investigated whether the phosphoinositides function in the signal transfer in mammalian auditory sensory cells. Live isolated outer hair cells synthesize the lipids of the phosphoinositide cycle, phosphatidic acid (Ptd), phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdInsP) and phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$). After one hour of incubation with [^{32}P]orthophosphate, Ptd constituted $21 \pm 7\%$ of labeled lipids, PtdIns $19 \pm 5\%$, PtdInsP $28 \pm 7\%$ and $PtdInsP_2$ $33 \pm 7\%$. Contractions were elicited in isolated cells after their membrane was permeabilized with Triton X-100. Addition of calcium and ATP resulted in contractions (average, 7.2% of initial cell length) in 81% of the cells studied; the omission of ATP or calcium (i.e. the presence of the calcium-chelator EGTA) reduced the incidence of contractions to 0% and 17%, respectively. In the absence of calcium, the putative second messenger, $InsP_3$, caused contractions in 63% of the observed cells while the physiologically inactive agents inositol and inositol bisphosphate failed to elicit responses. The results are compatible with the suggestion that $InsP_3$ mediates contractility in outer hair cells.

Hair cell, outer; Motility; Calcium; Phosphoinositide; Second messenger

Introduction

Auditory transduction in the mammalian cochlea involves two types of sensory cells, the inner and the outer hair cells. While the inner hair cells are the primary transducers of afferent acoustic information, the outer hair cells are postulated to modulate the transduction process. In doing so they may alter the micromechanics of the basilar membrane (Kemp, 1978; Kim et al., 1980; LePage and Johnstone, 1980; Dallos, 1981; Khanna and Leonard, 1982; Sellick et al., 1982) through their motile properties (Zenner et al., 1985b; Brownell et al., 1985).

Direct studies on auditory sensory cells in vitro have recently become possible with the development of techniques for the isolation of single, viable outer hair cells from the organ of Corti in guinea pigs (Zenner et al., 1985b; Brownell et al., 1985) and other species (Zajic and Schacht, 1987). The sensory cells isolated by microsurgery as in this study (Fig. 1) exhibit intracellular potentials as low as -70 mV (Zenner et al., 1985a), a value in good agreement with in situ measurements (Dallos et al., 1982). Other advantages of this microsurgical procedure are the high yield of viable cells and their ability to survive for several hours in culture thus permitting biochemical experiments.

A most striking feature of outer hair cells in vitro is their motility. Contractions have been

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observed both with fast time constants in the microsecond range (Brownell et al., 1985; Kachar et al., 1986) and slow time constants in the millisecond range (Zenner et al., 1985b; Flock et al., 1986). The slow contractions are ATP-dependent and actin-mediated and apparently controlled by intracellular calcium levels (Zenner, 1986; Flock, 1986). In a number of biological systems, physiological processes that are ultimately mediated by calcium engage the phosphoinositide cascade as a second messenger system (Berridge, 1984; Nishizuka, 1984). In the phosphoinositide cascade, the physiological stimulus initiates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) liberating inositol trisphosphate (InsP$_3$) and diacylglycerol as second messengers; InsP$_3$ in turn triggers the release of calcium from intracellular stores. The calcium dependency of outer hair cell motility and the fact that aminoglycoside antibiotics which impair the function of outer hair cells preferentially bind to PtdInsP$_2$ (Schacht, 1986) suggested the possibility that the phosphoinositide second messenger system controls the contractility of these cells. If this system functions in outer hair cells, PtdInsP$_2$ should be present and InsP$_3$ should elicit a contractile response.

Materials and Methods

Outer hair cells were isolated from the guinea pig cochlea essentially as described (Zenner et al., 1985b; Zajic and Schacht, 1987). The apical turn of the organ of Corti isolated from the guinea pig was placed in Hank's medium (adjusted to 300 mosM) in a Petri dish and further dissected by micromanipulation in the absence of proteolytic enzymes. Isolated hair cells were placed in a Petri dish (Nunc) and the outer membrane permeabilized with 0.1% Triton X-100 (Shell) for 5 s. This was followed by several rinses with Hank's medium in the absence of calcium and in the presence of ATP until cells maintained a stable length. At this time the test solution was added to a final volume of 50–150 μl.

All experiments were carried out at room temperature, pH 7.2, and 300 mosM. pH and osmolarity were measured before and after the experiment. Only hair cells that became attached to the bottom of the Petri dish were monitored and scored. An inverted microscope (Leitz Diavert) with bright field optics was used for observations. Dimensions of cells were quantitated from single frames of video recordings (Sony) with a resolution of 0.5–0.8 μm.

For studies of lipid metabolism, cells were separated from other tissue fragments by transfer with microcapillaries into incubation wells. One hundred to 200 cells were incubated for 60 min at room temperature in 75 μl of Hank's medium supplemented with 200–500 μCi [^{32}P]orthophosphate. Lipids were extracted with acidified chloroform/methanol, separated by thin layer chromatography, located by radioautography, and quantitated by liquid scintillation counting (Schacht, 1978).

Results

For the identification of phospholipids outer hair cells were labeled in vitro with [^{32}P]orthophosphate. The inorganic radiotracer is first incorporated into [γ-^{32}P]ATP which then serves as precursor for phospholipid synthesis. Analysis of lipids after extraction and thin layer chromatography showed the incorporation of ^{32}P primarily into phosphatidic acid (Ptd), phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdInsP) and PtdInsP$_2$. The absolute magnitude of the ^{32}P incorporation was somewhat variable between experiments owing to the difficulty of controlling the exact number of viable hair cells but the relative distribution of radioactivity between the lipids was remarkably consistent (Table I). The pattern of labeling with PtdInsP and PtdInsP$_2$ as the most highly labeled lipids is qualitatively and quantitatively similar to the typical labeling pattern of nervous tissue and also in good agreement with our previous studies of phospholipids in the whole organ of Corti (Orsulakova et al., 1976; Schacht, 1984).

Motility of isolated outer hair cells can be studied directly in a Petri dish under video-assisted microscopic observation. External calcium was removed by washing the hair cells with calcium-free Hank's medium and the cell plasma membrane was permeabilized with 0.1% Triton X-100 for 5 s. This treatment provided the necessary membrane permeability for the subsequent

TABLE I

^{32}P-LABELED LIPIDS OF OUTER HAIR CELLS

Outer hair cells were isolated and labeled as described in Materials and Methods. Radioactivity (cpm) in total lipids ranged from 200 to 800 per incubation and averaged 547 ± 357. Numbers are means ± S.D. of six incubations.

Lipid	^{32}P incorporation	
	cpm	as % of total labeled lipid
Phosphatidate	111 ± 84	21 ± 7
Phosphatidylinositol	112 ± 82	19 ± 5
Phosphatidylinositol 4-phosphate	152 ± 89	28 ± 7
Phosphotidylinositol 4,5-bisphosphate	172 ± 102	33 ± 7

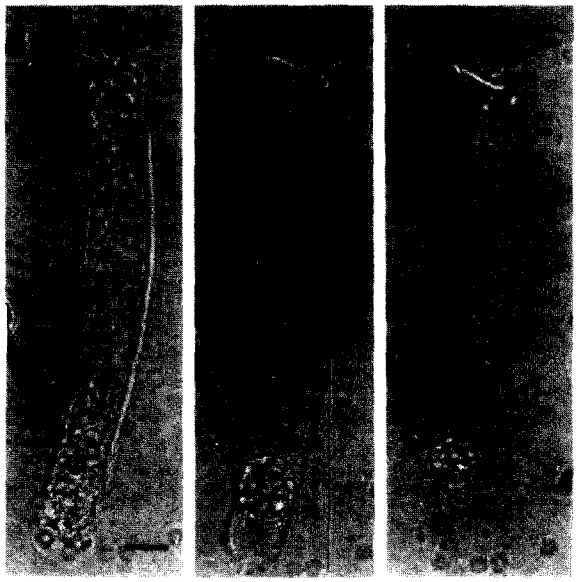

Fig. 1. Isolated outer hair cells. (a) Outer hair cell from the apical turn of the guinea pig (bright field; bar, 10 μm). (b) Cell permeabilised with Triton X-100 in the absence of calcium. (c) Same cell as in b in the presence of ATP and $InsP_3$ (0.1 mM each).

addition of calcium or $InsP_3$ while retaining the ability of the cells to contract. Exposure to calcium (> 1 μM) and ATP-containing medium caused 'slow' contractions of the cells in more than 80% of the experiments with an average response of 7.2% of their length (Table II). The contractions started upon addition of calcium/ATP without apparent lag and were essentially complete after 30 s. Both ATP and calcium were required since the response was essentially abolished when either ATP was omitted or the concentration of calcium lowered to below 1 μM through the addition of EGTA. In the absence of added calcium, $InsP_3$ induced contractions in about 60% of the cases with average responses of 2%. These contractions showed the same time course as those elicited by calcium, i.e., essentially complete within 30 s. In a limited number of experiments, the presence of GTP along with $InsP_3$ appeared to provide an even more consistent response (5 of 6 experiments). GTP has been suggested to enhance the release of intracellular calcium by $InsP_3$ (Dawson, 1985) or to release calcium by an independent mechanism (Gill et al., 1986). The additional presence of EGTA reduced the percentage of $InsP_3$-induced contractions. *myo*-Inositol 2-phosphate and *myo*-inositol were ineffective substitutes for $InsP_3$. The effects of $InsP_3$ (as well as of calcium) on the length of the hair cells were statistically significant at $P > 0.01$ (paired t-test).

TABLE II

CONTRACTILITY OF OUTER HAIR CELLS

The agents were applied to permeabilized cells as described in Materials and Methods. Concentrations applied were: 0.1 mM ATP; 1.25 mM $CaCl_2$; 1 mM EGTA; 0.01–1 mM $InsP_3$; 0.1 mM GTP; 1 mM Ins, $InsP_2$.

Additions	Responses/ experiments	Length [a] (μm)	Contraction [b] (%)
ATP + Ca^{2+}	83/102	62.5 ± 0.6	7.2 ± 0.7
Ca^{2+} alone	0/66	63.8 ± 0.6	0
ATP + EGTA [c]	3/18	57.5 ± 2.4	1.3 ± 0.6
ATP + $InsP_3$	20/34	59.7 ± 1.0	2.0 ± 0.6
ATP + $InsP_3$ + GTP	5/6	56.6 ± 3.1	2.0 ± 0.6
ATP + $InsP_3$ + EGTA	4/10	64.6 ± 2.2	1.8 ± 0.9
ATP + $InsP_2$	0/7	59.1 ± 2.1	0
ATP + Ins	0/6	60.2 ± 1.0	0

[a] Mean length ± S.E. of cells after Triton-treatment and before additions.

[b] Mean percent contraction from original length ± S.E. in response to additions.

[c] Resulting in an estimated free (Ca^{2+}) of 0.14 μM.

Discussion

The present study demonstrates that in outer hair cells from the guinea pig cochlea intracellular applications of $InsP_3$ elicit a slow motile response. In addition, we have shown that live outer hair cells possess the molecular machinery to synthesize $PtdInsP_2$ and the other lipids involved in the phosphoinositide cycle. These results are compatible with the idea that the phosphoinositide cascade is a second messenger system in outer hair cells. Specifically, our experiments suggest that: (i) like other cells, outer hair cells have the capacity to synthesize lipids via the phosphoinositide cycle, which would allow the second messenger, IP_3, to be released from the outer hair cell membrane into the cytoplasma following an adequate stimulus; (ii) $InsP_3$ can act as a second messenger to elicit a motile response, the molecular basis of which is thought to be an activation of the Ca^{2+}-dependent actin skeleton of the sensory cell. The latter suggestion is emphasized by the fact that the response is specific for IP_3: the hydrolysis products of $InsP_3$, *myo*-inositol (Ins) and *myo*-inositol bisphosphate ($InsP_2$) which are without physiological function in the phosphoinositide cascade, are also without effect on the contractility of the hair cells. The requirement of ATP is clearly evident and the involvement of calcium is indicated by the reduction of the number of responses to calcium and $InsP_3$ by the calcium chelator, EGTA. The fact that the IP_3-induced contractions are smaller than those induced by the addition of calcium can be understood from the assumed mechanism of action. The concentration of exogenously added calcium should be sufficient to saturate the system and elicit a maximal contractile response while the amount of calcium liberated by IP_3 from intracellular stores may be a limiting factor.

In the proposed sequence of events resulting in hair cell contraction the adequate stimulus remains unknown. It has been suggested, that the efferent transmitter, acetylcholine, is an appropriate physiological stimulus for outer hair cell motility in vivo (Brownell et al., 1985). This suggestion and our results are compatible as muscarinic actions of acetylcholine can be mediated through the phosphoinositide cascade (Nishizuka, 1984). However, while acetylcholine is rather well established as the transmitter, the pharmacology of the postsynaptic receptor on the outer hair cells has not yet been unambiguously determined. Neither muscarinic nor nicotinic properties have been ruled out and a 'mixed' receptor has also been postulated (Klinke, 1986).

Regardless of the precise nature of the primary stimulus, the evidence presented here is consistent with the hypothesis that the phosphoinositide cascade constitutes a second messenger system in outer hair cells. While more criteria need to be investigated (e.g., hydrolysis of $PtdInsP_2$ induced by the physiological stimulus; reversibility of the contraction), our results satisfy two basic requirements: the demonstration of the synthesis of polyphosphoinositides and the ability of the putative second messenger, $InsP_3$, to induce contractions in isolated outer hair cells. Thus, $InsP_3$ could play a key role in the intracellular control of slow active mechanical processes which are postulated to correct alterations in the position of the basilar membrane resulting from fluctuations in pressure and volume of the perilymph and endolymph (LePage and Johnstone, 1980). Furthermore, such a process could play a role during efferent neurotransmitter-evoked responses of outer hair cells as well as in the protection (adaptation, temporary threshold shift) of the basilar membrane during pronounced vibrations induced by high sound pressure.

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