Vasopressin, ATP and catecholamines differentially control potassium secretion in inner ear cell line

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Abstract
A strict control of endolymph composition (high potassium, low sodium fluid) and volume is instrumental for a proper functioning of the inner ear. Alteration of endolymph homeostasis is proposed in the pathogenesis of Menière’s disease. However, the mechanisms controlling endolymph secretion remain elusive. By using the vestibular EC5v cells, we provide evidence for the presence of vasopressin, catecholamine and purinergic signaling pathways, coupled to adenylyl cyclase, phosphoinositidase C and Ca2+ activation. We demonstrate that vasopressin and catecholamines stimulate while ATP inhibits apical potassium secretion by EC5v cells. These results open new interesting perspectives for the management of inner ear diseases.

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1. Introduction
The inner ear houses the hearing (cochlea) and balance (saccule, utricule, semicircular canals) organs. Endolymph, the fluid that bathes the hair cell bundles of the sensory cells, is unique with a high K+ concentration (170 mM), and virtually no Na+ (1 mM). Furthermore, the transepithelial potential (endocochlear potential) is +100 mV, lumen positive. Homeostasis of the volume, pressure, and electrochemical composition of endolymph is pivotal for an accurate functioning of the sensory cells. Indeed, it has been proposed that vertigo and hearing loss, such as in Menière’s disease, a syndrome linked to an increase of endolymph volume (hydrops), may result from dysfunctions in inner ear epithelial ion transport, notably K+. For example, the most frequent etiology of congenital deafness, due to connexin 26 mutations, is related to K+ recycling dysfunction [1]. Jervell and Lange–Nielsen syndrome, a long QT syndrome, associated to hearing loss, is caused by mutations in KCNE1 and KCNQ1 genes, encoding the K+ channel involved in endolymphatic K+ secretion [2]. Thus, investigations on the molecular mechanisms controlling ion composition of endolymph, and on water transport through the labyrinthine epithelium are critical to open new therapeutic options in the management of inner ear defects.

The development of a new vestibular cell line (EC5v) [3] that expresses all transporters known to be involved in endolymph...
secretion prompted us to investigate hormonal mechanisms controlling ionic transport via the two main regulatory pathways, phosphoinositidase C (PLC)/Ca²⁺ and adenylate cyclase pathways. Indeed, evidence has been provided for a regulating role of extra- cellular Adenosine Triphosphate (ATP) and Uridine Triphosphate (UTP), which act through a PLC stimulation, on both neurotransmission and endolymph homeostasis [4]. Furthermore, the putative effects of polypeptic hormones, coupled to adenylate cyclase activation, have been largely studied in the inner ear, specifically antidiuretic hormone Arginine Vasopressin (AVP) that has been proposed to be involved in Menière’s disease [2,5].

The present study aimed at investigating, in EC5v, the hormonal regulatory pathways mediated by transmembrane G Protein-Coupled Receptors (GPCR) via the stimulation of PLC or adenylate cyclase. For comparison, similar experiments were performed in renal KC3AC1 cells, derived from the cortical collecting duct (CCD). Indeed, several ionic transporters are expressed in both the inner ear and kidney, and their defects result in simultaneous dysfunctions of these seemingly unrelated organs [6]. This study provides direct evidence that AVP and ATP regulate K⁺ secretion on inner ear epithelium, and may be involved in the regulation of endolymph ionic composition and volume.

## 2. Materials and methods

### 2.1. Cell culture

The inner ear EC5v (from the ampulla of semicircular canals) and the renal KC3AC1 cells (from microdissected CCD) were established by a targeted oncogenesis strategy in mice, and were further characterized [3,7]. Both cell lines from passage 10 to 40 were cultured in renal KC3AC1 cells, derived from the cortical collecting duct (CCD). Indeed, several ionic transporters are expressed in both the inner ear and kidney, and their defects result in simultaneous dysfunctions of these seemingly unrelated organs [6]. This study provides direct evidence that AVP and ATP regulate K⁺ secretion on inner ear epithelium, and may be involved in the regulation of endolymph ionic composition and volume.

### 2.2. Products

Radioactive products were purchased from GE Life Sciences (Les Ulis, France), drugs and chemicals were from Sigma (Saint-Louis, MO, USA), except when indicated.

### 2.3. RT-PCR

Total RNA was extracted from cells with TRIZOL reagent (Invitrogen) according to the manufacturer’s recommendations and RNA was thereafter processed for RT-PCR, as previously described [3]. Supplementary Table 2 indicates primer sequences.

### 2.4. Western blot

Total protein extracts were prepared as previously described [7]. Immunoblots were incubated overnight at 4 °C with anti-vasopressin type 2 receptor (V2R) and anti-α-tubulin antibodies (see Supplementary Table 3). After 1 h incubation at room temperature with fluorescent secondary antibodies, membranes were scanned using the Odyssey infrared imaging system and images were processed with the Image Studio Software (LI-COR Biosciences).

### 2.5. cAMP assay

As previously described [8], confluent cells were scrapped and incubated for 5 min with the various compounds. cyclic Adenosine Monophosphate (cAMP) contents (fmol/5 min/μg protein) were measured by radioimmunoassay (Biotrack™ assay system, GE Life Sciences).

### 2.6. Phosphoinositidase C assay

Enzymatic studies were performed as described earlier [9]. Briefly, confluent cells were loaded overnight with 0.5 MBq myo-[³H] inositol (2.6 TBq/mmole). A 30 min incubation at 37 °C was started by adding cells (20 μg protein) in PLC incubation medium (Supplementary Table 1) in presence of LiCl to inhibit the inositol phosphates hydrolysis and various compounds. Radioactivity associated to free inositol, glycerophosphoinositol (GroPs), total inositol phosphates (ΣInsPs), and incorporated in total (Σ) phosphoinositides were determined. Results (mean ± SE) were expressed as radioactive ratios between specific inositol-containing pool and total labeled inositol-containing cellular pools (the latter were 2215 ± 694 cpm, n = 10, and 8330 ± 1727 cpm, n = 11) for KC3AC1 and EC5v cells, respectively.

### 2.7. Ca²⁺ assay

Cytosolic free calcium concentration [Ca²⁺], was measured on cellular suspensions as previously described [10]. Cells were loaded with Fura-2 for 45 min at 37 °C. Fluorescence was monitored in a F2000 spectrofluorometer (Hitachi Scientific Instruments, Mountain View, CA, USA). [Ca²⁺], was calculated using a dissociation constant (K₅ᵢ) of 224 nM.

#### 2.7.1. ⁸⁶Rb transport

As previously described [3], 10⁶ cells seeded on Transwell filters, were cultured for 5 d in the epithelial culture medium. ⁸⁶Rb (185 kBq/well) was added in the basolateral compartment. ⁸⁶Rb flux was evaluated by sampling 5 μl of apical fluid at 15 min intervals. Fluxes (nmol/cm²) were calculated as a function of K⁺ concentration and the specific radioactivity (18.5–37.0 MBq/mg Rb), assuming that ⁸⁶Rb is a strict marker of K⁺. Results are means of at least 6 independent filters.

### 2.8. Calculations

The kinetics agonist-induced cyclic AMP productions and/or PLC activation are calculated as previously described [11]. Results were given as means ± SE of n independent replicates performed in the same experiment or from n separated experiments. Differences between groups were analyzed using ANOVA followed by Dunnett’s multiple comparison test or Student’s t test when indicated. The significance was achieved for a P < 0.05.

## 3. Results

### 3.1. Expression of AVP and P2Y receptors

RT-PCR analyses revealed the presence of transcripts coding for GPCR V1a, V2, Oxt, P2Y₂, and P2Y₄ receptors in EC5v while KC3AC1 expressed transcripts for V2, P2Y₂ and P2Y₄ receptors. Specific amplicons were identified at the expected sizes and their sequences confirmed (Supplementary Table 1). Western blot analysis confirmed the presence of V2R protein at the expected molecular mass (~60 KDa) in both cell lines (Supplementary Fig. 2).

### 3.2. Pharmacological characterization of adenylate cyclase stimulation

In EC5v, cAMP production was 218 ± 44, and 1281 ± 202 fmol cAMP/5 min/μg protein (n = 7), in basal and forskolin (FSK)-stimulated conditions, respectively. The corresponding values were 608 ± 116 and 13 758 ± 6360 fmol cAMP/5 min/μg protein (n = 6) for KC3AC1, consistent with the presence of a functional adenylate cyclase system in both epithelial cell lines.
### 3.3. Pharmacological characterization of PLC stimulation

The basal production of total [3H]InsPs was not modified by pharmacological concentrations of either angiotensin II, bradykinine, phenylephrine, AVP, the V1a agonist [Phe2,Orn8]VT, or carbachol (Supplementary Table 4). In contrast, Inositol Phosphates (InsPs) production was markedly and similarly enhanced by 1 mM ATP (4.65 ± 0.24 and 7.35 ± 0.17% of the total incorporated [3H]InsP production in EC5v and KC3AC1, respectively) or UTP (4.69 ± 0.17 and 7.17 ± 0.22% in EC5v and KC3AC1, respectively).

In both cell lines, ATP increased PLC activities in a dose-dependent fashion according to Michaelian kinetics with the following kinetic parameters: Threshold response for 0.10 µM, pKₐ values 6.1 and 6.2, Hill coefficient nH = 0.82 and 0.98, and maximal stimulating factors 3.4 and 4.0, for EC5v and KC3AC1 cells, respectively (Fig. 2A and B).

The sensitivity of PLC to structural ATP analogues shares some similarities between EC5v and KC3AC1: Their apparent activation constant (Kₐ) for ATP, adenosine 5'-O-(3-thiophosphate) (ATP₇S), and UTP were lower than those of the other tested analogues, and, considering their intrinsic activities (Max/ATPmax), ADP-β-S exhibited weak agonistic potencies whereas Uridine Diphosphate (UDP), Uridine Monophosphate (UMP), AMP, adenosine, uridine and cAMP were almost devoid of activities (Supplementary Table 5).

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**Fig. 1.** Hormonal sensitivity of adenylate cyclase in EC5v (A, B) and KC3AC1 (C, D) cells. Results are mean ± SE of five independent determinations. (A) cAMP production were measured under basal conditions or in presence of either 1 mM dDAVP, 5 µM calcitonin, 5 µM human parathormone (hPTH), 0.1 mM isoproterenol (Iso), ±10 µM propranol (Propra), 0.5 mM PGE₂, or 5 µM FSK. ***: differences between basal and drug induced cAMP production (P < 0.001, ANOVA); $: propranolol decreased the cAMP response to isoproterenol (P < 0.0001, Student). (B) Dose-dependent stimulation of cAMP production by isoproterenol. Apparent activation constant Kₐ = 70 nM (arrow), and Hill coefficient nH = 1.9. (C) cAMP production were measured under basal conditions or in presence of either 10 nM dDAVP, ±10 µM OVTA, ±10 µM desGly, 5 µM calcitonin, 5 µM hPTH, 0.1 µM Iso, ±10 µM Propra, 0.5 µM PGE₂, or 5 µM FSK. Differences between basal and drug-induced cAMP production: ***: P < 0.001, : P < 0.05 (ANOVA); $: OVTA and desGly decreased response to dDAVP (P < 0.01 and 0.001, respectively, Student); £: propranolol decreased the cAMP response to isoproterenol (P < 0.005, Student). (D) Dose-dependent stimulation of cAMP production by dDAVP. Apparent activation constant Kₐ = 2.6 nM (arrow), and Hill coefficient nH = 1.7.
The main differences in the recognition patterns of nucleotides between cell lines resulted mainly in the potencies of the potent marker of P2X7 receptors 2'-0- and 3'-0-(benzoyl benzyl)-ATP (Bz-ATP) that stimulated PLC of KC3AC1 with an apparent affinity one order of magnitude higher than that for the EC5v, and the potent agonist of P2Y4 receptors β,γ-methylene ATP (β,γ-Me-ATP) that stimulate enzyme in EC5v with a $K_a$ value 10 times lower than that of KC3AC1 (Supplementary Table 5). The results observed in the two cell lines suggest different coupling procedures probably due to distinct functional properties of the cells.

Considering the PLC pathway, no major difference on stimulating hormone pattern was observed between the two cell lines, except subtle variations in PLC sensitivity to structural ATP analogues.

3.4. Intracellular Ca$^{2+}$ response to AVP and ATP

Owing to results of RT-PCR and PLC studies, we further investigate [Ca$^{2+}$], variations. In both cell lines, no Ca$^{2+}$ response occurred in presence of AVP, or the potent V1a agonist [Phe$^{2}$, Orn$^{8}$]VT whereas ATP exposure induced a rapid increase in [Ca$^{2+}$], followed by a sustained plateau (Fig. 2C and D), suggesting an efficient coupling between ATP receptors, PLC activation, and cellular Ca$^{2+}$ release.

3.5. K$^+$ transport

In EC5v, K$^+$ secretion, from basolateral to apical compartments, was clearly increased by 2-fold upon dDAVP or isoproterenol stimulation, and by 3-fold upon FSK stimulation, and was slightly, but significantly, inhibited by ATP (Fig. 3A). In KC3AC1, K$^+$ secretion was increased by 3-fold upon dDAVP and by 2-fold upon FSK, but was inhibited by isoproterenol (Fig. 3B). At variance with that observed for EC5v, K$^+$ transport was decreased by 50% by isoproterenol.

These results demonstrate that dDAVP, isoproterenol, and ATP, clearly differentially regulate K$^+$ transport, in both EC5v and KC3AC1.

4. Discussion

Epithelial cells receive hormonal inputs that regulate volume and electrolyte homeostasis. This regulation is of major importance in the kidney to control ionic transports and plasma composition. This fine tuning control is also crucial in the inner ear to strictly maintain the endolymph composition and volume, thereby preventing any hearing and balance dysfunction. In the present study, we provide first evidence that three different signaling cascades are functional in the inner ear. Indeed, we demonstrate that the β-adrenergic catecholamines (isoproterenol), the antidiuretic hormone (AVP), and the nucleotides (ATP and its agonists) activate their effectors, adenylate cyclase or PLC in vestibular EC5v cells. We also show that apical K$^+$ secretion in EC5v is markedly yet differentially affected by these stimuli.

4.1. Catecholamine regulation

In the inner ear, β-adrenergic receptors are present in the endolymph secretory structures, stria vascularis in the cochlea and dark cells in the vestibule [12]. The stimulating effect of isoproterenol on adenylate cyclase/CAMP system has been extensively studied [13,14]. Furthermore, isoproterenol has been shown to stimulate the short circuit current in isolated dark cell epithelium, suggesting a stimulating effect on endolymph secretion [13]. Our results demonstrate the presence of this regulatory system and the stimulation effect of isoproterenol on K$^+$ secretion in vestibular EC5v cells.
of K⁺ secretion in EC5v, suggesting the implication of catecholamine signaling pathway in balance and, possibly in hearing. Catecholamines could induce acute disturbance of endolymph homeostasis, and may be involved in Menière’s disease crisis, often triggered by acute stress.

In the late nephron, the main regulatory site for K⁺ excretion, basal-lateral Na⁺,K⁺-ATPase activity is pivotal to develop innovative therapeutic approaches controlling K⁺ secretion and water movements.

4.3. Purinergic regulation

Purines, particularly ATP, act as neurotransmitters and paracrine factors in different organs. Endolymph homeostasis is regulated by ATP, released during noise exposure or hypoxia, acting on both ionotropic (P2X) and metabotropic (P2Y) receptors [21]. ATP inhibits the KCNE1/KCNQ1 channel involved in endolymph K⁺ secretion, through activation of P2Y₄ receptor/PLC/Protein Kinase C pathway [22]. In EC5v, P2Y₄ have been identified, and ATP was shown to stimulate PLC pathway and to slightly decrease K⁺ secretion as previously shown in native tissue [23].

Although P2Y₂ receptors have been identified in CCD [24], inhibiting ENaC-dependant Na⁺ transport [25], their impact on K⁺ secretion remains questionable.

This study demonstrates the presence of regulatory mechanisms in endolymph secretory cells, supporting a precise regulation of high K⁺ concentration in endolymph that slightly differs from renal K⁺ excretion. Given that modification of endolymph composition induces hearing and/or balance disturbances, a better characterization of the signaling pathways involved in endolymph homeostasis is pivotal to develop innovative therapeutic approaches controlling K⁺ secretion and water movements.

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Appendix A. Supplementary data


References


