

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 adenylate cyclase, phosphoinositidase C and Ca^{2+} 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.

Keywords: endolymph, vasopressin receptors, Menière's disease, vestibular cells, purinergic receptors

LIST OF ABBREVIATIONS

α,β -Me-ATP: α,β -methylene-ATP

β,γ -Me-ATP: β,γ -methylene ATP

Σ InsPs: total inositol phosphates

Asn¹, Val⁵-angiotensin II: angiotensin II

ATP: Adenosine Triphosphate

ATP γ S: adenosine 5'-0-(3-thriophosphate)

AVP: Arginine Vasopressin

BSA: Bovine Serum Albumin

Bz-ATP: 2'- and 3'-0-4-(benzoyl benzyl)-ATP

cAMP: cyclic Adenosine Monophosphate

CCD: Cortical Collecting Duct

dDAVP: 1-deamino-8D-arginine vasopressin

desGly: [1-(β -mercapto- β,β -cyclopentamethylene propionic acid), 2-0-ethyltyrosine, 4-valine, 8-arginiamide] vasopressin [desgly⁹-d (CH₂)₅-Tyr (Et)² VAVP]

FSK: Forskoline

GroPns: glycerophosphoinositol

hPTH: human Parathormone

InsPs: Inositol phosphates

OVTA: Ornithine Vasotocin Analogue

PGE₂: Prostaglandin E₂

PLC: Phosphoinositidase C

poVT: Phe², Orn⁸- vasotocin: [Phe², Orn⁸]VT

UDP: Uridine Diphosphate

UMP: Uridine Monophosphate

V₂R: Vasopressin type 2 receptor

1. Introduction

The inner ear houses the hearing (cochlea) and balance (sacculae, utricle, semicircular canals) organs. Endolymph, the fluid that bathes the apical hair 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 ionic 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 ionic 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)/ Ca^{2+} and adenylyate cyclase pathways. Indeed, evidence has been provided for a regulating role of extracellular ATP and UTP, which act through a PLC stimulation, on both neurotransmission and endolymph homeostasis [4]. Furthermore, the putative effects of polypeptidic hormones, coupled to adenylyate cyclase activation, have been largely studied in the inner ear, specifically antidiuretic hormone (AVP) that has been proposed to be involved in Menière's disease [2].

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 adenylyate 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 [5]. 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, 6]. Both cell lines from passage 10 to 40 were cultured at 37°C in an humidified incubator with 5% CO₂ on collagen I-coated Petri dishes, or, when indicated, on Transwell filters with an epithelial culture medium (Supplemental Table 1).

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]. Supplemental Table 2 indicates primer sequences.

2.4. Western blot

Total protein extracts were prepared as previously described [6]. Immunoblots were incubated overnight at 4°C with anti-vasopressin type 2 receptor (V2R) and anti- α -tubulin antibodies (see Supplemental 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 [7], confluent cells were scrapped and incubated for 5 min with the various compounds. cAMP contents (fmol/5 min/ μ g protein) were measured by radioimmunoassay (BiotrackTM assay system, GE Life Sciences).

2.6. *Phosphoinositidase C assay*

Enzymatic studies were performed as described earlier [8]. Briefly, confluent cells were loaded overnight with 0.5 MBq myo- ^3H inositol (2.6 TBq/mmol). A 30 min incubation at 37°C was started by adding cells (20 μg protein) in PLC incubation medium (Supplemental Table 1) in presence of LiCl to inhibit the inositol phosphates hydrolysis and various compounds. Radioactivity associated to free inositol, glycerophosphoinositol (GroPns), total inositol phosphates (ΣInsPs), and incorporated in total (Σ) phosphoinositides were determined. Results (mean \pm 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. Ca^{2+} -assay

Cytosolic free calcium concentration $[\text{Ca}^{2+}]_i$ was measured on cellular suspensions as previously described [9]. 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). $[\text{Ca}^{2+}]_i$ was calculated using a dissociation constant (K_D) of 224 nM.

2.8. ^{86}Rb transport

As previously described [3], 10^6 cells seeded on Transwell filters, were cultured for 5 d in the epithelial culture medium. ^{86}Rb (185 kBq/well) was added in the basolateral compartment. ^{86}Rb flux was evaluated by sampling 5 μl of apical fluid at 15 min interval. Fluxes (nmol/cm^2) were calculated as a function of K^+ concentration and the specific radioactivity (18.5-37.0 MBq/mg Rb), assuming that ^{86}Rb is a strict marker of K^+ . Results are means of at least 6 independent filters.

2.9. *Calculations*

The kinetics agonist-induced cyclic AMP productions and/or PLC activation are calculated as previously described [10]. Results were given as means \pm 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, Oxtr, P2Y₂, and P2Y₄ receptors in EC5v while KC3AC1 expressed transcripts for V2, P2Y₂ and P2Y₆ receptors. Specific amplicons were identified at the expected sizes and their sequences confirmed (Supplemental Fig 1). Western blot analysis confirmed the presence of V2R protein at the expected molecular mass (~ 60 KDa) in both cell lines (Supplemental Fig 2).

3.2. Pharmacological characterization of adenylate cyclase stimulation

In EC5v, cAMP production was 218 ± 44 , and 1281 ± 202 fmol cAMP/5 min/ μ g protein (n=7), in basal and forskolin (FSK)-stimulated conditions, respectively. The corresponding values were 608 ± 116 and 13758 ± 6360 fmol cAMP/5 min/ μ g protein (n=6) for KC3AC1, consistent with the presence of a functional adenylate cyclase system in both epithelial cell lines.

In EC5v, basal cAMP generation was not modified by 1-deamino-8D-arginine-vasopressin (dDAVP), calcitonin, parathormone (PTH), or prostaglandin E2 (PGE₂) exposure, but was drastically increased by FSK and isoproterenol, this latter effect being inhibited by propranolol (Fig 1A). The isoproterenol-induced activation of cAMP production was dose-dependent (Fig 1B) with a threshold response for about 10 nM, an apparent activation constant $K_a = 70$ nM, a Hill coefficient $n_H = 1.9$, and a maximal response observed for about 0.4 μ M (maximal stimulating factor SF: 4.9).

In KC3AC1, basal cAMP production remained unchanged upon PTH and calcitonin administration, but was slightly increased by isoproterenol and PGE₂, and markedly enhanced by dDAVP and FSK (Fig 1C). The isoproterenol-induced cAMP production was inhibited by propranolol, and the enzyme stimulation elicited by dDAVP was reduced in the presence of antagonists OVTA or desGly⁹,Tyr(Et)²VAVP (desGly). The dDAVP-induced activation was dose-dependent (Fig 1D) with the following kinetic parameters: threshold response for 5 nM, $K_a = 2.8$ nM, $n_H = 1.7$, and SF=18. Maximal responses to saturating amounts of dDAVP, isoproterenol and PGE₂ were not additive (Supplemental Fig 3).

Comparing these two cell lines, it is worth noting that, whereas V2 receptors are expressed in both cell lines, dDAVP did not induce an adenylate cyclase stimulation in EC5v at variance to what is observed in KC3AC1.

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 (Supplemental Table 4). In contrast, InsPs production was markedly and similarly enhanced by 1 mM ATP (4.65 ± 0.24 and $7.35 \pm 0.17\%$ of the total incorporated [³H]InsP production in EC5v and KC3AC1, respectively) or UTP (4.69 ± 0.17 and $7.17 \pm 0.22\%$ 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 μ M, 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, 2B).

The sensitivity of PLC to structural ATP analogues shares some similarities between EC5v and KC3AC1: Their apparent activation constant (Ka) for ATP, ATP γ S, 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 UDP, UMP, AMP, adenosine, uridine and cAMP were almost devoid of activities (Supplemental Table 5).

The main differences in the recognition patterns of nucleotides between cell lines resulted mainly in the potencies of the potent marker of P2X7 receptors 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)-Me-ATP that stimulate enzyme in EC5v with a Ka value 10 times lower than that of KC3AC1 (Supplemental 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 $[Ca^{2+}]_i$ variations. In both cell lines, no Ca^{2+} response occurred in presence of AVP, or the potent V1a agonist [Phe², Orn⁸]VT whereas ATP exposure induced a rapid increase in $[Ca^{2+}]_i$ 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 [11]. The stimulating effect of isoproterenol on adenylate

cyclase/cAMP system has been extensively studied [12,13]. Furthermore, isoproterenol has been shown to stimulate the short circuit current in isolated dark cell epithelium, suggesting a stimulating effect on endolymph secretion [12]. Our results demonstrate the presence of this regulatory system and the stimulation 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, β -adrenergic agonists inhibits K^+ secretion [14], an effect that was clearly observed in KC3AC1.

Even though, β -adrenergic agonists stimulate cAMP production in both EC5v and KC3AC1 cells, the functional consequences of activated adrenergic cascade on apical K^+ secretion clearly differ between the inner ear and renal cells. Nevertheless, β -adrenergic agonists act in order to maintain low or high K^+ concentration in plasma or endolymph, respectively.

4.2. Antidiuretic hormone regulation

The putative effect of AVP in the inner ear has been extensively studied [15], mainly because of possible therapeutic applications in Menière's disease. However, the presence of V2R and AVP effect on endolymph homeostasis still remain debatable. In vestibular EC5v, AVP receptors have been clearly identified at both mRNA and protein levels, and AVP exposure led to a marked increase in apical K^+ secretion. Nevertheless, V2R activation was not efficiently coupled to cAMP production. The underlying coupling mechanisms remain to be elucidated, but could be related to the receptor number and their localization in microdomains of the plasma membrane or, more likely, to GPCR linked effectors, specifically expressed in these inner ear cells.

In sharp contrast, the presence of functional V2 receptors has been unambiguously demonstrated in CCD [16,17]. AVP increased the number and the activity of basolateral Na^+,K^+ -ATPase [18], promoting Na^+ absorption and K^+ secretion [19]. In KC3AC1, we clearly showed that AVP, presumably *via* V2R activation, dose-dependently stimulated cAMP production and increased apical K^+ secretion.

Collectively, the exact cellular mechanisms by which AVP stimulates K^+ conductance and controls K^+ homeostasis remain elusive but clearly differ between renal excretion and endolymphatic secretion of K^+ .

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 [20]. ATP inhibits the KCNE1/KCNQ1 channel involved in endolymph K^+ secretion, through activation of P2Y₄ receptor/PLC/PKC pathway [21]. 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 [22].

Although P2Y₂ receptors have been identified in CCD [23], inhibiting ENaC-dependant Na^+ transport [24], 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.

5. Acknowledgments

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7. Figures legends

Figure 1. Hormonal sensitivity of adenylate cyclase in EC5v (A, B) and KC3AC1 (C, D) cells. Results are mean \pm SE of 5 independent determinations.

A: cAMP production were measured under basal conditions or in presence of either 1 μ M dDAVP, 5 μ M calcitonin, 5 μ M hPTH, 0.1 μ M isoproterenol (Iso), \pm 10 μ M propranol (Propra), 0.5 μ M PGE₂, or 5 μ M 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 K_a=70 nM (arrow), and Hill coefficient n_H=1.9.

C: cAMP production were measured under basal conditions or in presence of either 10 nM dDAVP, \pm 10 μ M OVTA, \pm 10 μ M desGly, 5 μ M calcitonin, 5 μ M hPTH, 0.1 μ M Iso, \pm 10 μ M Propra, 0.5 μ M PGE₂, or 5 μ M 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 K_a=2.8 nM (arrow), and Hill coefficient n_H=1.7.

Figure 2. ATP-sensitive phosphoinositidase C (A, B), and Ca²⁺-dependent pathway (C, D), in EC5v (A, C) and KC3AC1 (B, D) cells.

A, B: Results are means \pm SE of 5 independent determinations (See Supplemental Table 4).

C, D: Intracellular free Ca²⁺ mobilization were monitored with Fura-2 on cell suspension after the addition (arrows) of either 10 μ M poVT, 10 μ M AVP, or 1 mM ATP.

Figure 3. Calculated basolateral to apical transepithelial K⁺ flux with ⁸⁶Rb determination in basal condition, and in presence of FSK (5 μ M), dDAVP (0.1 μ M), Iso (1 μ M) and ATP (1 mM). Values are means \pm SE of three independent determinations, the experiment has been repeated twice for each tested drug, and 4 times for ATP in EC5v.

8. Supplemental data

Supplemental Table 1. Composition of the different media.

Epithelial culture medium

DMEM/Ham's F12 medium (1:1); 2 mM glutamine; 50 nM dexamethasone (Sigma, St Louis, MO, USA); 50 nM sodium selenite (Sigma); 5 µg/ml transferrin; 5 µg/ml insulin (Sigma); 10 ng/ml EGF (Tebu, Le Perray en Yvelines, France); 2 nM T3 (Sigma); 100 U/ml penicillin/streptomycin; 20 mM HEPES; pH 7.4; dextran charcoal-treated serum: 2% for EC5v cells, 5% for KC3AC1 cells.

cAMP incubation medium

20 mM HEPES-NaOH, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.33 mM Na₂HPO₄, 0.44 mM Na₂HPO₄, 4 mM NaHCO₃, 1 mM MgCl₂, 1mM CaCl₂, 5 mM glucose, 3 mM lactic acid, 10 mM sodium acetate, 0.1 % Bovine Serum Albumin

PLC incubation medium

35 mM HEPES-NaOH, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.8 mM MgSO₄, 0.33 mM Na₂HPO₄, 0.44 mM Na₂HPO₄, 4 mM NaHCO₃, 1 mM MgCl₂, 1mM CaCl₂, 5 mM glucose, 3 mM lactic acid, 10 mM sodium acetate, 10 mM LiCl, 0.1 % bacitracin; 0.1 % Bovine Serum Albumin

Supplemental Table 2. Primer sequences of genes analyzed in RT-PCR.

Gene	Primers sense / <i>antisense</i>	Amplicon cDNA (bp)	Accession numbers
V1a	CCTACATGCTGGTGGTGATG <i>TCTTCACTGTGCGGATCTTG</i>	474	NM 016847
V1b	ACAGCTTGCCTATGGCCTAT <i>TGGTGAAAGCCACATTGGTA</i>	310	NM 011924
V2	GTCTCCTCGGAGTTGCGTAG <i>TGGGTGTGCGAATCTTTGTA</i>	582	NM 019404
Oxtr	CACCTACCTGCTGTTGCTGA <i>TGTAGATCCATGGGTTGCAG</i>	668	XM 144956
P2Y1	CTGGGACTCGGAAAAACAA <i>AAGTGGCATAAACCCCTGTCTG</i>	342	NM 008772
P2Y2	CTTCCTGTTTCCTGCCTCAG <i>TGCTGCAGTAGAGGTTGGTG</i>	484	NM 008773
P2Y4	ACTAGGTCCCAGCCCAAGTT <i>GCACCATGATTGTGGAAGCTG</i>	575	NM 020621
P2Y6	CGAGCATAGGAAAGGCTGAC <i>GGTAGCGCTGGAAGCTAATG</i>	404	XM 133678
P2Y12	GCTGCCTTGCTGAAGTCTCT <i>ATGAAGGCCAGATGACAAC</i>	628	NM 027571

The abbreviations of the receptor genes, their GenBank or NCBI accession number and 5' to 3'-nucleotide sequences of the sense and antisense primers are presented.

Supplemental Table 3. Western blot antibodies

		Species	Dilution	References	
Primary antibodies	Anti-V2R	Rabbit	1:200	Sc-30027	Santa Cruz
	Anti- α -tubuline	Mouse	1:5000	T6199	Sigma
Secondary antibodies	Anti-rabbit	Goat	1:15 000	680 Dylight™	LI-COR Biosciences
	Anti-mouse	Goat	1:15 000	800 Dylight™	

Total protein extracts were prepared from EC5v or KC3AC1 cells cultured on collagen I-coated Petri dishes or filters. Briefly, cells were homogenized in lysis buffer, as described previously [25]. Twenty micrograms of protein were separated by electrophoresis on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (ScienceTec, Courtaboeuf, France). Thereafter, immunoblots were incubated for 1 h at room temperature in 5% fat free milk-Tris buffer saline – 0.1% Tween 20 (TBS-T) before an overnight incubation at 4°C with rabbit anti-V2R antibody (1:200, H80, sc-30027, Santa Cruz, Heidelberg, Germany) and mouse anti- α -tubulin antibody (1:5000, Sigma, St Louis, MO, USA). After extensive washes, membranes were incubated simultaneously with goat anti-rabbit IgG Dylight™ 680 antibody (1:15000) and goat anti-mouse IgG Dylight™ 800 antibody (1:15000) (Thermo, Fischer Scientific, Illkirch, France) for 1 h at room temperature. After extensive washes, membranes were scanned at 700 nm and 800 nm, respectively using the Odyssey infrared imaging system (LI-COR Biosciences, Bad Homburg, Germany). Images were processed with the Image Studio Software (LI-COR Biosciences). Amounts of V2R on the blot were normalized to the amount of α -tubulin.

Supplemental Table 4. Hormonal sensitivity of phosphoinositidase C in EC5v and KC3AC1 cell lines expressed as [³H]InsPs production in % of the total incorporated radioactivity.

	EC5v	KC3AC1
Basal	0.94 ± 0.07	0.64 ± 0.08
Angiotensin II (10 µM)	0.99 ± 0.02	0.66 ± 0.05
Bradykinine (10 µM)	1.00 ± 0.07	0.84 ± 0.03
Phenylephrine (10 µM)	1.03 ± 0.04	0.75 ± 0.06
AVP (10 µM)	0.99 ± 0.07	0.68 ± 0.08
[Phe ² ,Orn ⁸]VT (10 µM)	0.96 ± 0.03	0.64 ± 0.06
Carbachol (1mM)	1.04 ± 0.04	0.68 ± 0.07
ATP (1 mM)	4.65 ± 0.24*	7.35 ± 0.17*
UTP (1 mM)	4.69 ± 0.17*	7.17 ± 0.22*

Data are the mean values ± SE obtained from 5 independent determinations performed during the course of the same experiment. [³H] inositol loaded cells were incubated for 30 min at 37°C with 10 mM LiCl in absence or presence of agonist before [³H]InsPs extraction. *: Difference between nucleotide-induced enzyme stimulation and corresponding basal activities are highly significant (P<0.001, Student's t-test).

Supplemental Table 5. Kinetic parameters for nucleotide-induced phosphoinositidase C activation in EC5v and KC3AC1 cell lines.

	pKa	95% confidence interval	Hill coefficient	Max/ATP max
EC5v				
ATP	6.16	6.07 - 6.24	0.9	1.0
ATP γ S	6.06	5.74 - 6.38	0.9	1.2
UTP	5.55	5.24 - 5.86	0.6	0.7
(β,γ)-Me-ATP	4.56	4.09 - 5.02	0.9	1.0
ADP	4.10	3.89 - 4.32	1.1	1.0
2-MeS-ATP	3.65	2.91 - 4.38	0.5	0.5
Bz-ATP	3.26	2.51 - 4.01	0.6	0.6
(α,β)-Me-ATP	2.74	2.65 - 2.84	1.5	0.9
ADP- β -S	<2	-	-	0.2
UDP / UMP / AMP	<2	-	-	0
Adenosine / Uridine / cAMP	<<2	-	-	0
KC3AC1				
ATP	6.25	6.07 - 6.43	1.1	1.0
ATP γ S	6.22	6.12 - 6.32	1.2	1.5
UTP	6.03	5.85 - 6.21	1.1	0.9
Bz-ATP	4.54	4.47 - 4.61	1.6	1.1
2-MeS-ATP	4.34	4.18 - 4.50	0.9	1.0
ADP	4.13	3.96 - 4.30	1.4	0.3
(β,γ)-Me-ATP	3.52	3.39 - 3.65	1.1	1.1
(α,β)-Me-ATP	3.09	2.79 - 3.39	1.1	0.5
ADP- β -S	<3	-	-	0.1
UDP / UMP / AMP	<2	-	-	0
Adenosine / Uridine / cAMP	<<2	-	-	0

Apparent activation constants are given as $pK_a = -\log K_a$ in which K_a values are expressed as molar concentration for each tested analogue. The 95% confidence interval variation range of pK_a value was calculated by computerized analysis of the corresponding dose-response curves (see Materials and Methods). The lowest and highest r^2 value of the fits for calculation of 95% confidence intervals are 0.93 and 0.96 for (α,β)-Me-ATP in EC5v and KC3AC1 cells, respectively, 0.99 for ATP in EC5v cells, and 0.99 for ATP γ S in KC3AC1 cells.

In EC5v cells, computations give the following rank order for stereospecificity: ATP = ATP γ S > UTP > (β,γ)-Me-ATP = ADP > 2 MeS-ATP = Bz ATP > (α,β)-Me-ATP > ADP β S = UDP = UMP = AMP > adenosine = uridine = cAMP.

In KC3AC1 cells, computations give the following rank order for stereospecificity: ATP = ATP γ S = UTP > Bz ATP = 2 MeS-ATP = ADP > (β,γ)-Me-ATP = (α,β)-Me-ATP > ADP β S > UDP = UMP = AMP >> adenosine = uridine = cAMP.

Supplemental Figure 1. Amplification of vasopressin and P2Y receptors from EC5v and KC3AC1 cells, and from mice whole kidney extracts, used as positive control. PCR was performed in the absence (NTC) or presence of RNA extracts prepared either without (-) or in presence (+) of reverse transcriptase during the reverse transcription step. The expected sizes were 474, 310, 582, 668, 342, 484, 575, 404 and 628 bp, for the V1a, V1b, V2, OXY, P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12 receptors, respectively.

Supplemental Figure 2. Western blot of EC5v and KC3AC1 cells cultured on filters or Petri dishes. For electrophoresis, 12% polyacrylamide gels were performed, and blots were probed with NH₂-terminal anti-V2R and α -tubulin antibodies. A 60 kDa protein band was detected in both cell lines for V2R.

Supplemental Figure 3. Lack of additivity in adenylate cyclase response upon vasopressin, isoproterenol, and PGE₂ in KC3AC1 cells. Data obtained in the same experiment were summarized and results are means \pm SE of 5 independent determinations. KC3AC1 cells were incubated with 1 mM IBMX in the absence or presence of 1 μ M dDAVP, 10 μ M isoproterenol, 0.5 μ M PGE₂, 1 μ M dDAVP + 10 μ M isoproterenol, 1 μ M dDAVP + 0.5 μ M PGE₂, 10 μ M isoproterenol + 0.5 μ M PGE₂, or 5 μ M forskolin before cAMP extraction. All activities measured in presence of agonists were highly different from basal cAMP production ($P < 0.001$) whereas cAMP production determined in presence of dDAVP + isoproterenol or dDAVP + PGE₂ did not differ from activity measured in presence of dDAVP alone, and cAMP production observed with isoproterenol + PGE₂ was not different from cAMP generation assayed in either isoproterenol alone or PGE₂ alone (Student's t-test).