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ATP activates P2X receptors to mediate gap junctional coupling in the cochlea

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Abstract

ATP is an important extracellular signaling molecule and can activate both ionotropic (P2X) and metabotropic purinergic (P2Y) receptors to influence cellular function in many aspects. Gap junction is an intercellular channel and plays a critical role in hearing. Here, we report that stimulation of ATP reduced gap junctional coupling between cochlear supporting cells. This uncoupling effect could be evoked by nanomolar physiological levels of ATP. A P2X receptor agonist benzoylbenzoyl-ATP (BzATP) but not a P2Y receptor agonist UTP stimulated this uncoupling effect. Application of P2X receptor antagonists pyridoxalphosphate-6-azophenyl-2′, 4′-disulfonic acid (PPADS, 50 μM) or oxidized ATP (oATP, 0.1 mM) eliminated this uncoupling effect. We further found that ATP activated P2X receptors in the cochlear supporting cells allowing Ca^{++} influxing, thereby increasing intracellular Ca^{++} concentration to mediate gap junctions. These data suggest that ATP can mediate cochlear gap junctions at the physiological level by the activation of P2X receptors rather than P2Y receptors. This P2X receptor-mediated purinergic control on the cochlear gap junctions may play an important role in the regulation of K^+-recycling for ionic homeostasis in the cochlea and the reduction of hearing sensitivity under noise stress for protection.

Keywords

ATP; gap junction; purinergic receptor; potassium; inner ear

Introduction

Gap junctions in the cochlea play a critical role in hearing. Dysfunction of inner ear gap junctions can induce a high incidence of hearing loss [1]. Gap junctions in the cochlea only exist in supporting cells [2–5]. Hair cells have neither gap junctions nor connexin expressions [2, 5–8]. The hypothesized function of gap junctions in the inner ear includes K^+-recycling [2–3, 7, 9–10], intercellular signaling [11–13], nutrient supplies to hair cells [14–15], and the gain control on outer hair cell (OHC) electromotility to regulate hearing sensitivity [8, 16–17].

ATP is an important extracellular signaling molecule and activates purinergic (P2) receptors to influence cellular function [18–19]. P2 receptors have ATP-gated ionotropic (P2X) and G protein-coupled metabotropic (P2Y) subgroups. Both P2X and P2Y receptors are

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extensively expressed in the cochlear hair cells and supporting cells [20]. ATP physiologically exists in the cochlear endolymph and perilymph [21] via gap junction hemichannel release [16], and plays important roles in many aspects, including elevation of the intracellular Ca^{++} concentration in hair cells to modify sound transduction and neurotransmission [22–25], reduction of the endocochlear potential (EP) to mediate hearing sensitivity [26–27], synchronization of auditory nerve activity in the cochlear development [28–29], and the propagation of Ca^{++} wave in the organ of Corti [30]. We previously demonstrated that ATP can mediate OHC electromotility to regulate active cochlear amplification [16–17] and K^+-sinking in the cochlear supporting cells for K^+-recycling [10] by the activation of P2X receptors.

High concentration of ATP (0.1 mM) has also been reported to reduce the current-coupling ratio between cochlear Hensen cells [31]. However, the detailed mechanism underlying this effect remains undetermined. Also, it remains unclear whether the reduction of the current ratio resulted from gap junction uncoupling or ATP-evoked inward current, which can also reduce the current ratio. In this study, we used input capacitance (C_{in}) measurement to examine the effect of ATP on the cochlear gap junction and the underlying mechanism. We found that ATP can mediate cochlear gap junctions even at the physiological level. We also found that ATP mediates gap junctions in the cochlea through the activation of P2X receptors rather than P2Y receptors.

Materials and Methods

Animal preparation and isolation of cochlear supporting cells

The animal preparation and cochlear cell isolation have been reported elsewhere [10, 32]. Adult guinea pigs (250–400 g) were used. Guinea pigs were decapitated after intraperitoneal injection of a lethal dose of sodium pentobarbital (200 mg/kg). The cochlea was isolated after decapitation and dissected in a standard extracellular solution (130 NaCl, 5.37 KCl, 1.47 $MgCl_2$, 2 $CaCl_2$, 25 Dextrose, and 10 HEPES in mM; 300 mOsm and pH 7.2). After the removal of the embedded bone, the sensory epithelium was micro-dissected by a sharpened needle. The isolated sensory epithelium was dissociated by enzyme with trypsin (1 mg/ml) for 3–5 min. The dissociated cells were then transferred to a recording dish for recording. All experimental procedures were conducted at room temperature (23 °C) in accordance with protocols approved by University of Kentucky's Animal Care & Use Committee.

Patch-clamp recording and input capacitance measurement

Cochlear supporting cells can be distinctly identified and selected under microscope (Fig. 1A). Classical whole-cell recording was performed using Axopatch 200B (Molecular Devices, CA) [8, 10, 17]. Patch pipettes were filled with an intracellular solution (140 KCl, 5 EGTA, 2 $MgCl_2$, and 10 HEPES in mM, pH 7.2) with initial resistance of 2.5–3.5 MΩ in bath solution. Data collection was performed with jClamp (SciSoft, CT). The signal was filtered by a 4-pole low-pass Bessel filter with a cut-off frequency of 2 kHz and digitized utilizing a Digidata 1322A (Molecular Devices, CA).

Gap junctional coupling between cells was monitored by input capacitance (C_{in}) [32–33]. C_{in} was continually recorded online at 1–3 Hz from the transient charge induced by small (−10 mV) test pulses with duration of 18X the time constant at the holding potential. The transient charge was calculated from the integration of capacitance current with time [32]. Membrane potential (V_m) was corrected for pipette series resistance (R_s). Data analysis was performed with jClamp and SigmaPlot (SPSS Inc. Chicago, IL).

Ca^{++} fluorescence imaging and measurement

The cochlear supporting cells were incubated in normal extracellular solution with 5 μM Fluo-4 AM (F-14217; Molecular Probes) for 30 min at room temperature. After washout and de-esterification for 30 min, the intensity of the Fluo-4 fluorescence emission was continuously recorded online by a photometer (Photon Tech. Int., Birmingham, NJ) under a Nikon microscope [13].

Chemicals, chemical perfusion, and extracellular Ca^{++} removing

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA). ATP and other chemicals were applied by a Y-tube or a bath perfusion system [10, 17]. To remove extracellular Ca^{++}, a Ca^{++}-free extracellular solution with 10 mM EGTA (130 NaCl, 5.37 KCl, 1.47 $MgCl_2$, 10 EGTA, and 10 HEPS in mM, 300 mOsm and pH 7.2) was applied with a Y-tube [17].

Results

ATP uncouples gap junctions between cochlear supporting cells

ATP uncoupled gap junctions between cochlear supporting cells and reduced C_{in} (Fig. 1). Fig. 1C shows that the C_{in} of 3 Hensen cells (HCs) was reduced to 1/3 from 75 pF to 25 pF of the single cell level after perfusion of 36 μM ATP. The average of C_{in} of single, double, and triple HCs was 20.3±0.9, 38.4±1.1, and 54.9±4.9 pF, respectively, with a 20 pF step increase (Fig. 1E). After uncoupled, the C_{in} of 2–3 HCs was reduced to the single cell level (Fig. 1E). ATP also uncoupled gap junctions between other types of cochlear supporting cells. The average of C_{in} of a pair of Deiters cells (DCs), pillar cells (PCs), and Claudius cells (CCs) was 64.3±9.1, 49.3±3.5, and 23.8±2.8 pF, respectively (Fig. 1F). After stimulation with ATP, the C_{in} was reduced to 32.7±1.1 (n=27), 24.8±1.2 (n=19), and 12.3±0.8 pF (n=36), respectively. The reduction was 50%, corresponding well to 2-coupled cells uncoupled.

ATP in the cochlear endolymph and perilymph is at the submicromolar level [21]. Fig. 1D shows that stimulation of nanomolar ATP uncoupled gap junctions between HCs. The C_{in} of a pair of HCs was reduced from 43 pF to a half value of 21 pF under the application of 36 nM ATP.

However, after cells were completely uncoupled, re-application of ATP (indicated by the 2nd horizontal bar in Fig. 1D) did not further reduce C_{in}. ATP also did not reduce C_{in} in single cells even the ATP-evoked inward current is clearly visible (Fig. S1). This indicates that C_{in} measurement is independent of current measurement and that C_{in} reduction resulted from gap junctional uncoupling.

P2X agonists rather than P2Y agonists uncouple gap junctions between supporting cells

Both P2X and P2Y receptors are extensively expressed in the cochlear supporting cells. Immunofluorescent staining shows that the cochlear supporting cells had intense labeling for P2X2, P2X7, and P2Y4 receptors (Fig. S2 and S3). Benzoylbenzoyl-ATP (BzATP) is an agonist to P2X7 receptors and can activate other P2X receptors as well. However, it can not activate P2Y receptors at concentrations less than 7 μM [18]. Fig. 2A&B shows that application of 3 μM BzATP uncoupled gap junctions between cochlear supporting cells. The uncoupling was also reversible (Fig. 2B). After washout of ATP, the gap junctional coupling was restored and the C_{in} returned to the multi-cell level.

However, UTP which is a P2Y agonist and cannot activate P2X receptors [18] had no effect on gap junctions between cochlear supporting cells (Fig. 2C&D). C_{in} was stable and not

reduced during UTP treatment. Fig. 2D shows that the C_{in} of a pair of HCs was not reduced even a high concentration of UTP (1 mM) was used (Fig. 2D). However, subsequent application of 36 μM ATP (indicated by the 2nd horizontal bar in Fig. 2D) reduced the C_{in} to a half value, indicating that these HCs were well-coupled.

P2X antagonists block ATP uncoupling effect

The ATP-evoked uncoupling effect on gap junctions between cochlear supporting cells can be blocked by P2X receptor antagonists. Fig. 3A shows that ATP uncoupled a pair of HCs and the C_{in} was reduced to a half value. Subsequent application of a P2X blocker, pyridoxalphosphate-6-azophenyl-2′, 4′-disulfonic acid (PPADS, 50 μM), blocked this ATP-evoked uncoupling effect and ATP-evoked inward current (Fig. 3B). Oxidized ATP (oATP) is an irreversible P2X7 receptor antagonist and also can block other P2X receptors [18]. Fig. 3C shows that pre-incubation of 0.1 mM of oATP for 45 min abolished the ATP-evoked uncoupling effect. The C_{in} was not reduced for application of 36 μM ATP and also no inward current was visible (Fig. 3D). However, the recorded C_{in} was ~74 pF, indicating that there was a good electrical-coupling between these DCs.

Extracellular Ca^{++} is required for ATP uncoupling effect

One important characteristic of P2X receptors is permeability to Ca^{++} [18–19], which can close inner ear gap junctional channels [34]. Fig. 4A shows the ATP elevated intracellular Ca^{++} concentration in a DC. The elevation is also reversible and repeatable. Re-application of ATP elevated the Ca^{++} concentration again. P2X receptor antagonists also eliminated this ATP-evoked Ca^{++} elevation in the cochlear supporting cells. Pre-treatment with 50 μM PPADS completely abolished this ATP-evoked Ca^{++} elevation (Fig. 4B).

P2X receptors but not P2Y receptors have ionic permeability allowing Ca^{++} ions influxing to elevate intracellular Ca^{++} concentration [18–19]. Fig. 4C&D show that the removal of extracellular Ca^{++} eliminated ATP-evoked uncoupling effect. After application of a Ca^{++}-free extracellular solution with 10 mM EGTA, the C_{in} was not reduced and remained stable at ATP application. Fig. 4D shows that after ending of perfusion of the Ca^{++}-free extracellular solution with EGTA, re-application of ATP (indicated by the 2nd horizontal bar in Fig. 4D) uncoupled cells and reduced C_{in} from 30 pF to 10 pF, corresponding well to 3-coupled CCs uncoupled.

Discussion

In this study, we found that ATP mediated gap junctions between cochlear supporting cells even at the nanomolar physiological level of ATP (Figs. 1–3). P2X agonists but not P2Y agonists stimulated the uncoupling effect (Fig. 2). P2X antagonists also eliminated this uncoupling effect (Fig. 3). These data indicate that ATP activates P2X receptors rather than P2Y receptors to mediate gap junctions between cochlear supporting cells under the physiological conditions.

P2X receptors but not P2Y receptors are permeable to K^+ and Ca^{++} ions [18–19]. We found that ATP elevated intracellular Ca^{++} concentration in the cochlear supporting cells (Fig. 4A). PPADS could block this ATP-evoked Ca^{++} elevation (Fig. 4B). We also found that removal of extracellular Ca^{++} eliminated the ATP-evoked uncoupling effect (Fig. 4C&D). Combined with the fact that the P2X agonist rather than the P2Y agonist stimulated the uncoupling effect (Fig. 2), these data strongly suggest that ATP activate P2X receptors rather than P2Y receptors allowing extracellular Ca^{++} influx into the cochlear supporting cells to increase the intracellular Ca^{++} concentration, thereby blocking gap junction channels [34–35].

This ATP-mediated uncoupling effect can control K^+-recycling and help K^+-sinking in the cochlea [10]. The supporting cells sink K^+ ions, which hair cells release during the mechano-electrical transduction process, then transfers them back to the endolymph via gap junctions [3, 9]. ATP activates P2X receptors sinking K^+ [10] and also allowing Ca^{++} influx, which elevates intracellular Ca^{++} concentration (Fig. 4) and consequently blocks gap junctions (Figs. 1–2). This can protect K^+ ions flowing backward from the neighboring cells. After cessation of acoustic stimulation, the ATP release is decreased. Gap junctions between supporting cells return to normal. Absorbed K^+ ions then flow through gap junction channels to neighboring cells along the chemical gradient.

This P2X receptor-mediated control may also play an important role in the protection of hearing sensitivity from noise stress. It has been reported that noise can increase ATP concentration in the endolymph, which can activate P2X receptors in tissues lining the endolymphatic compartment to reduce the EP and the resistance of the endolymphatic compartment [26–27]. Uncoupling of gap junctions between the cochlear supporting cells can further enhance the reduction of this resistance. Moreover, we previously reported that alternation of gap junctions between cochlear supporting cells can mediate OHC electromotility to reduce the active cochlear amplification [8]. Thus, this ATP-mediated uncoupling effect may not only mediate K^+-recycling in supporting cells but also eventually influence active amplification in the cochlea.

P2X receptors are extensively expressed in the inner ear (Fig. S2) [16, 25]. In this study, we found that ATP can activate P2X receptors to mediate gap junctions between cochlear supporting cells at the physiological levels (Figs. 1–3). These new data further indicate that ATP-gated P2X receptors may play an important role in the cochlear ionic homeostasis and hearing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. ATP reduces inner ear gap junctional coupling
2. This reduction can occur at the nanomolar physiological level of ATP
3. This uncoupling effect is dependent on P2X rather than P2Y receptor activation
4. ATP activates P2X receptors allowing Ca influx to mediate gap junctions
5. This purinergic control may play an important role in K-recycling and hearing

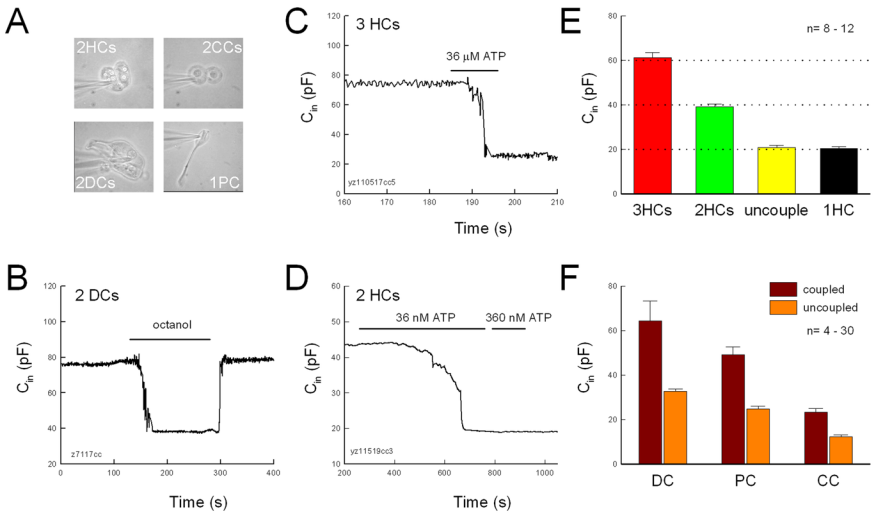

Fig. 1.

Uncoupling effect of ATP on gap junctions between cochlear supporting cells. A: Captured images of patch clamp recording in different types of cochlear supporting cells. HC: Hensen cell; DC: Deiters cell; PC: pillar cell; CC: Claudius cell. B: Reduction of C_{in} by perfusion of a well-known gap junction uncoupling agent octanol. The C_{in} of a couple of DCs was reduced to a half value. C: Uncoupling of 3 HCs by ATP stimulation. A horizontal bar represents ATP perfusion. C_{in} is reduced to the single cell level after perfusion of ATP. D: The uncoupling response evoked by nanomolar ATP. Note that re-application of ATP after cell uncoupled did not further reduce C_{in}. E: Step-reduction of C_{in} for HCs uncoupling evoked by ATP. F: The reduction of C_{in} of a pair of DCs, PCs, and CCs by ATP stimulation. After uncoupled, C_{in} is reduced by 50%.

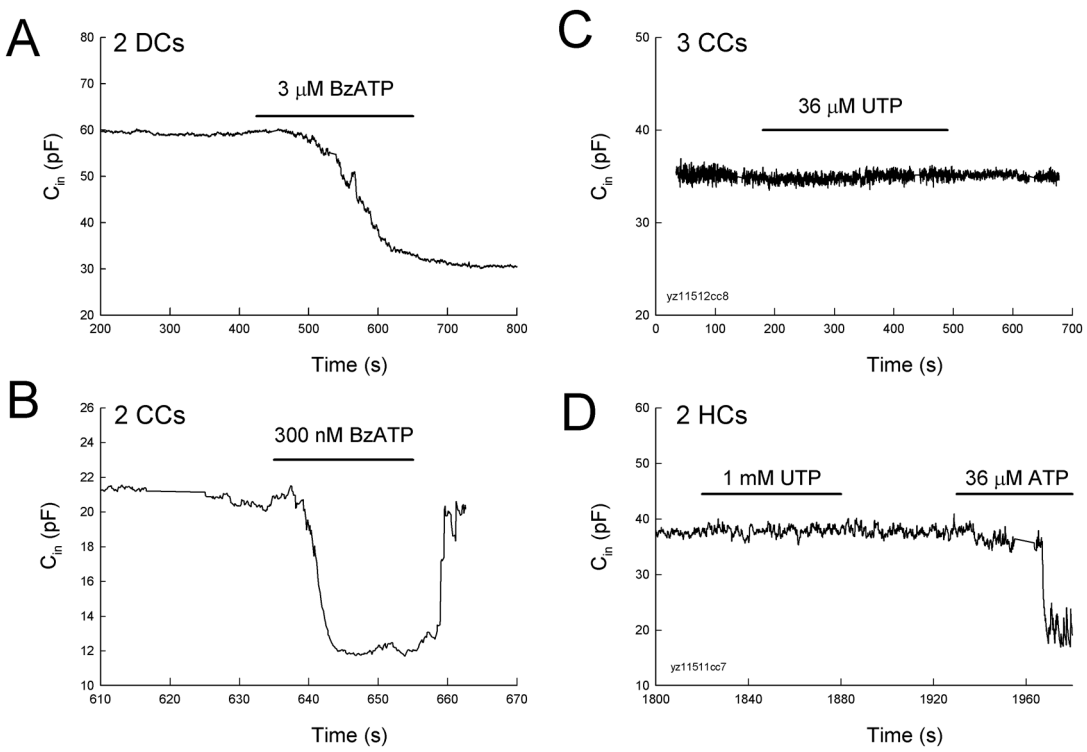

Fig. 2.

A P2X agonist BzATP but not a P2Y agonist UTP uncouples gap junctions between cochlear supporting cells. A–B: Uncoupling effect of BzATP on gap junctions between cochlear supporting cells. C–D: Ineffectiveness of UTP on gap junctions between cochlear supporting cells. C_{in} remains stable and unchanged for UTP stimulation. The 2nd horizontal bar in panel D represents the subsequent perfusion of 36 μM ATP to uncouple cells.

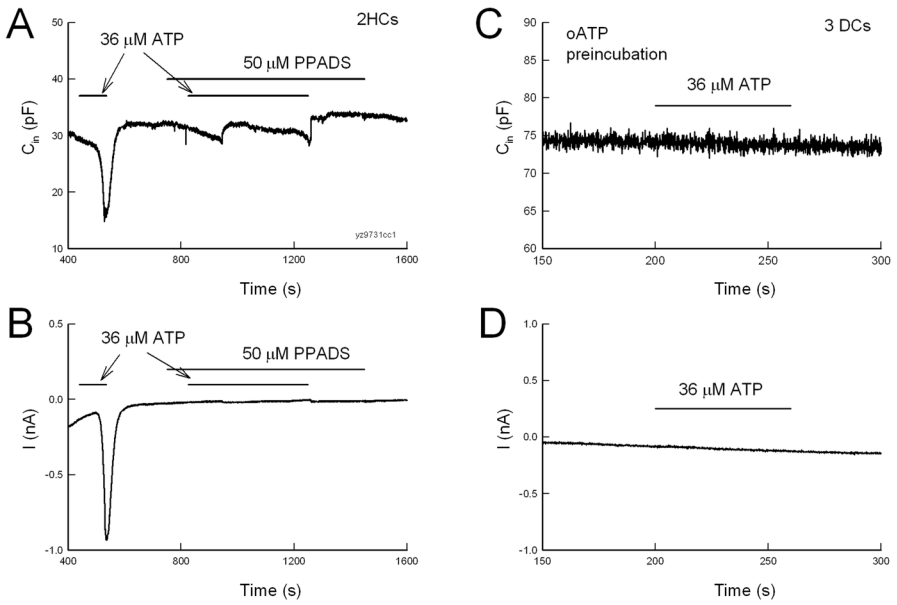

Fig. 3.

Blockage of ATP-evoked uncoupling effect by P2X antagonists. A&B: Elimination of the ATP-evoked uncoupling and inward current by PPADS. Horizontal bars represent perfusions of ATP (36 μM) and PPADS (50 μM). C–D: Pre-incubation of oATP (0.1 mM) blocked ATP-evoked uncoupling effect in 3 DCs. C_{in} is 75 pF, indicating that these cells are still well-coupled by gap junctions.

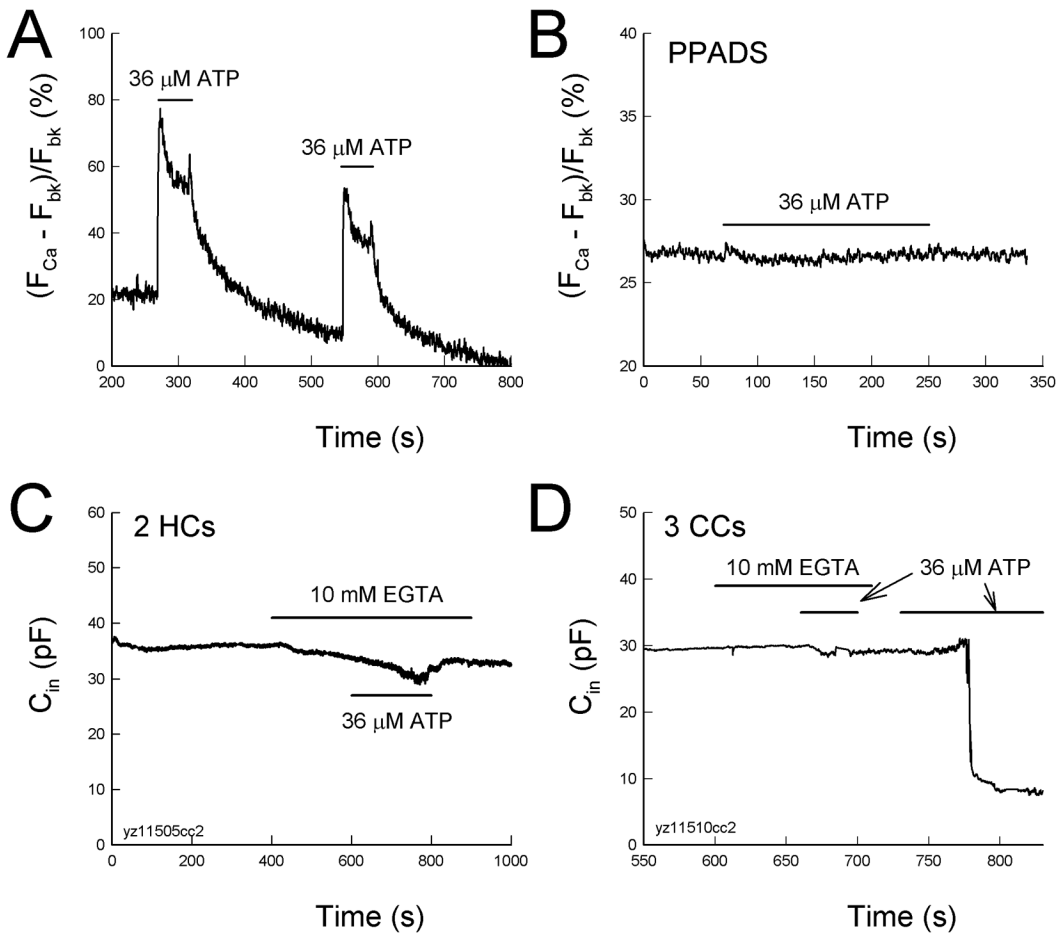

Fig. 4.

Extracellular Ca^{++} is required for ATP-evoked uncoupling effect on gap junctions between cochlear supporting cells. A: ATP-evoked intracellular Ca^{++} rising in a DC. B: Blockage of ATP-evoked intracellular Ca^{++} elevation in 2CCs by PPADS. The cells were incubated by PPADS (50 μM). A horizontal bar represents the perfusion of 36 μM ATP. C–D: Removal of extracellular Ca^{++} eliminates the ATP-evoked uncoupling effect on gap junctions between cochlear supporting cells. Upper-horizontal bar represents perfusion of a Ca^{++}-free extracellular solution with 10 mM EGTA to remove extracellular Ca^{++}. Lower-horizontal bars represent ATP stimulations. Cells were held at −80 mV.