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Purinergic signalling in rat GFSHR-17 granulosa cells: an *in vitro* model of granulosa cells in maturing follicles

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Abstract Purinergic signalling in rat GFSHR-17 granulosa cells was characterised by Ca^{2+}-imaging and perforated patch-clamp. We observed a resting intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) of 100 nM and a membrane potential of −40 mV. This was consistent with high K^+– and Cl^- permeability and a high intracellular Cl^- concentration of 40 mM. Application of ATP for 5–15 s every 3 min induced repeated $[Ca^{2+}]_i$ increases and a 30 mV hyperpolarization. The phospholipase C inhibitor U73122 or the IP_3-receptor antagonist 2-aminoethoethyl diphenyl borate suppressed ATP responses. Further biochemical and pharmacological experiments revealed that ATP responses were related to stimulation of $P2Y_2$ and $P2Y_4$ receptors and that the $[Ca^{2+}]_i$ increase was a prerequisite for hyperpolarization. Inhibitors of Ca^{2+}-activated channels or K^+ channels did not affect the ATP-evoked responses. Conversely, inhibitors of Cl^- channels hyperpolarized cells to −70 mV and suppressed further ATP-evoked hyperpolarization. We propose that $P2Y_2$ and $P2Y_4$ receptors in granulosa cells modulate Cl^- permeability by regulating Ca^{2+}-release.

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Introduction

Granulosa cells form a monolayer that surrounds oocytes in primary follicles. Upon external stimulation, granulosa cells begin to proliferate simultaneously with an increase in oocyte volume. Proliferating granulosa cells express FSH receptors as this hormone begins to be secreted by the pituitary gland. The proliferation of the granulosa cells and the volume increase in the oocyte correlate with the reorganization of the follicle. An antral cavity is formed, which contains a fluid consisting of water, ions and many other components such as peptides. The antral fluid is secreted by the granulosa cells via a mechanism that remains to be elucidated. The granulosa cells of the maturing follicles express receptors for pituitary hormones and other physiological ligands such as ATP. It has been shown that ATP, at physiologically relevant concentrations, stimulates an increase in the intracellular free Ca^{2+}-concentration ($[Ca^{2+}]_i$) (Tai et al. 2000) and that ATP modulates K^+ as well as Cl^- channels in cumulus cell-enclosed oocytes mainly composed of granulosa cells (Arellano et al. 2002). These findings demonstrate the expression of purinergic receptors by granulosa cells.

Two families of purinergic receptors are known. The P2X receptors ($P2X_{1–7}$), which are not sensitive to UTP, form a family of related ionotropic receptors (King and Townsend-Nicholson 2003). The binding of ATP to P2X receptors opens the channels and allows the flow of cations through the membrane, which can lead to increases in $[Ca^{2+}]_i$. The P2Y receptor family consists of eight subtypes ($P2Y_1$,

$P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11–14}$) and belongs to the class of seven-transmembrane G-protein-coupled receptors (7TM-GPCRs). These receptors share a common membrane topology and the ability to stimulate various G-proteins. Like other metabotropic 7TM-GPCRs, the P2Y purinergic receptors stimulate various intracellular signalling pathways (Abbracchio et al. 2006; King and Townsend-Nicholson 2003; von Kügelgen 2006). For instance, $P2Y_{12–14}$ receptors block the synthesis of cAMP by inhibiting adenylyl cyclase via the activation of G_i proteins. The $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors act via the activation of G_q proteins, stimulate phospholipase C (PLC), and can be distinguished by their different agonists and inhibitors (Abbracchio et al. 2006; von Kügelgen 2000, 2006). $P2Y_1$ is not sensitive to UTP (Abbracchio et al. 2006; von Kügelgen 2000, 2006). $P2Y_{11}$ is sensitive to UTP and inhibited by reactive blue or suramin (Abbracchio et al. 2006; King and Townsend-Nicholson 2008; von Kügelgen 2000, 2006; White et al. 2003). However, $P2Y_{11}$ receptor gene is absent in murine and rat genome (Abbracchio et al. 2006). In rat cells such as GFSHR-17 granulosa cells, $P2Y_2$ and $P2Y_4$ can be distinguished from other G_q coupled P2Y receptors by their sensitivity to UTP and insensitivity to UDP. Conversely, $P2Y_6$ can be stimulated by UDP and inhibited by reactive blue as well as by pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) (Abbracchio et al. 2006; Burnstock 2007a, b; von Kügelgen 2000, 2006). At low ATP concentration (<10 μM), the stimulation of $P2Y_2$ and $P2Y_4$ receptors can be antagonized by reactive blue (Wildman et al. 2003). $P2Y_2$ and $P2Y_4$ can be distinguished by the $P2Y_4$ sensitivity to Zn^{2+}. Applied together with ATP, Zn^{2+} blocked $P2Y_4$ but does not affect $P2Y_2$ (Wildman et al. 2003). The P2Y receptors that activate G_q stimulate PLC and thereby induce hydrolysis of the membrane phosphoinositol-4,5-bisphosphate (PIP_2) to yield inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). As an intracellular second messenger, IP_3 activates the IP_3 receptor, a ligand-gated Ca^{2+} channel expressed in the ER membrane that releases Ca^{2+} into the cytosol when activated. The increased $[Ca^{2+}]_i$ is involved in stimulating various signal transduction pathways and, along with DAG, activates protein kinase C (PKC), which in turn stimulates various cellular activities such as proliferation. Ca^{2+} can also activate or inhibit Ca^{2+}-sensitive ion channels and thus alter the membrane potential. These alterations can be registered as hyperpolarization or depolarization of the cell membrane.

We used the perforated patch-clamp technique coupled with imaging of $[Ca^{2+}]_i$ by the Fura 2/AM ratiometric method (Grynkiewicz et al. 1985) in rat GFSHR-17 granulosa cells expressing FSH receptors. These cell line represents an in vitro model for rat granulosa cells in maturing follicle (Keren et al. 1993). We observed a high permeability of the cells to K^+ and Cl^-, which could be involved in the secretion of antral fluid. We also found that Cl^- permeability is modulated by $P2Y_2$ and $P2Y_4$ receptors via regulation of Ca^{2+}-release from intracellular stores.

Materials and methods

Chemicals

If not otherwise stated, all chemicals and cell culture media were obtained from Sigma–Aldrich (Taufkirchen, Germany).

Cell culture

The rat GFSHR-17 granulosa cells (Keren et al. 1993) were seeded ($2–5\times10^5$ cells/ml) on cover slips in petri dishes containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% foetal calf serum, penicillin and streptomycin. Cells were cultivated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was renewed every 2–3 days. Cells were used for the experiments 2–5 days after plating. The GFSHR-17 cells were used up to a total of 25 passages.

Electrophysiological measurements

A cover slip with cells was transferred to a superfusion chamber containing 0.5 ml of a bath solution composed of (in mM) 121 NaCl, 5 KCl, 6 $NaHCO_3$, 5.5 glucose, 0.8 $MgCl_2$, 1.8 $CaCl_2$, and 25 HEPES. The pH was adjusted to 7.4 by addition of 10–15 mM NaOH. The chamber was mounted on a Zeiss inverted microscope (Oberkochen, Germany). Cells were washed with 10 ml (2 ml/min) of the bath solution and allowed to adapt to room temperature (20–24°C) for at least 30 min. Perforated patch-clamp configuration was established on single cells using a patch-clamp amplifier EPC 7 (List Medical, Darmstadt, Germany). A stock solution of 50 mg/ml amphothericin B in DMSO was diluted to 250 μg/ml in the pipette filling solution composed of (in mM) 140 KCl, 5 NaCl, 1 $MgCl_2$, 0.25 $CaCl_2$, 0.5 EGTA, 1 glucose, and 10 HEPES (pH 7.4). A stable perforated patch-clamp configuration was achieved within 3–5 min of the establishment of the cell-attached patch-clamp configuration. The membrane potential was registered in current-clamp mode. The data were filtered at 3 kHz and digitised at 10 kHz via an interface ITC 16 (Instrutech, Minnesota, USA). Data acquisition and off-line analyses were performed using the software Pulse Pulsefit (HEKA Electronics, Lamprecht, Germany), Excel (Microsoft, USA) and Origin (Microcal Software, Inc, Northampton, USA).

Measurement of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ concentration were performed as described previously (Grynkiewicz et al. 1985; Ngezahayo et al. 2003). Cells were loaded with Fura 2/AM (Calbiochem-Novabiochem, Schwalbach am Taunus, Germany) for 20–30 min at room temperature. The Fura 2/AM loaded cells were then transferred to the superfusion chamber mounted on an inverted microscope (see above). Cells were then washed with the bath solution (2 ml/min) for at least 5 min to remove external Fura 2/AM. The dye in the cells was excited at 340 nm and 380 nm using a monochromator polychrome II (T.I.L.L. Photonics GmbH, Planegg, Germany) equipped with a 75 W XBO xenon lamp. The fluorescent images and intensities at 510 nm were registered with a digital CCD camera (C4742-95, Hamamatsu Photonics K.K.; Japan) and used to calculate the fluorescence ratio (F_{340}/F_{380}). $[Ca^{2+}]_i$ was estimated from F_{340}/F_{380} ratio as described by Grynkiewicz et al. (1985) using the program Aquacosmos (Hamamatsu Photonics K.K.; Japan). Agonists or inhibitors were applied during electrophysiological as well as Ca^{2+}-imaging experiments using a Small Volume Perfusion System setup (Bioscience Tools, San Diego, USA).

Western blot

To isolate the proteins, cells were collected from culture dishes in ice cold phosphate buffered solution (PBS) containing (in mM): 137 NaCl, 2.7 KCl, 10 Na_2HPO_4, and 1.8 KH_2PO_4 (pH 7.4). After centrifugation at 500 *g* at 4°C for 5 min, the supernatant was discarded and the cells were diluted in a lysis buffer containing (in mM): 10 NaCl, 25 HEPES, 2 EDTA, and protease inhibitors (aprotinin and phenylmethylsulphonyl fluoride), (pH 7.5). Cells were then sonicated at 4°C for 10 min followed by a centrifugation step at 15,000 *g* at 4°C for 30 min. The supernatant was again discarded and the pellet was dissolved in 30–50 µl of a solubilization buffer containing (in mM): 200 NaCl, 50 HEPES, protease inhibitors (pH 7.5). An equal volume of a 2% Chaps solution was added to the solubilization buffer, and a centrifugation step was performed at 6,500 *g* at 4°C for 10 min. The protein concentration in the supernatant was estimated using the Bradford technique. For each experiment, samples containing 5–10 µg of protein were applied to the SDS polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred to a nitrocellulose membrane using 1.2 mA/cm^2 for 120 min. Staining the nitrocellulose membrane was performed by overnight incubation at 4°C with the corresponding primary anti-P2Y receptor antibodies (Alomone Labs Ltd., Jerusalem, Israel) diluted to 1:1000 ($P2Y_2$, 0.8 mg/ml) or 1:500 ($P2Y_4$, 0.3 mg/ml). The membrane was washed with TBST containing (in mM): 137 NaCl, 20 Tris-HCl, 0.1% Tween (pH 7.5) and then incubated for 1–2 h with goat-anti-rabbit IgG secondary antibodies conjugated with alkaline phosphatase and diluted to 1:500. Proteins were visualised using Sigma Fast BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) followed by a final washing step in H_2O. During all washing steps and the incubations with primary and secondary antibody, milk (3%) was used to neutralize non-specific binding.

Results

Stimulation of rat GFSHR-17 granulosa cells with ATP

Repeated pulse applications of ATP (10–50 µM) to rat GFSHR-17 granulosa cells every 3 min for 3–15 s stimulated a repetitive increase in $[Ca^{2+}]_i$ (Fig. 1a). This Ca^{2+} signal was characterized by an increase from a non-stimulated $[Ca^{2+}]_i$ of about 100 nM to a maximum concentration of approximately 200 nM within 5–10 s (Fig. 1b). The increase was followed by a decrease to the initial concentration within 30 s. The ATP-related stimulation of $[Ca^{2+}]_i$ increase could be repeated every 3 min by applying ATP for less than 15 s (Fig. 1a). Simultaneous Ca^{2+}-imaging and membrane potential measurement using the perforated patch-clamp technique revealed a resting membrane potential of approximately −40 mV. Application of ATP induced a 10 mV depolarization of the cells (Fig. 1c) followed by a hyperpolarization of approximately 30 mV (Fig. 1c, Table 1). The depolarization and hyperpolarization corresponded to inward and outward current (results not shown). The GFSHR-17 are strongly coupled via gap junctions (Ngezahayo et al. 2003) which renders difficult to adequately space-clamp in order to measure the currents through the single cell membrane. We therefore measured the membrane potential in the current clamp mode. The depolarization could also be induced by pressure (superfusion with control bath solution). This was not observed in all experiments, and was most likely related to pressure-dependent opening of gap junction hemichannels (Bintig et al. 2007).

Extending the duration of the presence of ATP or reducing the intervals between successive ATP applications compromised the ability to stimulate the cells, indicating a desensitization of the receptors (Fig. 1d). For a ATP presence longer than 1 min, the desensitization was characterised by a continuous reduction of the Ca^{2+} signal. The decline of the signal began even when ATP was still present. The removal of ATP was not followed by a spontaneous recovery of purinergic sensitivity. The recovery from the desensitization took a long time of 30–60 min (Fig. 1d).

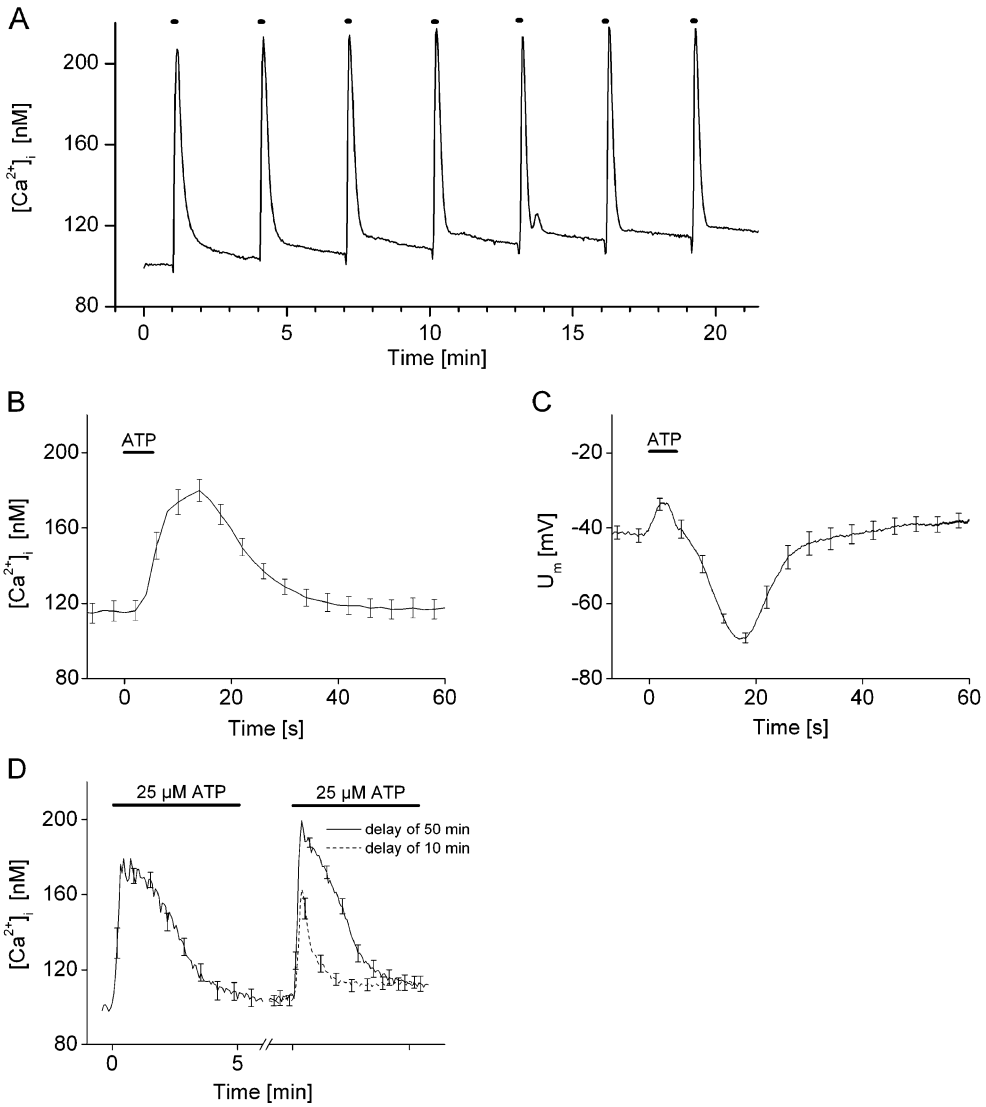

Fig. 1 Intracellular Ca^{2+} homeostasis in rat GFSHR-17 granulosa cells. **a** Repeated application of 25 µM ATP (*bars*) for 5–15 s every 3 min induced a repetitive Ca^{2+} signal. **b** Applying 25 µM ATP induced an increase in $[Ca^{2+}]_i$ and **c** a depolarization of approximately 10 mV followed by a hyperpolarization of approximately 30 mV. A detailed analysis showed that the depolarization was most likely unrelated to ATP. The results in **b** and **c** are averages, the *error bars* represent the SEM for n=25 experiments, respectively. The result in **a** is an average for n=42. For clarity, the errors are not indicated but are comparable to those shown in **b**. **d** Ca^{2+} signals evoked by consecutive ATP application for 5 min each. Depending on the delay of the second application, the amplitude and duration of the induced Ca^2 signal was reduced in comparison to the first one, indicating a desensitization of the receptors. To evoke a signal comparable to the first one, a delay of 45–60 min was requested

The purinergic receptors of rat GFSHR-17 granulosa cells

Two families of purinergic receptors are known. There are the ionotropic P2X receptors, which allow a Ca^{2+} influx from the extracellular space, and the metabotropic P2Y receptors, which induce the release of Ca^{2+} from intracellular stores. To analyse whether the ATP-evoked $[Ca^{2+}]_i$-increase was related to an influx of Ca^{2+} from the extracellular solution (i.e., P2X receptors) or to a Ca^{2+}-release from intracellular stores (i.e., P2Y receptors), ATP was applied in

Table 1 The values of the membrane potential predicted by the Goldmann–Hodgkin equation and measured values at various $[K^+]_o$ in non-stimulated cells and in ATP-stimulated cells (25 µM)

$[K^+]_o$ (mM)	Control		25 µM ATP		
	Predicted U_m (mV)	Measured U_m (mV)	Predicted U_m (mV) with assumption that pK^+ was increased (pK^+=10)	Predicted U_m (mV) with assumption that pCl^- was decreased (pCl^-=0.01)	Measured U_m (mV)
5	−43.8	**−40.6±0.2** (n = *25*)	−71.1	−71.1	**−67.5±2.1** (n = 25)
20	−36.9	**−34.8±0.4** (n = 8)	−45.8	−45.5	**−45.0±2.9** (n = 8)
35	−31.5	**−35.2±0.2** (n = 12)	−34.5	−33.0	**−31.6±2.4** (n = 12)
60	−24.5	**−24.2±0.1** (n = 15)	−22.0	−20.3	**−21.7±1.2** (n = 15)

For the calculation of U_m, $pK^+ = pCl^- = 1$, $pNa^+ = 0.025$, as well as intracellular concentrations for K^+ =140 mM, Na^+ =10 mM, Cl^- =40 mM, and external concentrations for K^+ =5–60 mM, Na^+ =140 mM and Cl^- =130 mM were assumed for non-stimulated cells. The results are average ± SEM

the presence of EGTA in the external bath solution. We found that the absence of Ca^{2+} in the bath solution did not alter the ATP-evoked increase in $[Ca^{2+}]_i$, indicating a Ca^{2+}-release from intracellular stores (Fig. 2a). This result suggests that the observed ATP-evoked response of rat GFSHR-17 granulosa cells was mainly due to P2Y receptors and not P2X receptors. Consistent with this conclusion, the application of P2X receptor agonists such as α, β-methyleneadenosine 5′-triphosphate lithium salt (α, β-meATP) did not induce any measurable changes in the $[Ca^{2+}]_i$ concentration of the cells (results not shown). Moreover, UTP which does not stimulate P2X receptors, was equally efficient in stimulating the rat GFSHR-17 cells (Figs. 1b, 2b).

In rat cells, $P2Y_2$, $P2Y_4$ and to a minor extend $P2Y_6$ can be stimulated by UTP (Abbracchio et al. 2006; von Kügelgen 2006). The $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors activate G_q proteins, which are linked to PLC, PIP_2 hydrolysis and increases in $[Ca^{2+}]_i$. We found that the Ca^{2+} signal could be completely suppressed by the PLC inhibitor U73122 or by the IP_3 receptor blocker 2-aminoethoethyl diphenyl borate (2-APB; Calbiochem-Novabiochem, Schwalbach am Taunus, Germany) (Fig. 2 c). These results

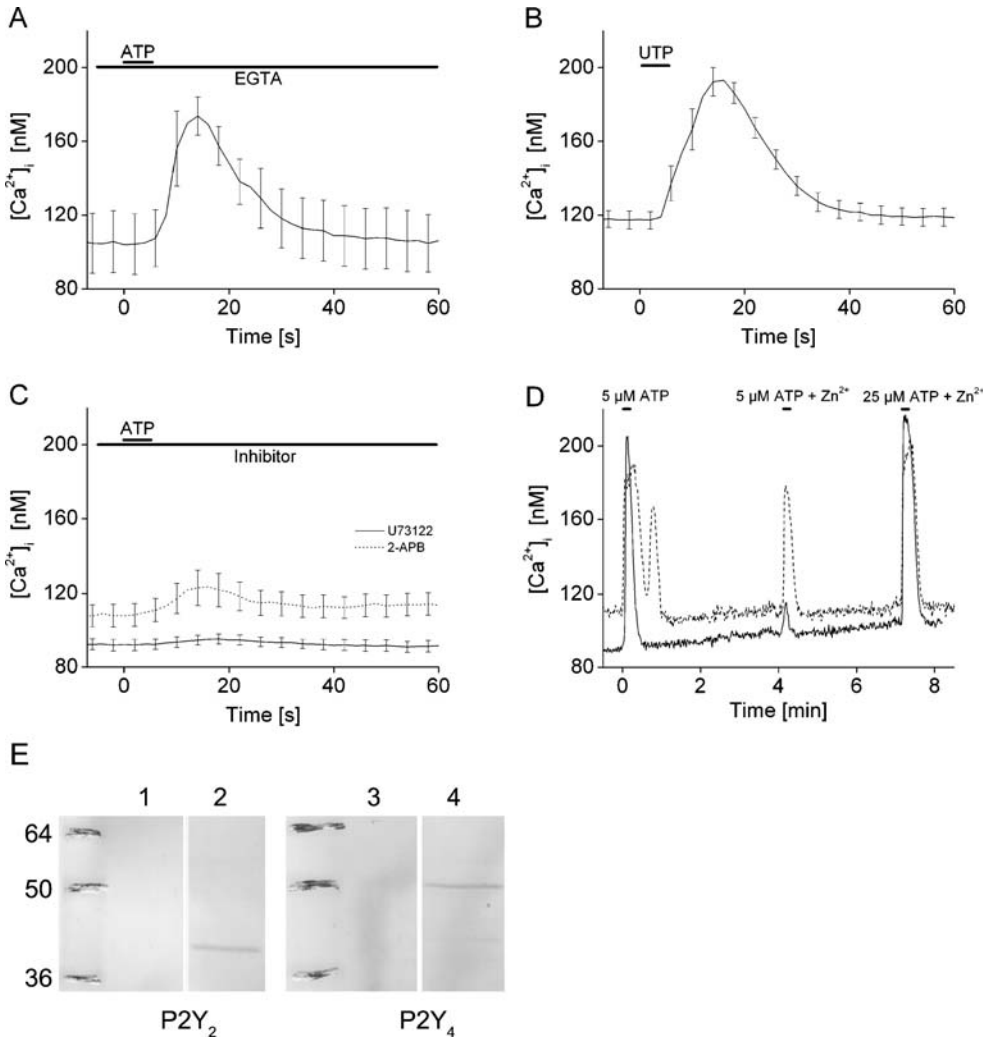

Fig. 2 Pharmacological analysis of the ATP-related Ca^{2+} response in rat GFSHR-17 granulosa cells. **a** EGTA in the extracellular solution did not alter the Ca^{2+} response to 25 µM ATP. **b** UTP (25 µM) was equally efficient in stimulating the Ca^{2+} response. **c** The presence of U73122 (10 µM), an inhibitor of PLC or 2-APB (100 µM), an IP_3 receptor antagonist, suppressed the Ca^{2+} response to ATP. The results are averages and the error bars represent the SEM for at least seven experiments for each treatment. **d** Stimulation of the rat GFSHR-17 granulosa cells by 5 µM ATP. Some cells could be stimulated while others could not. Within the responding population Zn^{2+} could suppress the ATP (5 µM) related stimulation. Zn^{2+} did not suppress the stimulation by 25 µM ATP which stimulated almost the whole cell population. The experiments are representative of 36 cells (note the oscillating Ca^{2+} signal elicited by 5 µM ATP. This was observed in some cells. Currently we do not understand how such oscillations are regulated). (E) $P2Y_2$ and $P2Y_4$ expression was analyzed by Western blot. The $P2Y_2$ and $P2Y_4$ antibodies stained bands at ~42 kDa and ~50 kDa, respectively (lanes 2 and 4). The bands were absent when the primary antibodies were pre-absorbed with the respective antigenic peptides (lanes 1 and 3)

suggest the presence of only $P2Y_{2,\ 4\ and\ 6}$ receptor types. An extended pharmacological battery showed that antagonists of $P2Y_6$ receptors such as reactive blue or PPADS (Abbracchio et al. 2006; von Kügelgen 2006) did not affect ATP-evoked responses. Furthermore, UDP, the agonist of the $P2Y_6$ receptor, was able to stimulate the cells, but only at a high concentration of 200 μM (results not shown). These pharmacological experiments indicate that the observed ATP-dependent stimulation of the rat GFSHR-17 granulosa cells was mainly related to the $P2Y_2$ and $P2Y_4$ receptors. Working with low ATP concentration (5–10 μM), we observed that the likelihood to stimulate the cells with ATP was reduced. Some cells responded while others did not. Within the responding cell population, Zn^{2+} which is known to inhibit $P2Y_4$ receptors (Wildman et al. 2003) blocked the response of some cells to stimulation with 5–10 μM ATP. At 25 μM however, ATP was able to stimulate almost all cells even in presence of 300 μM Zn^{2+} (Fig. 2d). We also analyzed the expression of both molecules with western blot experiments. Monoclonal antibodies against $P2Y_2$ and $P2Y_4$ receptors recognized molecules of about 42 kDa and 50 kDa, respectively (Fig. 2e).

Simultaneous $[Ca^{2+}]_i$ imaging and electrophysiological measurements showed that the suppression of the $[Ca^{2+}]_i$ signal using a PLC inhibitor or an IP_3 receptor antagonist (Fig. 2c) correlated with the suppression of hyperpolarization (Fig. 3a). Additionally, it was found that applying the SERCA pump inhibitor cyclopiazonic acid (CPA) in the absence of any ligand induced an increase in $[Ca^{2+}]_i$ followed by the hyperpolarization of the cells (Fig. 3b, c).

Hyperpolarization of rat GFSHR-17 granulosa cells and ion flow

In the perforated patch-clamp configuration, rat GFSHR-17 granulosa cells showed a resting membrane potential (U_m) of approximately −40 mV (Table 1). We estimated the U_m using the Goldmann–Hodgkin equation:

$$U_m = \frac{RT}{F} \ln \frac{p_{K^+}[K^+]_0 + p_{Na^+}[Na^+]_0 + p_{Cl^-}[Cl^-]_i}{p_{K^+}[K^+]_i + p_{Na^+}[Na^+]_i + p_{Cl^-}[Cl^-]_0}$$

where p is the coefficient of membrane permeability to the respective ion and indices $_i$ and $_o$ indicate intracellular and extracellular spaces, respectively. The gas constant (8.314 J mol^{-1} K^{-1}), the temperature in Kelvin (room temperature: 295°K) and the Faraday constant (96,485 C mol^{-1}) are represented by R, T, and F, respectively. In our experiments, the extracellular bath solution contained 5 mM K^+, 140 mM Na^+, and 130 mM Cl^-. In the intracellular space, the values of 140 mM K^+ and 10 mM Na^+ were assumed. To achieve the observed resting potential of approximately −40 mV, the values $pK^+ = pCl^- = 1$ and $pNa^+ = 0.025$ as well as 40 mM Cl^- in the intracellular space were estimated.

The ATP-stimulated increase of $[Ca^{2+}]_i$ was followed by a hyperpolarization to −67.5 mV (Fig. 1c, Table 1). The

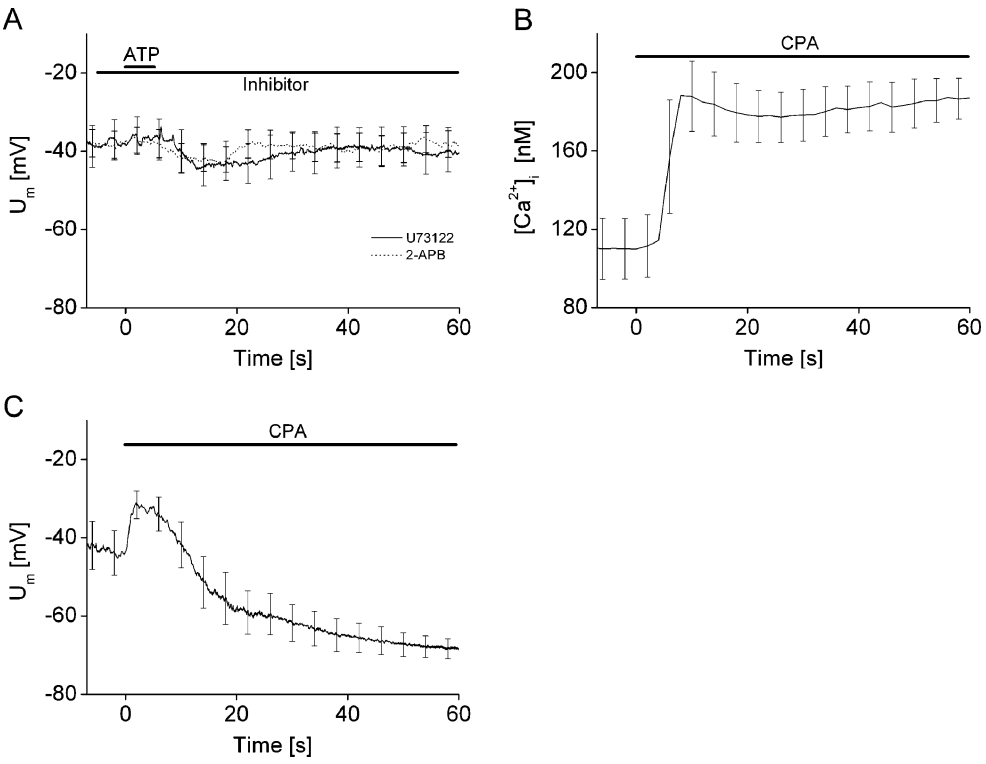

Fig. 3 The relationship between the increase in $[Ca^{2+}]_i$ and hyperpolarization. **a** The presence of U73122 (10 μM) or 2-APB (100 μM) suppressed both the ATP-induced Ca^{2+} response and hyperpolarization. **b** Applying CPA (50 μM) induced an increase in $[Ca^{2+}]_i$ that was followed by **c** hyperpolarization of the membrane potential. The results are averages and the error bars represent the SEM for at least five experiments for each treatment. It is noteworthy that CPA induced an increase in $[Ca^{2+}]_i$ and a hyperpolarization with amplitudes similar to those stimulated by 25 μM ATP

ATP-induced hyperpolarization was suppressed by U73122 (an inhibitor of PLC) as well as by 2-APB (IP_3-receptor antagonist) (Fig. 3a). Furthermore, inhibition of SERCA pumps using CPA stimulated an increase in $[Ca^{2+}]_i$ and was able to induce hyperpolarization of the cells (Fig. 3b, c), indicating that the $[Ca^{2+}]_i$-increase was a prerequisite for the hyperpolarization. The hyperpolarization could be achieved by opening K^+ channels or by inhibiting Cl^- channels. To achieve the observed U_m of −67.5 mV by ATP application, the Goldmann–Hodgkin equation would predict that ATP induces a increase of pK^+ to 10 or a decrease of pCl^- to 0.01.

Changes in the external concentrations of K^+ ($[K^+]_o$) or the substitution of Cl^- with gluconate in the extracellular solution affected the U_m as predicted by the Goldmann–Hodgkin equation (Tables 1, 2), if we assume a permeability of gluconate of 0.3 (Kim et al. 2003). It was not possible to distinguish whether the ATP-related hyperpolarization was due to the activation of K^+ channels or the inhibition of Cl^- permeability by application of ATP in presence of various $[K^+]_o$ concentrations (Fig. 4a, Table 1). When NaCl was replaced by Na-gluconate in external solution, however, the application of ATP hyperpolarized the cell to −80 mV. If ATP were increasing the pK^+ to 10 or reducing the pCl^- to 0.01, the Goldmann–Hodgkin equation would predict a U_m of −69 mV and −77 mV, respectively (Table 2). The comparison between the measured and the estimated values for U_m suggests that an inhibition of Cl^- permeability likely produced the ATP-evoked hyperpolarization. Accordingly, apamine, clotrimazole (CLT) or iberiotoxin (IbTx), the blockers of Ca^{2+}-activated K^+-channels with low conductance (SK-channels), intermediate conductance (IK-channels) or high conductance (BK-channels), respectively, did not alter ATP-related stimulation individually or as a three-drug cocktail (Fig. 4b). Other inhibitors of K^+ channels such as tetraethylammonium chloride (TEA) also failed to affect the ATP-stimulated hyperpolarization (results not shown). Pharmacological inhibition of Cl^- channels with DIDS, mibefradil or diphenylamine-2-carboxylic acid (DPC) hyperpolarized the cells to a level comparable to that achieved by ATP application under control conditions and suppressed further ATP-induced hyperpolarization (Fig. 4c, Table 3).

Discussion

This report characterises the link between purinergic receptors and the regulation of membrane potential in granulosa cells of maturing follicles using the rat GFSHR-17 granulosa cell line. The GFSHR-17 granulosa cells express the FSH receptor and are therefore a suitable in vitro model for this purpose (Keren et al. 1993). The aim of this study was to elucidate the role of purinergic receptors in the granulosa cells during follicular maturation.

Purinergic receptors are classified in two groups: ionotropic P2X receptors and metabotropic P2Y receptors. The P2X receptors are mainly permeable to cations when activated by purines and can induce depolarization as well

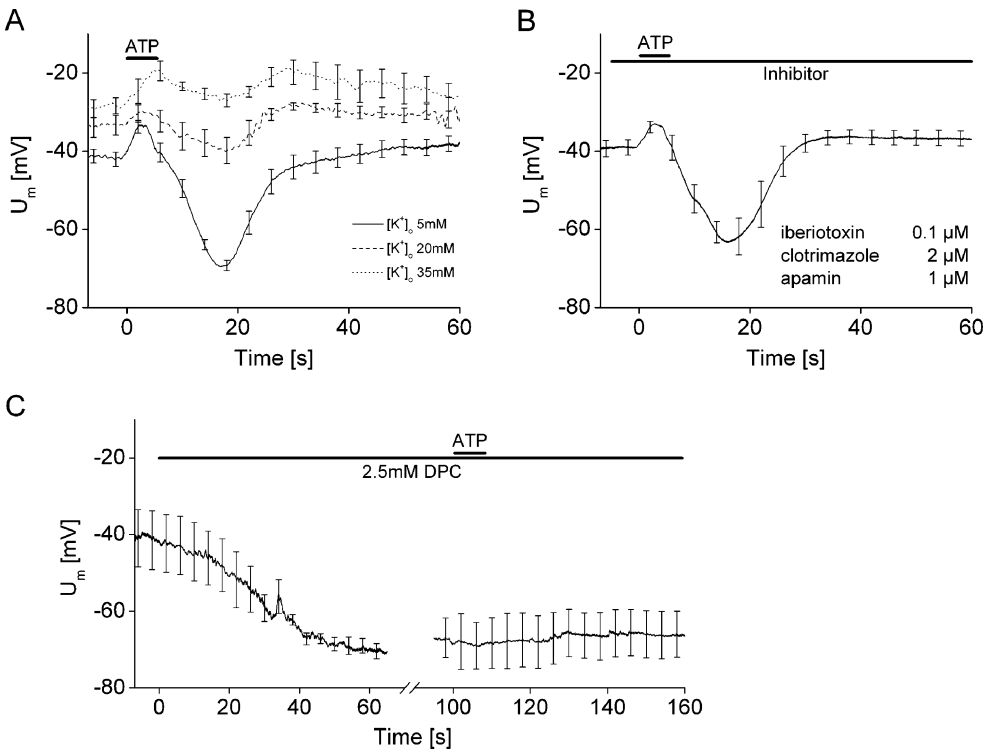

Fig. 4 **a** Increasing $[K^+]_o$ from 5 mM to 35 mM shifts the membrane potential as predicted by the Goldmann–Hodgkin equation (Table 1) and suppressed ATP-induced hyperpolarization. **b** The continuous presence of a cocktail containing the inhibitors of Ca^{2+}-activated K^+ channels (0.1 µM iberiotoxin, 2 µM clotrimazole, 1 µM apamin) did not alter the ATP induced Ca^{2+} release (not shown) or hyperpolarization. **c** The inhibition of Cl^- channels by DPC (2.5 mM) induced hyperpolarization. Similar results were obtained using DIDS (500 µM) or mibefradil (30 µM). The results are averages and the error bars represent the SEM for at least five experiments

Table 2 The values of the membrane potential predicted by the Goldmann–Hodgkin equation and measured values under ATP (25 μM) and non-ATP-induced stimulation of the cells when NaCl in external solution was replaced by Na-Gluconate (*pGluconate*=0.3; $[Cl^-]_o$=10 mM)

Substitution of NaCl by Na-Gluconate (*pGluconate*=0.3)		Na-Gluconate + 25 μM ATP		
Predicted U_m (mV)	Measured U_m (mV)	Predicted U_m (mV) with assumption that pK^+ was increased (pK^+=10)	Predicted U_m (mV) with assumption that pCl^- was decreased (pCl^-=0.01)	Measured U_m (mV)
−34.7	**−36.9±0.3** (*n* = 5)	−69.7	−76.7	**−81.5±1.9** (*n* = 5)

The results are average ± SEM

as increases in $[Ca^{2+}]_i$ due to the influx of cations, primarily Na^+ and Ca^{2+} from the external space. It is shown that applying ATP stimulates depolarization of the rat GFSHR-17 granulosa cells (Fig. 1c). As proposed by Bintig et al. (2007), however, it seems that this depolarization is not related to the activation of P2X receptors but to opening gap junction hemichannels. This is consistent with observations by other authors. It was recently shown that granulosa cells express gap junction hemichannels (Tong et al. 2007). Additionally, the hemichannels are mechanosensitive and function as ATP-release channels (Bintig et al. 2007; Gomes et al. 2005; Romanello et al. 2001). Moreover, it is shown that the ATP-related Ca^{2+} signal could be elicited even in the presence of EGTA in the extracellular solution (Fig. 2a). These results strongly suggest that the ATP-related increase in $[Ca^{2+}]_i$ does not depend on the stimulation of P2X receptors, but on the activation of P2Y receptors.

Two physiological processes can be evoked that explain the rise of ATP in the extracellular space of the maturing follicle: paracrine secretion and nervous co-stimulation (Aguado 2002; Burnstock 2007a, b; Tai et al. 2000).

As for co-stimulation, the survival and maturation of the follicle are controlled by neurotransmitters such as acetylcholine and norepinephrine, as well as neuropeptides secreted by the ovarian nerve (Aguado 2002). It can therefore be assumed that ATP appears in the extracellular space of the maturing follicle as a co-transmitter with other neurotransmitters, as suggested by Tai et al. (2000). Upon arriving in the follicular interstitial space by either paracrine secretion or co-stimulation, we suggest that ATP binds to the P2Y receptors and induces the cascade of reactions to increase $[Ca^{2+}]_i$ and stimulate hyperpolarization. Maturing follicle are characterized by a strong proliferation of the granulosa cells. Whether purinergic stimulation is involved in regulation of the proliferative activity of the granulosa cells is at moment matter of speculation. We estimated the doubling time of the rat GFSHR-17 granulosa cells. For cell cultivated under control conditions and in presence of ATP or the non hydrolysable ATP-γ-S, doubling times of 25.8 h, 24.5 h and 24.2 h were respectively found. These values are not significantly different. Because of desensitization, a long lasting presence of ATP induced a single Ca^{2+} signal (Fig. 1d). It could therefore be expected that cultivation of the cells with ATP would not affect the proliferation activity. However, the doubling times show a tendency that the purinergic stimulation could increase the proliferative activity of granulosa cells. A careful study combining analysis of frequency of ATP application and the whole duration of stimulation is needed for a definitive conclusion.

$P2Y_2$ and $P2Y_4$ receptors are expressed in rat GFSHR-17 granulosa cells

The different subtypes of P2Y receptors can be distinguished by their pharmacology as well as by their intracellular signalling cascades. We observed that P2Y receptors of the rat GFSHR-17 granulosa cells could be stimulated by UTP (Fig. 2b) but not by ADP (results not shown). We also found that blocking the IP_3 pathway inhibits ATP stimulation in the cells (Figs. 2c and 3a). It can therefore be assumed that rat GFSHR-17 granulosa cells express P2Y receptor subtypes that are sensitive to UTP and are linked to the IP_3 pathway. Only $P2Y_2$, $P2Y_4$ and $P2Y_6$ are UTP-sensitive and are linked to the activation of

Table 3 The values of the membrane potential predicted by the Goldmann–Hodgkin equation and measured values under ATP (25 μM) and non-ATP-induced stimulation of the cells, when Cl^- permeability was inhibited with DPC (2.5 mM)

DPC		DPC + 25 μM ATP		
Predicted U_m (mV)	Measured U_m (mV)	Predicted U_m (mV) with assumption that pK^+ was increased (pK^+=10)	Predicted U_m (mV) with assumption that pCl^- was decreased (pCl^-=0.01)	Measured U_m (mV)
−71.1	**−68.2±0.5** (*n* = 8)	−83.0	−71.1	**−72.9±1.1** (*n* = 8)

The results are average ± SEM. Similar results were obtained with Mibefradil (30 μM) or DIDS (500 μM)

the IP_3-Ca^{2+}-cascade at the intracellular site (Burnstock and Williams 2002; King and Townsend-Nicholson 2003). The agonist of $P2Y_6$ UDP (Abbracchio et al. 2006; King and Townsend-Nicholson 2003; von Kügelgen 2000, 2006), however, could stimulate the rat GFSHR-17 granulosa cells only at concentrations of 200 μM. Moreover, reactive blue, an inhibitor of $P2Y_6$ (Abbracchio et al. 2006; von Kügelgen 2000, 2006) did not affect the ATP evoked response (results not shown). These results suggest a minor role of $P2Y_6$ receptor and a major role of $P2Y_2$ and $P2Y_4$ receptors in ATP-induced activity in rat GFSHR-17 granulosa cells. This assumption is supported by Western blot experiments, which showed that the rat GFSHR-17 granulosa cells expressed molecules specifically recognized by anti-$P2Y_2$ and anti-$P2Y_4$ antibodies (Fig. 2e). The anti-$P2Y_2$ antibody stained a molecule with ~42 kDa (Fig. 2e, lane 2), which correlates to the predicted size of 42.0 kDa for the $P2Y_2$ receptor Sage and Marcus 2002). A size of 40.7 kDa was predicted for $P2Y_4$ receptor. The anti-$P2Y_4$ antibody, however, recognized a molecule of approximately 50 kDa (Fig. 2e, lane 4). These bands disappeared when the antibodies were blocked with the corresponding antigenic peptides (Fig. 2e, lane 1 and 3). The discrepancy between the predicted band at 40.7 kDa for $P2Y_4$ and the specifically stained band at 50 kDa can be explained by posttranslational modifications such as glycosylation (Sage and Marcus 2002). It is therefore tempting to assume that the ATP-evoked Ca^{2+} and electrical responses in the rat GFSHR-17 granulosa cells are related to the activation of $P2Y_2$ and $P2Y_4$ receptors. Further pharmacological experiments revealed that the density of each receptor type could be variable. Using low ATP concentration (5–10 μM), there were cells which could not be stimulated. Within the responding cell population, Zn^{2+} (300 μM) which inhibited $P2Y_4$ receptors was able to suppress the Ca^{2+} evoked response by 5–10 μM ATP in some cells, while other cells were not affected (Fig. 2d). At 25 μM ATP the inhibitory effect of Zn^{2+} could not be observed (Fig. 2d). These results could be related to a various density of the $P2Y_2$ and $P2Y_4$ receptors in the membrane of the rat GFSHR-17 granulosa cells. How both receptor subtypes participate to the regulation of the ATP-evoked response in granulosa cells remains an interesting question. A combination of biochemical analysis and molecular biological dissection is needed to determine the contribution of each receptor to the observed ATP-evoked response in granulosa cells.

ATP stimulates hyperpolarization in rat GFSHR-17 granulosa cells

The measured resting potential suggests a high pCl^- and pK^+ (≅1) and an elevated $[Cl^-]_i$ of about 40 mM under control conditions. This suggestion is supported by the following experimental observations: (i) Gradual increase of $[K^+]_o$ from 5 mM to 20 mM, 35 mM and 60 mM and substitution of NaCl in the external solution with Na-gluconate depolarized the cells as predicted by the Goldmann-Hodgkin equation from −40 mV to −34.8 mV, −35.2 mV, −24.2 mV and −37 mV, respectively (with assumed *pGluconate* = 0.3 suggested by Kim et al. 2003) (Fig. 4a, Tables 1, 2). (ii) Inhibitors of Cl^- channels such as DPC, DIDS or mibefradil induced hyperpolarization of the cells to approximately −70 mV (Fig. 4c, Table 3). (iii) $[Cl^-]_i$ in the range of 40 mM has been measured in various non-excitable cells such as astrocytes and in rat GFSHR-17 granulosa cells under the whole-cell patch-clamp configuration using a pipette solution containing 140 mM K^+, 10 mM Na^+ and 30–40 mM Cl^- (Ngezahayo et al. 2003). Since the theoretically predicted U_m under various conditions and the measured U_m are very close, it can be assumed that non-stimulated rat GFSHR-17 granulosa cells are equally permeable to K^+ and Cl^-. This permeability to ions must be compensated by a large permeability to water. Since rat GFSHR-17 granulosa cells are an in vitro model for the granulosa cells of maturing follicle, we propose that this mechanism is involved in the secretion of the antral fluid during follicle maturation.

The ATP-induced hyperpolarization can be associated with an increase in membrane permeability to K^+ or a decrease in membrane permeability to Cl^-. To achieve the observed U_m of −67.5 mV by ATP application, the

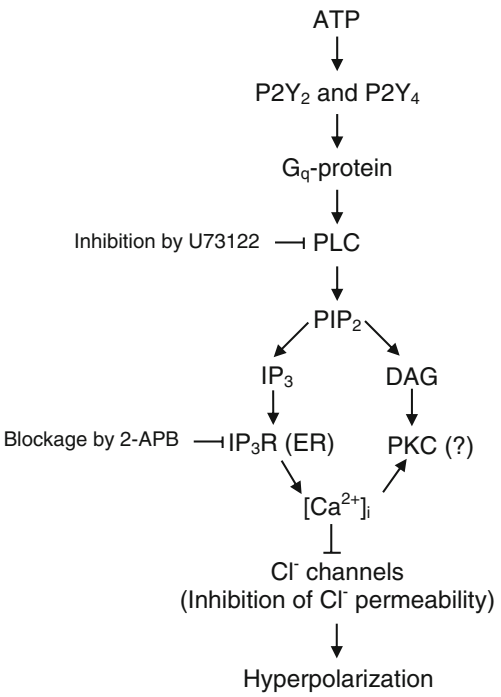

Fig. 5 Schematic representation of ATP signalling in granulosa cells of maturing follicle. The *question mark* shows possible pathways that remain to be elucidated

Goldmann–Hodgkin equation would predict that ATP induces an increase of pK^+ to 10 or a decrease of pCl^- to 0.01. Accordingly, we predicted the U_m that would be achieved by application of ATP under various conditions with the assumption that ATP affects the permeability of either K^+ or Cl^- (Tables 1, 2, and 3). At various $[K^+]_o$, the Goldmann–Hodgkin equation predicted values that were very close when we assumed that ATP affected either K^+ or Cl^- permeability. The measured values agree with the prediction of the Goldmann–Hodgkin equation; however, these experiments cannot decipher which ion is affected by ATP-application. After substitution of external NaCl with Na-gluconate, ATP-application hyperpolarized the cells to a membrane potential of −81 mV (Table 2). If we assume that ATP was activating K^+-channels, a U_m of −69 mV would be predicted by the Goldmann–Hodgkin equation. Conversely, if we assume that ATP reduced pCl^-, the Goldmann–Hodgkin equation would predict a U_m of −77 mV. The results therefore suggest that ATP induced an inhibition of Cl^- permeability. Furthermore, the inhibition of Ca^{2+}-release with inhibitors of PLC or IP_3-receptors blocked the hyperpolarization (Fig. 3a). The SERCA pump inhibitor CPA yielded an increase in $[Ca^{2+}]_i$ (Fig. 3b) and thereby induced hyperpolarization (Fig. 3c). If we assume that the ATP-evoked hyperpolarization was related to the activation of K^+ channels, it would be correct to assume that these channels are Ca^{2+}-activated. Pharmacological inhibition of the putative Ca^{2+}-activated BK, IK and SK channels by IbTx, CLT, apamin or a cocktail containing all these inhibitors (Fig. 4a) did not alter the ATP-induced hyperpolarization, nor did inhibitors of K^+ channels such as TEA. The Cl^- channel inhibitors DPC, DIDS or mibefradil hyperpolarized the cells to −70 mV as predicted by the Goldmann–Hodgkin equation (Table 3). An additional application of ATP was not able to provoke a further hyperpolarization, even though the Goldmann–Hodgkin equation predicted a reinforcement of hyperpolarization to −83 mV under the assumption that the permeability for K^+ would be increased by ATP ($pK^+ = 10$) (Fig. 4c, Table 3). Biophysical analysis, combined with pharmacological dissection, allow us to postulate that ATP-induced stimulation of $P2Y_2$ and $P2Y_4$ receptors in rat GFSHR-17 granulosa cells induces a Ca^{2+}-dependent inhibition of Cl^- channels and thereby promotes hyperpolarization (Fig. 5). We propose that an ATP-dependent inhibition of Cl^- permeability in granulosa cells of the maturing follicle is a key mechanism in regulating the secretion of antral fluid.

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