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The Effects of Extracellular Nucleotides on $[Ca^{2+}]_i$ Signalling in a Human-Derived Renal Proximal Tubular Cell Line (HKC-8)

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ABSTRACT

HKC-8 cells are a human-derived renal proximal tubular cell line and provide a useful model system for the study of human renal cell function. In this study, we aimed to determine $[Ca^{2+}]_i$ signalling mediated by P2 receptor in HKC-8. Fura-2 and a ratio imaging method were employed to measure $[Ca^{2+}]_i$ in HKC-8 cells. Our results showed that activation of P2Y receptors by ATP induced a rise in $[Ca^{2+}]_i$ that was dependent on an intracellular source of Ca^{2+}, while prolonged activation of P2Y receptors induced a rise in $[Ca^{2+}]_i$ that was dependent on intra- and extracellular sources of Ca^{2+}. Pharmacological and molecular data in this study suggests that TRPC4 channels mediate Ca^{2+} entry in coupling to activation of P2Y in HKC-8 cells. U73221, an inhibitor of PI-PLC, did not inhibit the initial ATP-induced response; whereas D609, an inhibitor of PC-PLC, caused a significant decrease in the initial ATP-induced response, suggesting that P2Y receptors are coupled to PC-PLC. Although P2X were present in HKC-8, The P2X agonist, α,β me-ATP, failed to cause a rise in $[Ca^{2+}]_i$. However, PPADS at a concentration of 100 μM inhibits the ATP-induced rise in $[Ca^{2+}]_i$. Our results indicate the presence of functional P2Y receptors in HKC-8 cells. ATP-induced $[Ca^{2+}]_i$ elevation via P2Y is tightly associated with PC-PLC and TRP channel. J. Cell. Biochem. 109: 132–139, 2010.

KEY WORDS: PROXIMAL TUBULAR CELL; HKC-8; ATP; P2Y; GPCR; PLC

P2 receptors, a major subgroup of the purinergic receptors, are cell membrane receptors that are activated by extracellular ATP and its analogues [Burnstock, 2006a,b]. Based on their structure and mode of action P2 receptors are subdivided into two groups: P2X and P2Y receptors. P2X receptors are ionotropic ATP-gated receptor channels. P2Y receptors are G-protein coupled receptors that bind nucleotides, such as ATP, activating phospholipase C (PLC) signalling pathways. Activation of either type of P2 receptors leads to a rise in $[Ca^{2+}]_i$. Activation of P2X receptors causes a rise in $[Ca^{2+}]_i$ due to Ca^{2+} entry through the membrane-spanning pore region of the P2X receptor, which is permeable to both monovalent and divalent cations. Nucleotide binding to P2Y receptors leads to an activation of membrane-bound PLC, resulting in increased formation of breakdown products of membrane phospholipids. These products are capable of stimulating release of Ca^{2+} from intracellular stores and/or causing an influx of Ca^{2+} through membrane-spanning ion channels [Hardie and Minke, 1993; Clapham, 1996; Ramsey et al., 2006]. The G protein-coupled P2Y receptors comprise at least eight mammalian subtypes (P2Y(1,2,4,6,11,12,13,14)), while the P2X receptors comprise at least seven subtypes (P2X(1,2,3,4,5,6,7)) [Bailey et al., 2008; Robert and Steven, 2008].

P2 receptors are present in epithelial cells of the nephron in the luminal and basolateral membranes [Nilius et al., 1995; McCoy et al., 1999; Insel et al., 2001; Shirley et al., 2005; Bailey and Shirley, 2009]. Activation of these receptors has been shown to occur in an autocrine and paracrine fashion through release of ATP from the epithelial cell [Schwiebert and Kishore, 2001; Schwiebert et al., 2002; Leipziger, 2003; Hovater et al., 2008]. In addition, ATP release has been shown to occur onto the luminal and basolateral sides of epithelial cell monolayers [Schwiebert and Kishore, 2001; Bailey et al., 2008].

The proximal tubule is the segment of the duct system of the nephron lying between the Bowman's capsule and the loop of Henle. Approximately two-thirds of the salt and water in the filtrate that is reabsorbed during its passage through the duct system of the nephron occurs in the proximal tubule. Experiments performed in proximal tubule cells have demonstrated the presence of P2X and P2Y receptors [Bouyer et al., 1998; Paller et al., 1998; Bailey et al.,

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2000, 2001]. Schwiebert et al. [2002] have demonstrated the abundant expression of P2X4 and P2X5, and lesser amount of P2X7, in human proximal tubule primary cultures [Schwiebert, 2001]. Other experiments have demonstrated the presence of P2Y1, P2Y2 and P2Y6 receptors in proximal tubule cells [Bailey et al., 2000, 2001]. Furthermore, activation of the P2Y receptor by extracellular nucleotides, such as ATP, in cells of the proximal tubule has been shown to increase $[Ca^{2+}]_i$. While evidence for the functional consequences of P2 receptor stimulation in the proximal tubule is limited, several studies have shown that nucleotide activation of the P2Y receptor in cells of the distal nephron and the cortical collecting duct inhibit salt and water absorption [McCoy et al., 1999; Thomas et al., 2001; Leipziger, 2003; Matos et al., 2007]. Perfusion of the pertitubular capillaries with a solution containing ATP stimulated proximal bicarbonate transport [Diaz-Sylvester et al., 2001] whereas inhibition of Na^+/H^+ exchange by solution containing of ATP was due to activation of luminal P2Y1 receptors in rat proximal tubule [Bailey, 2004]. Moreover ATP acts as a modulator in regulation of renal proximal tubule cell proliferation [Lee and Han, 2006] by increasing $[Ca^{2+}]_i$, p44/42 MAPKs and CDks [Lee et al., 2005]. However, it remains unclear of the pathway which underlies ATP-induced $[Ca^{2+}]_i$ elevation.

HKC-8 cells are a cell line derived from human renal proximal tubular, which displays many of the characteristics, such as polarisation and channel expression, of cells of the proximal tubule. HKC-8 cells therefore are a useful model system for the study of function of human renal proximal tubular cell [Hara et al., 2000; Breznan et al., 2004]. HKC-8 cells were employed in this study to investigate P2 receptor $[Ca^{2+}]_i$ signalling in the renal epithelium of the proximal tubule. In particular, $[Ca^{2+}]_i$ signalling mediated by activation of P2X and P2Y receptors in response to extracellular nucleotides were investigated.

METHODS

CELL CULTURE

Cells from a human proximal tubule cell line (HKC-8) were cultured in standard culture medium (DMEM/Ham F12, 2 mM glutamine, 500 U/ml penicillin, 1 mg/ml streptomycin, and 5% FBS in 75 cm^2 flasks, at 37°C (95% air and 5% CO_2) until confluent. HKC-8 cells were transferred to 13-mm cover-slips (VWR, Lutterworth, Leicestershire, UK) for $[Ca^{2+}]_i$ measurements.

$[Ca^{2+}]_i$ MEASUREMENTS

Measurements of $[Ca^{2+}]_i$ in HKC-8 cells were carried out using the acetoxymethyl ester of fura-2 (Molecular Probes, Invitrogen, Paisley, UK). According to the protocol, 50 μg Fura-2 AM (F1221, Molecular Probe, Invitrogen, Paisley, UK) was dissolved in 20 μl 20% pluronic acid (0.01 g in 50 μl DMSO) to generate a stock solution. Prior to the experiment, a mixture of 1 μl stock dye in 200 μl EBSS was loaded onto the cells and incubated for at least 30 min at 37°C. Cells, grown on 13-mm cover-slips (VWR), were placed in a bath chamber mounted on the stage of a Nikon Eclipse TE-2000 U inverted microscope fitted with a 20× UV, 1.3 NA oil-immersion objective. A perfusion system was used to supply control and drug-containing solutions to the bath chamber. Measurements of the changes in $[Ca^{2+}]_i$ were made using a Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instrument Company, Novato, CA, USA) to excite fura-2 at 340 and 380 nm and a Electron Multiplier CCD Digital Camera (Hamamatsu Photonics, Welwyn Garden City, UK) to capture corresponding paired florescence emission signals at 510 nm at a time interval of every 4–5 s. Following each experiment multiple regions of interest (ROI) from were selected from captured fluorescence emission data and the changes in $[Ca^{2+}]_i$ were reported graphically as mean F340/F380 fluorescence ratio against time. N represented the number of coverslips.

STATISTICS

Data from one coverslip were averaged and presented in the figures. The same experiments were repeated on different coverslips (N). Data are presented as means ± SEM, and statistical differences were assessed using the Student's paired *t*-test. $^*P < 0.05$ was considered significant.

TOTAL RNA EXTRACTION AND PREPARATION OF cDNA

RNA was extracted from HKC-8 cells using an RNEasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Gel electrophoresis was performed using the purified RNA and the results were qualitatively checked using a Gene Genius Bio-Imaging system (Syngene, Cambridge, UK). Total RNA was denatured by heating to 70°C for 5 min. First-strand cDNA synthesis was performed by adding 0.5 μg $Oligo(dT)_{18}$, 50 units of Bioscript (reverse transcriptase), 2mM dNTP (final concentration), and 5× reaction buffer in a total volume of 20 μl. The reaction was performed at 37°C for 60 min and then stopped by heating at 70°C for 10 min.

Second-strand synthesis was performed with gene-specific primers designed to specific sequences in the coding region of the gene of interest. The following primers were employed (Table I). PCR was carried out in a Creacon T-CY Thermal Cycler using the following conditions: 1 cycle of denaturation at 94°C for 2 min followed by 35 cycles at 54°C (45 s), 72°C (60 s) and 94°C (30 s) with final elongation at 72°C (10 min). Amplification products were run by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

RESULTS

mRNA EXPRESSION OF PURINERGIC RECEPTORS AND TRANSIENT RECEPTOR POTENTIAL CHANNELS (TRPC) IN HKC-8 CELLS

Previous studies have shown the expression of P2X1 in cultured mouse proximal cells [Filipovic et al., 1998], expression of P2X4 and P2X7 in cultured human proximal cells [Schwiebert and Kishore, 2001], expression of P2Y1, 2, 4 and 6 in rat proximal tubule [Bailey et al., 2000, 2001]. We therefore focused our studies on these P2 receptors in HKC-8.

RT-PCR analysis showed expression of mRNA for P2Y2, P2Y6, P2X4 and P2X7 receptors (Fig. 1). Further RT-PCR experiments were performed and identified the presence of TRPC4 mRNA (Fig. 1).

TABLE I. Primer Sequence for PCR

Name	Forward	Reverse	Length
TRPC1	TCCTCCTCCCTGCCTTCC	AGCGTATTCTCCTCCTTCACC	92
TRPC3	GAGTAGTTAGCAACGGTATCG	GGACCTAATCAGTAGCAACG	157
TRPC4	AACTCAGCAGCGACTAAG	TATAGGGAGCATTAACATTTC	191
TRPC5	AGCGGAGAGAAGCAGGTC	GATTTCGTAGTTGTTGGTGTGG	108
TRPC6	CAACGAGAGCCAGGACTATC	GGTAGTAGCCGTAGCAAGG	89
TRPC7	CCTCCCTTTTCTCGCCATAG	GCATTCACAACTAATAATCCCAAG	135
P2Y1	GGGGTTCCTGAACTACGC	CTCCTCCCTACCTTAGAAACG	163
P2Y2	CGCTTCAACGAGGACTTC	TATATGTGGTGGACGCATTC	78
P2Y4	ACTGTTGGTTTGATGAGGATTTC	GAGGCGGAAGATGAAGAGC	116
P2Y6	ACCCACCACCTGTGTCTAC	AGGTCAGCCAGAGCAAGG	177
P2X1	CATCTATCTTTGGGAATTTATTTGTC	GAGGCACTTGGGTTGGAG	117
P2X4	CCAACATCACCACTACTTACC	CTGTGTCCTGCGTTCTCC	106
P2X7	AAGAGGAGATCGTGGAGAATGG	GGGATACTCGGGACACAACC	155
GADPH	GGAGTCCACTGGCGTCTTC	GGCATTGCTGATGATCTTGAGG	163

EXTRACELLULAR ATP CAN INDUCE A RISE IN THE INTRACELLULAR Ca^{2+} CONCENTRATION ($[Ca^{2+}]_i$) IN HKC-8 CELLS THAT IS CONCENTRATION-DEPENDENT

Bath application of ATP produced a rise in intracellular Ca^{2+} in HKC-8 cells that could be measured by monitoring the change in fluorescence of the ratiometric dye, fura-2. Figure 2a shows a response to 10 μM ATP, which consists of a increase in $[Ca^{2+}]_i$ upon ATP application followed by a return to baseline level upon wash-off of ATP. Figure 2b shows that ATP (1–30 μM) caused a dose-dependent increase in the peak Ca^{2+} response, with an EC_{50} of approximately 2.4 μM. The values were obtained by normalizing the increase in the fluorescence ratio measured at concentrations of 1, 2, 5 and 10 μM ATP to a concentration of 30 μM ATP, which was found to be a maximal concentration. Therefore we used this concentration in all experiments.

PROLONGED APPLICATION OF ATP INDUCES A RISE IN $[Ca^{2+}]_i$ THAT IS DEPENDENT ON INTRA- AND EXTRACELLULAR SOURCES

Figure 3a shows a response to the prolonged application of 10 μM ATP in HKC-8 cells, which consists of an initial increase in $[Ca^{2+}]_i$ followed by a decline to a plateau of elevated $[Ca^{2+}]_i$. Figure 3b demonstrates that when HKC-8 cells are stimulated with ATP (10 μM) in the absence of extracellular Ca^{2+} then the initial rise in $[Ca^{2+}]_i$ was still present. Furthermore, no significant reduction in the peak Ca^{2+} response was detected (N = 4). The subsequent plateau of elevated $[Ca^{2+}]_i$ however, that characterises the response in Figure 3a, was absent and over the time course of the experiment the

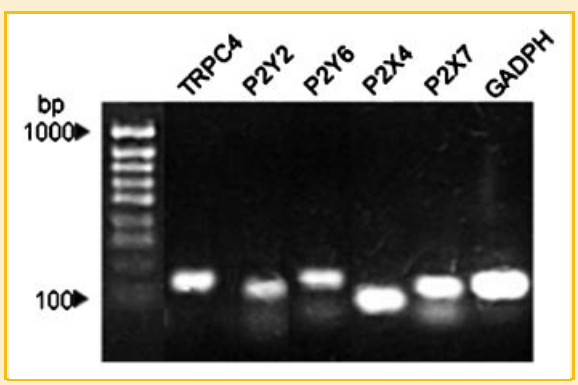

Fig. 1. Expression of P2 receptor and TRPC4 in HKC-8 cells. RT-PCR analysis showed expression of mRNA for P2Y2, P2Y6, P2X4, P2X7 receptors and TRPC4 in HKC-8 cells.

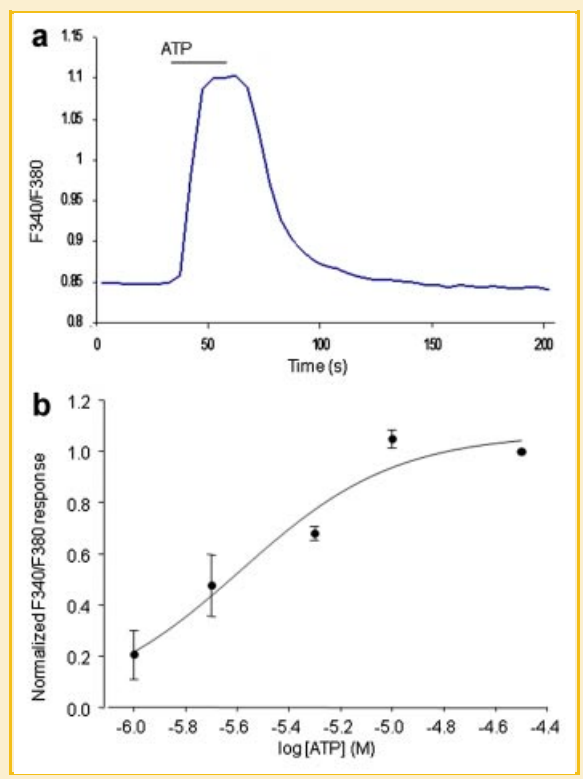

Fig. 2. Extracellular ATP induced a concentration-dependent $[Ca^{2+}]_i$ elevation. a: ATP at a concentration of 10 μM induced a significant $[Ca^{2+}]_i$ elevation and $[Ca^{2+}]_i$ returned to baseline level when ATP was washed off. b: There was a concentration dependent $[Ca^{2+}]_i$ elevation in response to ATP at a range of concentrations. The EC50 of the dosage response curve is 2.4 μM. Each point was obtained from four to five different coverslips.

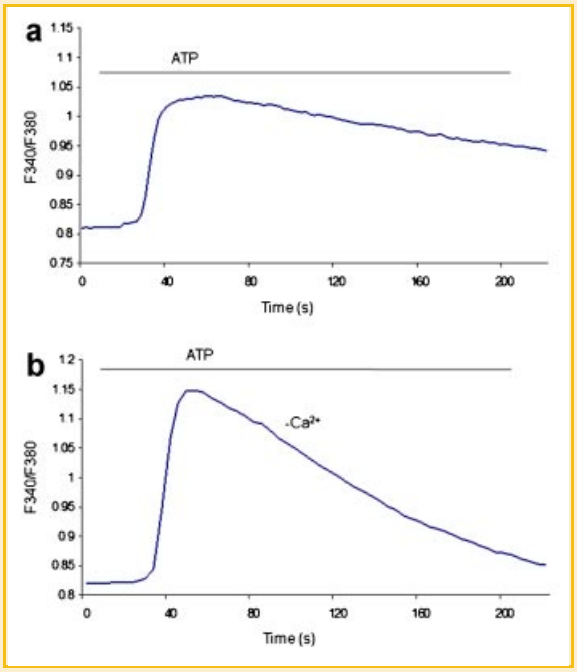

Fig. 3. Prolonged application of ATP induces a rise in $[Ca^{2+}]_i$ that is dependent on intra-and extracellular sources. a: It represented a prolonged application of 10 μM ATP in HKC-8 cells, which consists of an initial increase in $[Ca^{2+}]_i$ followed by a decline to a plateau of elevated $[Ca^{2+}]_i$. b: In the absence of extracellular Ca^{2+}, ATP (10 μM) still initiated $[Ca^{2+}]_i$ elevation in HKC-8 cells.

$[Ca^{2+}]_i$ returned to a level seen prior to application of ATP (N = 4). These results demonstrate that the rise in $[Ca^{2+}]_i$ in response to prolonged application of ATP, is derived from Ca^{2+}-containing stores within the cell and the extracellular environment.

EMPTYING OF Ca^{2+} FROM THE ENDOPLASMIC RETICULUM (ER) SIGNIFICANTLY REDUCES THE PEAK INCREASE IN $[Ca^{2+}]_i$ IN RESPONSE TO STIMULATION WITH ATP

Figure 3b suggests that the ATP-induced rapid initial rise in $[Ca^{2+}]_i$ in HKC-8 cells is due to release from intracellular Ca^{2+} stores as this response did not require extracellular Ca^{2+}. To demonstrate this more clearly we depleted Ca^{2+} levels within the ER, thought to be the main Ca^{2+}-containing store in the eukaryotic cell, with thapsigargin, a well characterised inhibitor of the ER Ca^{2+}-ATPase [Friedman et al., 1989], before stimulating the cells with ATP. Application of thapsigargin (1 μM) caused an increase in $[Ca^{2+}]_i$ that signifies emptying of Ca^{2+} from the ER (N = 4, Fig. 4). After 10 min of thapsigargin treatment ATP (10 μM) was applied to the cells to test for the effects of Ca^{2+} store depletion on the ATP-induced Ca^{2+} response. The peak rise in $[Ca^{2+}]_i$ in response to ATP treatment (10 μM) was inhibited by 78 ± 5% (N = 4) following treatment with thapsigargin as compared to cells which had not been treated with thapsigargin. Thapsigargin did not completely abolish the Ca^{2+} response to ATP (Fig. 4).

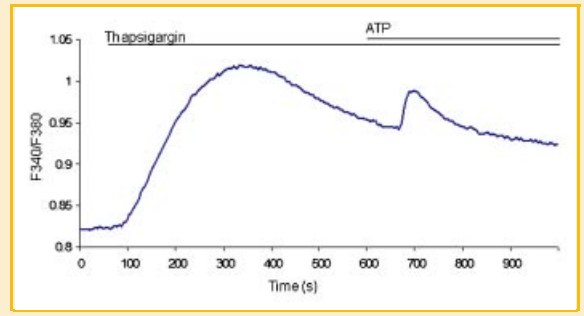

Fig. 4. Depletion of Ca^{2+} store attenuated ATP induced $[Ca^{2+}]_i$ elevation in HKC-8 cells. Ca^{2+} store was depleted by thapsigargin (1 μM) prior to ATP stimulation. The peak rise of $[Ca^{2+}]_i$ elevation was reduced by 78 ± 5% (N = 4) following treatment with thapsigargin.

CONTRIBUTION OF P2 RECEPTOR SUBTYPES TO THE ATP-INDUCED Ca^{2+} RESPONSE IN HKC-8 CELLS

Contribution of P2X receptors. The purinergic antagonist PPADS, at a concentration of 10 μM, believed to be selective for the P2X receptor subtype [Lambrecht et al., 1992; Bailey et al., 2008], was used to test for the involvement of P2X receptors in eliciting the ATP-induced rise in $[Ca^{2+}]_i$. Figure 5a demonstrates that 10 μM PPADS had no measurable effect on the ATP-induced rise in $[Ca^{2+}]_i$ (N = 3). α,β-methylene-ATP (α,β me-ATP), a non-hydrolyzable analogue of ATP, was used as an agonist of the P2X receptor. Application of 100 μM α,β me-ATP (Fig. 5b) did not result in any increase of $[Ca^{2+}]_i$ (N = 8). Finally, the P2 antagonist PPNDS, which is reported to have a preferential action at the $P2X_1$ and $P2X_7$ receptor [Wood and Hennessey, 2003; Xia et al., 2004; Donnelly-Roberts et al., 2009], inhibited the ATP-induced rise in $[Ca^{2+}]_i$ (Fig. 5c). PPNDS (10 μM) inhibited the peak Ca^{2+} response by 73 ± 5% (N = 4). After washout of the antagonist a partial recovery of the ATP response was observed (Fig. 5c).

Contribution of P2Y receptors. The P2Y receptor agonist UTP was used to test for the presence of P2Y receptors. Prolonged application of UTP (10 μM) gave rise to an increase in $[Ca^{2+}]_i$ followed by a decline to a plateau of elevated $[Ca^{2+}]_i$ (Fig. 6a). This response very closely resembled the response to 10 μM ATP. PPADS, at a concentration of ≥30 μM, can block some P2Y receptors [Ho et al., 1995; Schachter et al., 1996; von Kugelgen and Wetter, 2000]. Therefore, 100 μM PPADS was used to test for the involvement of P2Y receptors in eliciting the ATP-induced rise in $[Ca^{2+}]_i$. PPADS (100 μM) inhibited the peak Ca^{2+} response by 52 ± 6% (N = 7, Fig. 6b). After washout of the antagonist a partial recovery of the ATP response was observed (Fig. 6b).

EFFECTS OF ATP ON $[Ca^{2+}]_i$ IN HKC-8 CELLS ARE MEDIATED THROUGH PHOSPHOLIPASE C

The results previously described indicate that P2Y receptors play an important role in generating the ATP-induced rise in $[Ca^{2+}]_i$ in HKC-8 cells. To test for the coupling of G protein-phospholipase C activation in generating the ATP-induced Ca^{2+} responses in HKC-8 cells, ATP-induced responses were observed in the presence of phopholipase C inhibitors. Figure 7a shows two consecutive

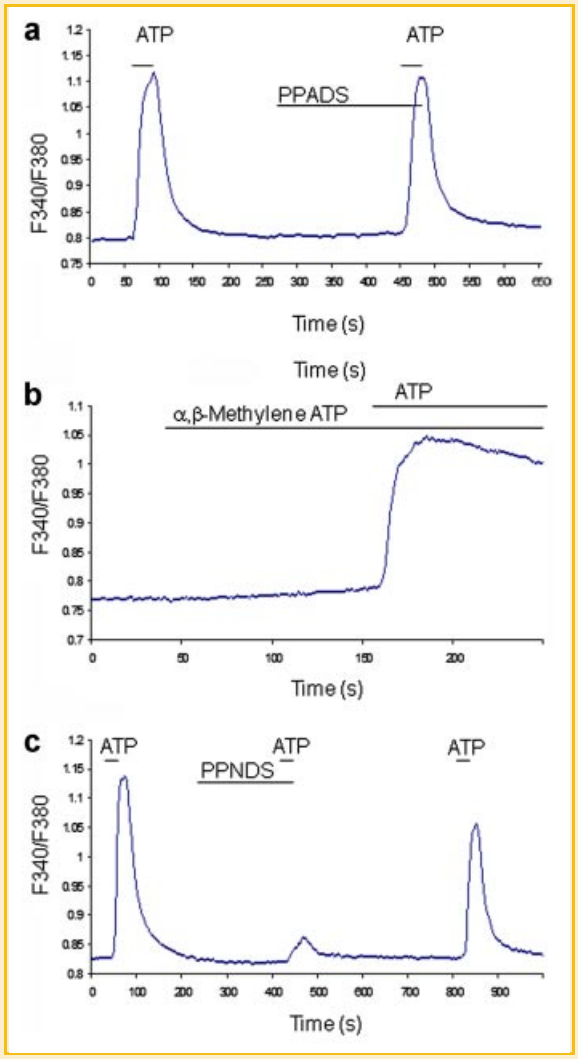

Fig. 5. Contribution of P2X receptor to the ATP-induced Ca^{2+} response in HKC-8 cells. a: 10 μM PPADS had no measurable effect on the ATP-induced rise in $[Ca^{2+}]_i$. b: Application of 100 μM α, β me-ATP, which is an agonist of P2X, did not result in any increase of $[Ca^{2+}]_i$. c: PPNDS (10 μM) inhibited the peak Ca^{2+} response.

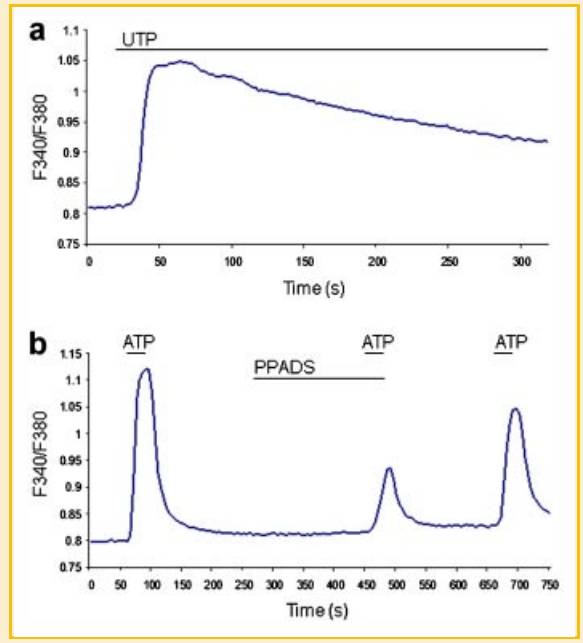

Fig. 6. Involvement of P2Y receptors into ATP-induced Ca^{2+} response in HKC-8 cells. a: Prolonged application of UTP (10 μM) gave rise to an increase in $[Ca^{2+}]_i$ followed by a decline to a plateau of elevated $[Ca^{2+}]_i$. b: PPADS (100 μM) inhibited the peak Ca^{2+} response by 52 ± 6%. After washout of the antagonist a partial recovery of the ATP (10 μM) response was observed.

responses to 10 μM ATP separated by an interval of 5 min. Both responses consist of a increase in $[Ca^{2+}]_i$ upon ATP application followed by a return to baseline level upon wash-off of ATP. As can be seen in Figure 7b pre-incubation with 10 μM U73122, an inhibitor of phosphatidylinositol-phospholipaseC (PI-PLC), had no effect on the ATP-induced rise in $[Ca^{2+}]_i$ (N = 8), whereas pre-incubation with 10 μM D609, an inhibitor of phosphatidylcholine-phospholipase C (PC-PLC), reduced the ATP-induced rise in $[Ca^{2+}]_i$ by 83 ± 23% (N = 7, Fig. 7c).

THE PLATEAU OF ELEVATED $[Ca^{2+}]_i$ FOLLOWING PROLONGED APPLICATION OF ATP IN HKC-8 CELLS IS DUE TO ACTIVATION OF STORE-OPERATED CHANNELS (SOCS)

From previous experiments, the results of which are shown in Figure 3, it was determined that the rise in $[Ca^{2+}]_i$ in response to prolonged application of ATP, is derived from Ca^{2+}-containing stores within the cell and the extracellular environment. This is also demonstrated in Figure 8a where prolonged application of ATP (10 μM) in the absence of extracellular Ca^{2+} caused an initial rise in $[Ca^{2+}]_i$ followed by a decline to the initial baseline level. The rise in $[Ca^{2+}]_i$ during this phase of the experiment is entirely dependent on Ca^{2+} release from an intracellular compartment, possibly the ER. Figure 8a demonstrates that the subsequent perfusion of a solution containing extracellular Ca^{2+} caused an increase in $[Ca^{2+}]_i$ that reached a peak before slowly declining over time. The phenomenon of Ca^{2+} entry following intracellular Ca^{2+} store depletion has been recorded in many cell types and is attributed to the opening of store-operated channels (SOCs) present in the plasma membrane. With this in mind known blockers of SOCs were used to confirm their presence. The calcium channel blocker lanthanum chloride completely inhibited Ca^{2+} entry through SOCs without effecting Ca^{2+} release from stores (N = 5, Fig. 8b). 2-Aminoethyl diphenyl-borinate (2-APB) (50 μM), inhibited Ca^{2+} entry through SOCs by 96 ± 1% (N = 4, Fig. 8c).

DISCUSSION

The pharmacological and molecular data presented in this study indicate the presence of functional P2Y receptors in the human renal proximal tubular cell line HKC-8. Short-term (<30 s) activation of P2Y receptors by extracellular nucleotides, in particular ATP, induces a rise in $[Ca^{2+}]_i$ that is dependent on an intracellular source

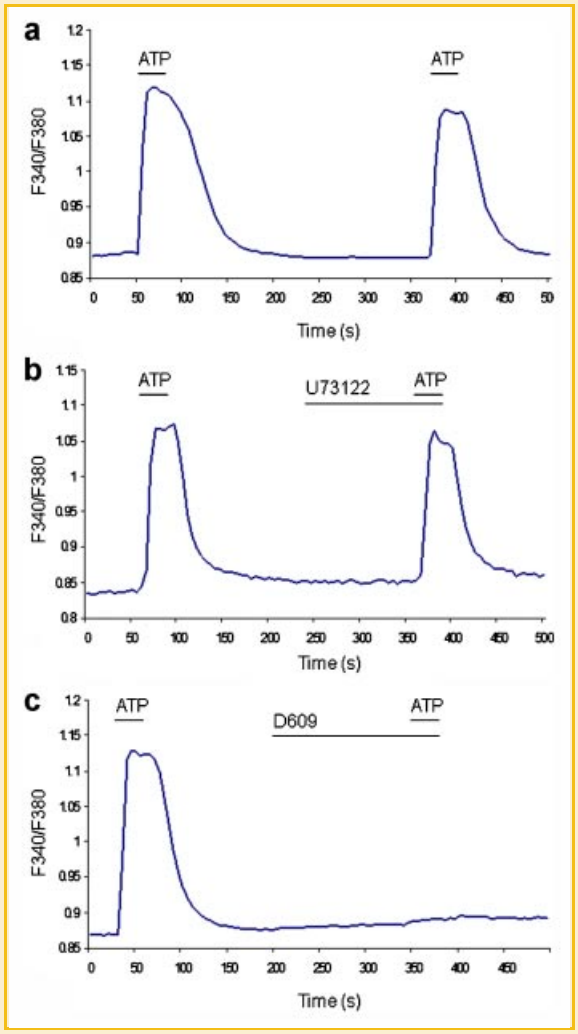

Fig. 7. Effects of ATP (10 μM) on $[Ca^{2+}]_i$ in HKC-8 cells are mediated through phospholipase C (PLC). a: It represented the reproducible ATP-induced $[Ca^{2+}]_i$ elevation over a period. b: Pre-incubation with 10 μM U73122, an inhibitor of phosphatidylinositol-phospholipaseC (PI-PLC), had no effect on the ATP-induced rise in $[Ca^{2+}]_i$. c: Pre-incubation with 10 μM D609, an inhibitor of phosphatidylcholine-phospholipase C (PC-PLC), reduced the ATP-induced rise in $[Ca^{2+}]_i$.

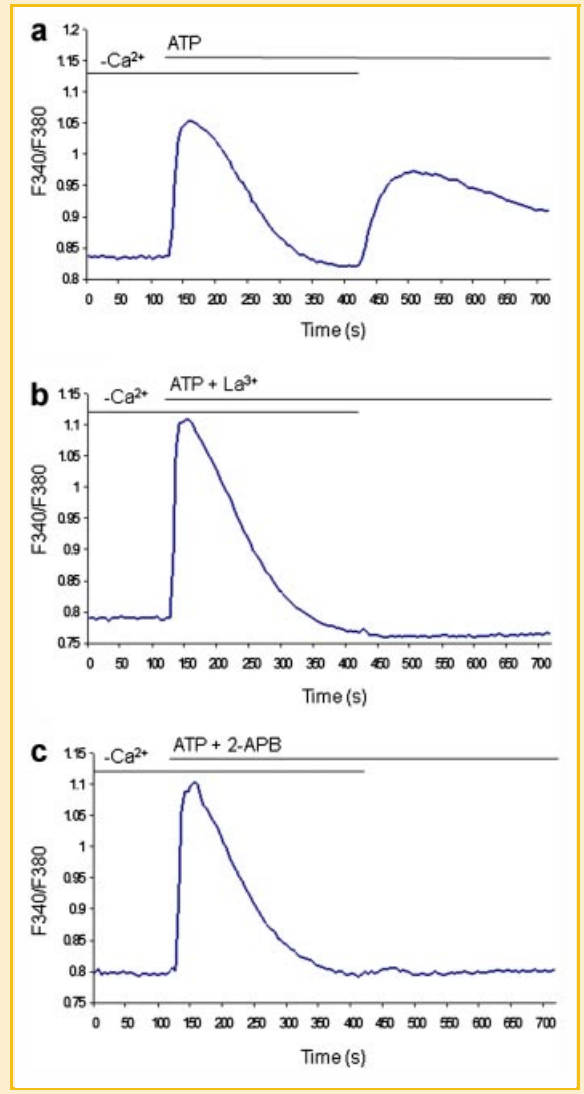

Fig. 8. Activation of "store-operated" channels mediated the plateau of ATP-induced $[Ca^{2+}]_i$ elevation in HKC-8 cells. a: Prolonged application of ATP (10 μM) in the absence of extracellular Ca^{2+} caused an initial rise in $[Ca^{2+}]_i$ followed by a decline to the initial baseline level. b: La^{3+} (100 μM) completely inhibited Ca^{2+} entry through SOCs without effecting Ca^{2+} release from stores. c: 2-APB (50 μM) inhibited Ca^{2+} entry through SOCs.

of Ca^{2+}, while prolonged activation of P2Y receptors induces a rise in $[Ca^{2+}]_i$ that is dependent on intra- and extracellular sources of Ca^{2+}. Pharmacological and molecular data in this study suggests that the ability of HKC-8 cells to utilize extracellular sources of Ca^{2+} in response to P2Y activation is due to activation of TRP channels. Finally, molecular data presented in this study indicates the presence of P2X receptors although the P2X agonist, α,β me-ATP, failed to cause a rise in $[Ca^{2+}]_i$.

Data from the present study indicates that the initial rise in $[Ca^{2+}]_i$ in response to extracellular ATP is dependent on intracellular Ca^{2+} stores as the response is not effected by removing external Ca^{2+}. This finding links the initial ATP-induced rise in $[Ca^{2+}]_i$ to activation of the G-protein coupled P2Y receptor. It is has been shown that activation of P2Y receptors will activate the membrane-bound enzyme phospholipase C, triggering the breakdown of the membrane constituents, PI and PC [Bucheimer and Linden, 2004]. The breakdown of PI leading to the production of inositol 1,4,5-trisphosphate (IP_3) and activation of the IP_3 receptor is thought to form the pathway leading to the rise in $[Ca^{2+}]_i$. However, findings from this study suggest that this pathway in HKC-8 cells may not be responsible for generating the initial rise in $[Ca^{2+}]_i$ as U73221, an inhibitor of PI-PLC, does not inhibit the initial ATP-induced response. Furthermore, an inhibitor of PC-PLC, D609, caused a significant decrease in the initial ATP-induced response, suggesting that P2Y receptors are coupled to PC-PLC in this model but may not be coupled to PI-PLC. A precedent for this finding has been reported in airway smooth muscle cells where it was found that D609, not U73122, inhibited ATP-induced short circuit current, which is

activated indirectly through the ATP-induced rise in $[Ca^{2+}]_i$ [Sato et al., 2005].

PCR data presented in this study indicates the presence of P2X receptors in HKC-8 cells. Consistent to a previous study [Takeda et al., 1998; Turner et al., 2003], the subtypes of P2X4 and P2X7, has also been detected in primary cultured human proximal tubules [Schwiebert, 2001]. PPADS as a general antagonist of P2X 1,2,3,4,5 [Bailey et al., 2008], has no effect on ATP-induced Ca^{2+} elevation and α,β me-ATP which activates P2X failed to induce a rise in $[Ca^{2+}]_i$ [Ralevic and Burnstock, 1998], suggesting stimulation of P2X alone in HKC-8 has less connection to cellular Ca^{2+} elevation. A similar mechanism presenting in miMCD-3 kidney tubular cells may occur, in which activation of P2X required P2 stimulation [Xia et al., 2004]. In addition, P2X receptor ion channels are permeable to Na^+ and K^+ ions, resulting in membrane depolarisation. Membrane depolarisation could affect the driving force of ion channels. In A6 cells basolateral P2X4 receptors are tightly involved to function of epithelial sodium channels [Zhang et al., 2007]. Studies in the primary cultured renal proximal tubule epithelial cells confirmed the ability of ATP to stimulate cellular proliferation as a consequence of stimulation of P2 receptors, possibly of the P2X subclass [Paller et al., 1998; Lee and Han, 2006]. It is intriguing in our results that PPNDS, as another P2X receptor antagonist, largely inhibited the ATP-induced rise in $[Ca^{2+}]_i$. If PPNDS only strongly affects P2X7 [Donnelly-Roberts et al., 2009], membrane depolarisation by P2X7 should not mask initial Ca^{2+} elevation due to Ca^{2+} release from store. Other potential effect of PPNDS on P2Y receptors may underline this big inhibition.

Inconsistent to previous studies [Takeda et al., 1998; Schwiebert and Kishore, 2001; Turner et al., 2003; Bailey, 2004; Lee and Han, 2006; Monaghan et al., 2006], we failed to detect the expression of P2Y1 and P2Y4 in HKC-8. The rational explanation for this discrepancy is counted upon the different species and the nature of proximal cell which deriving HKC-8. Most studies, which mapped the expression of P2 receptors in kidney, focused on rat tubules but not on human. Part of conclusion was drawn by the P2Y1 specific regulators, for example, MRS-2179 [Bailey, 2004] and 2MeSATP [Inglis et al., 1999]. In addition, the P2Y1 receptor was found on glomerular mesangial cells, the brush border membrane of the proximal straight tubule and on peritubular fibroblasts [Turner et al., 2003]. P2Y4 receptors were found on the tubule epithelium of the proximal convoluted tubule [Turner et al., 2003]. Therefore, nature of human proximal epithelial cell which derives HKC-8 is important to determine the expression of P2 receptors in HKC-8. With respect to function, P2Y2 receptors are activated fully by ATP and less potently by ADP whereas P2Y4 receptors are activated primarily by UTP and antagonised by ATP even at high receptor number [Bailey et al., 2008]. Nevertheless, our results demonstrated that P2Y receptor mediated ATP-induced Ca^{2+} elevation and it is associated with PC-PLC and TRP channel.

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