

## Extracellular ATP raises cytosolic calcium and activates basolateral chloride conductance in *Necturus* proximal tubule

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1. Extracellular nucleotides modulate ionic transport mechanisms in various epithelia. In the present study, we investigated the effects of extracellular ATP on the intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) and electrophysiological properties of *Necturus maculosus* proximal convoluted tubule (PCT).
2. ATP raised  $[\text{Ca}^{2+}]_i$  in microdissected fura-2-loaded PCTs (half-maximal effect,  $\sim 15 \mu\text{mol l}^{-1}$  ATP). The initial ATP-induced changes in  $[\text{Ca}^{2+}]_i$  were not blunted by the removal of external  $\text{Ca}^{2+}$  nor by the presence of  $\text{Ca}^{2+}$  channel blockers, but were abolished by thapsigargin and suramin. The sequence for the potency of various agonists on  $[\text{Ca}^{2+}]_i$  was 2-methylthioATP (2MeSATP) = ADP = ATP  $\gg$  UTP, 2',3'-O-(4-benzoylbenzoyl) ATP (BzATP),  $\alpha,\beta$ -methylene ATP (AMPCPP), adenosine.
3. *In vivo* electrophysiological measurements showed that  $100 \mu\text{mol l}^{-1}$  peritubular ATP added to a Ringer solution reduced the basolateral cell membrane potential ( $V_m$ ) and increased the cell membrane input conductance. In a low  $\text{Cl}^-$  solution, this ATP-induced depolarization was enhanced. These effects were inhibited by  $1 \text{ mmol l}^{-1}$  SITS, consistent with the activation of a basolateral  $\text{Cl}^-$  conductance.
4. The ATP-induced change in  $V_m$  was reproduced by ADP but not by UTP or adenosine, and was prevented by suramin.
5. The ATP-induced membrane depolarization was not influenced by thapsigargin, BAPTA AM, or staurosporin and was not reproduced by manoeuvres increasing  $[\text{Ca}^{2+}]_i$  or intracellular cAMP content.
6. We conclude that, in *Necturus* PCT, a P2y receptor mobilizes  $\text{Ca}^{2+}$  mainly from intracellular pools and increases a basolateral  $\text{Cl}^-$  conductance,  $G_{\text{Cl}}$ . The activation of  $G_{\text{Cl}}$  occurs by a mechanism which is not related either to an increase in  $[\text{Ca}^{2+}]_i$  or cAMP content, or to PKC activation.

Extracellular ATP concentration is known to be low compared with its intracellular level, but it may reach higher values locally since this nucleotide can be extruded from the cytosol by various mechanisms (Dubyak & El-Moatassim, 1993) and may thus act as a paracrine factor. There is growing evidence that extracellular ATP modulates ion transport systems in various cells, including epithelial cells. Most of these effects have been related to the binding of ATP to P2-type receptors or of its catabolite, adenosine, to P1 (also called A)-type receptors. In the case of ATP binding to P2 receptors, the modulation of ionic transport systems is either subsequent to the activation of a signal transduction system (ATP binds to a P2 receptor coupled to

a G protein) or direct (ATP binds to a channel–receptor), as detailed in the review by Dubyak & El-Moatassim (1993). In both cases, an increase in intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) is usually observed. Recently, it has been pointed out that extracellular ATP could activate  $\text{Cl}^-$  conductances in various secretory epithelia (Chan, Zhou & Wang, 1995; Hwang, Schwiebert & Guggino, 1996). In most cases, the transductional system underlying this response was identified, but in a few cases (Stutts, Chinet, Mason, Fullton, Clarke & Boucher, 1992; Stutts, Fitz & Paradiso, 1994; Guo, Merlin, Harvey, Laboisie & Hopfer, 1995), the effect of extracellular ATP on  $\text{Cl}^-$  conductance could not be related to any investigated signal transduction pathway, raising the

possibility of a direct effect of the nucleotide on  $\text{Cl}^-$  channels, or of the involvement of some unknown transduction system. In reabsorbing epithelia, it has also been observed that extracellular ATP modulates  $\text{Cl}^-$  transport in the distal part of the nephron (Middleton, Mangel, Basavappa & Fitz, 1993). In contrast, little is known concerning the effects of extracellular ATP on  $[\text{Ca}^{2+}]_i$  or on ionic transport in the proximal tubule of the nephron, the tubular segment of which reabsorbs about 60% of the filtered NaCl load. This relative lack of information might be due in part to the difficulty of applying  $\text{Ca}^{2+}$  measurement techniques in the intact proximal tubule (McCarthy & O'Neil, 1990). Thus, most studies focusing on the effects of extracellular ATP in proximal tubular cells have been performed on cell cultures: ATP increases  $[\text{Ca}^{2+}]_i$  in a primary culture of proximal cells (Cejka, Bidet, Tauc & Poujeol, 1993; Cejka, Le Maout, Bidet, Tauc & Poujeol, 1994) and in LLC-PK1, a cell line which shares common features with proximal tubular cells (Weinberg, Davis, Shayman & Knight, 1989). A recent study demonstrated that in isolated rabbit proximal tubule, extracellular ATP also increases  $[\text{Ca}^{2+}]_i$  via basolateral P2y receptors (Yamada, Seki, Taniguchi, Uwatoko, Suzuki & Kurokawa, 1996), without significant effect on the electrophysiological properties of the cell membrane.

We investigated the effects of extracellular ATP on  $[\text{Ca}^{2+}]_i$  and basolateral membrane potential ( $V_m$ ) in the proximal convoluted tubule (PCT) of the amphibian nephron. Our results are consistent with the presence of a P2y-type receptor and with the activation by extracellular ATP of a basolateral  $\text{Cl}^-$  conductance,  $G_{\text{Cl}}$ . However,  $G_{\text{Cl}}$  activation does not seem to be related to the  $[\text{Ca}^{2+}]_i$  increase or to the other investigated signal transduction systems.

## METHODS

### Biological material

Fluorimetric measurements were performed on microdissected PCTs from male *Necturus maculosus* kidneys (Nasco, WI, USA). Electrophysiological experiments were performed *in vivo* on PCTs of the same species. Animals were kept in tap water and fed twice a week with live *Chironoma*. Before experiments, the animals were anaesthetized by immersion in a 0.07% (w/v) solution of tricaine methane sulphonate. After the experiments, they were over-anaesthetized in a 0.7% (w/v) solution of tricaine methane sulphonate, then decapitated.

### Microdissection of PCTs

In order to prepare PCTs for microdissection, the abdominal cavity was exposed and the thoracic aorta cannulated to deliver 20 ml Ringer solution (see below) to rinse the blood from the kidneys. Iliac and mesenteric vessels were then ligatured, and the caudal vein was cannulated to deliver 10 ml Ringer solution supplemented with 220 U ml<sup>-1</sup> collagenase (Type 1A; Sigma). After removing and slicing the kidneys, enzymatic digestion of connective tissue was achieved by a 1 h incubation (at 26 °C) of kidney portions in a Ringer solution supplemented with 2200 U ml<sup>-1</sup> collagenase. Microdissection of PCTs was carried out at 4 °C in the Ringer solution. The viability of microdissected PCTs was attested by Trypan Blue extrusion.

### *In vivo* experiments

Anaesthesia was obtained as described above and maintained throughout the experiment by immersion of the branchiae in a 1/5th dilution of the anaesthetic solution. Dissection of the animal and exposure and superfusion of the kidneys with a Ringer solution during the experiment have been described elsewhere (Planelles, Kurkdjian & Anagnostopoulos, 1984).

### Measurement of $[\text{Ca}^{2+}]_i$ in microdissected PCTs

Microdissected PCTs were loaded with the  $\text{Ca}^{2+}$ -sensitive fluorescent probe fura-2 by a 45 min incubation in Ringer solution containing 10  $\mu\text{mol l}^{-1}$  fura-2 AM and 0.5% (v/v) Pluronic F-127, then rinsed in a large volume of Ringer solution for at least 15 min. One fura-2-loaded PCT was deposited onto a glass coverslip glued with silicone grease to a microperfusion chamber fixed to the stage of a microscope (Diaphot TMD; Nikon). The PCT adhered to the coverslip and was continuously superfused by one of six solutions delivered by gravimetry at a rate of  $\sim 350 \mu\text{l min}^{-1}$ , as detailed elsewhere (Paulais, Baudouin-Legros & Teulon, 1995).

Fura-2 fluorescence was monitored in ratio mode using a microfluorometer (Filterscan RF-F3010; Photon Technology International, South Brunswick, NJ, USA) as previously described (Paulais *et al.* 1995). Briefly, the light of a xenon arc lamp was alternately directed towards 340.8 nm (10.3 nm bandwidth) and 380 nm (9.5 nm bandwidth) interference filters (Oriol, Stratford, NJ, USA) then towards the specimen via a  $\times 40$ , 0.85 NA objective lens (CF Fluor; Nikon) coupled to a DM 400 dichroic mirror (Nikon). The light emitted at 510 nm (8.2 nm bandwidth) was detected by a photomultiplier tube, after restriction of its field of view to a  $\sim 250 \mu\text{m}^2$  rectangular area covering part of the tubule image. When long periods separated two consecutive measurements on the same preparation (see Results), unnecessary specimen illumination was cut in order to avoid fura-2 photobleaching. Fluorescence signals were collected for 8.5 ms at each excitation wavelength and integrated over 2 s periods. Data were stored on the hard disk of a personal computer (Brett Computers, France). All experiments were performed at room temperature ( $\sim 20$  °C). At the end of each experiment, the tubule was superfused with a  $\text{Ca}^{2+}$ -free (no  $\text{CaCl}_2$  added) Ringer solution supplemented with 5 mmol l<sup>-1</sup> MnCl<sub>2</sub> and 5  $\mu\text{mol l}^{-1}$  ionomycin. This manoeuvre quenches intracellular fura-2 fluorescence, so that background noise can be measured and subtracted from all fluorescence signals.

We calibrated fura-2 fluorescence *in situ* (Gryniewicz, Poenie & Tsien, 1985) on twenty fura-2-loaded PCTs superfused with 1  $\mu\text{mol l}^{-1}$  ionomycin in  $\text{Ca}^{2+}$ -containing (5 mmol l<sup>-1</sup>) Ringer solution, then in  $\text{Ca}^{2+}$ -free solution (no  $\text{Ca}^{2+}$  added, plus 5 mmol l<sup>-1</sup> EGTA). Experimental 340/380 fluorescence ratios ( $R$ ) were converted to  $[\text{Ca}^{2+}]_i$  according to the equation (Gryniewicz *et al.* 1985):

$$[\text{Ca}^{2+}]_i = K_d [(R - R_{\text{min}})/(R_{\text{max}} - R)] (S_{f380}/S_{s380}),$$

where  $K_d$ , the dissociation constant of fura-2 for  $\text{Ca}^{2+}$ , was 224 nmol l<sup>-1</sup> (Gryniewicz *et al.* 1985),  $R_{\text{max}}$  and  $R_{\text{min}}$  are the  $R$  values at saturating and  $\text{Ca}^{2+}$ -free conditions, respectively, and  $S_{f380}$  and  $S_{s380}$  are the signals emitted by the  $\text{Ca}^{2+}$ -free (f) and  $\text{Ca}^{2+}$ -saturated (s) forms of fura-2, respectively, when illuminated at 380 nm. We obtained successful calibration in nine tubules, yielding a resting  $[\text{Ca}^{2+}]_i$  of  $167 \pm 25 \text{ nmol l}^{-1}$ . Difficulty with *in situ* calibration of fura-2 has been reported by other groups on various tissues, including PCTs (McCarthy & O'Neil, 1990), and is sometimes circumvented by measuring calibration parameters from the fluorescence properties of fura-2 in solutions mimicking the intracellular ionic composition. We routinely used this latter procedure but obtained an unusually high mean  $[\text{Ca}^{2+}]_i$  value of

$306 \pm 22 \text{ nmol l}^{-1}$  ( $n = 140$ ). Thus, although the accuracy of extra-cellular calibration of fura-2 was established in mammalian renal tubules (Taniguchi, Marchetti & Morel, 1989; Nitschke, Fröbe & Greger, 1991), we did not extend it to *Necturus* PCTs, and reported our results as 340/380 ratios.

#### Electrophysiological measurements on PCT *in vivo*

**Microperfusions.** All experiments were performed at room temperature. Peritubular microperfusions were achieved by inserting a double- (or triple)-barrelled micropipette into a peritubular vessel with a micromanipulator (Leitz, Germany). Each barrel of the micropipette was connected to a catheter filled with an artificial solution. The solutions differed one from another by a single parameter. A gravimetric system allowed alternate delivery of the solutions.

Some experiments were performed in luminal oil-blocked tubules, in which the luminal compartment was virtually eliminated by injecting coloured castor oil via a single micropipette inserted in the glomerulus.

**Measurement of basolateral membrane potential.** Basolateral membrane potential,  $V_m$  (or transepithelial potential difference,  $V_{te}$ ) was measured by means of a microelectrode inserted into a cell (or into the lumen) of a PCT located in the microperfused area. Microelectrodes were pulled from capillary glass (o.d. 2 mm, i.d. 1.6 mm; Clarke, Pangbourne, UK) on a vertical puller (PE2; Narishige, Japan) and filled with 1 mol l<sup>-1</sup> KCl. Resistance of the microelectrodes was 40–60 MΩ. The microelectrode was placed on a micromanipulator (MM1; Narishige) and was connected via a Ag/AgCl pellet holder to the input of an electrometer (FD 223; WPI, USA). The output of the electrometer was connected to a multipen chart recorder (Servofram; Sefram, France). A 1 mol l<sup>-1</sup> KCl–Ag/AgCl macroelectrode placed in the peritoneal cavity closed the electrical circuit.

**Measurement of cell membrane input conductance.** Cell membrane input conductance was assessed by inserting two microelectrodes into the cellular layer of a single PCT. One microelectrode was connected to a current generator/electrometer delivering constant current pulses (intensity, 30 to 80 nA; time duration, 1 s; interval between two consecutive pulses, 4 s). The second microelectrode recorded  $V_m$  and the superimposed electrotonic potentials,  $dV_m$ , induced by the injected current pulses. In this experimental configuration,  $dV_m$  results from current distribution along the cellular cable and is a function of the cell membrane conductance and the distance between the recording microelectrode and the source of stimulation.  $dV_m$  was measured under control conditions (ctrl), then in the experimental condition (exptl). Since the experiments were performed in paired fashion (so that the distance between the two electrodes was constant), the ratio  $dV_{m,exptl}/dV_{m,ctrl}$  can be taken as a qualitative index of the change of cell membrane input conductance (Teulon, Planelles & Anagnostopoulos, 1978), a value of  $dV_{m,exptl}/dV_{m,ctrl} < 1$  indicating an increase in cell membrane input conductance, and conversely. By locating both microelectrodes in the tubular lumen and by recording  $V_{te}$  and  $dV_{te}$ , the change in transepithelial input conductance was estimated by the ratio  $dV_{te,exptl}/dV_{te,ctrl}$ .

**Measurement of the voltage divider ratio.** The ratio of the cell membrane resistances was estimated by inserting the punctual current source (a microelectrode connected to the current generator delivering rectangular pulses as described above, with a current intensity of 100–160 nA) into the lumen of a PCT. The recording microelectrode, sequentially inserted at the same location into the cell then advanced into the lumen, as previously described

(Anagnostopoulos, Teulon & Edelman, 1981), sequentially measured the induced  $dV_m$  and  $dV_{te}$ . In this configuration,  $dV_m$  results from current flow out of the lumen across the transcellular route. Thus, the voltage divider ratio, VDR ( $dV_m/dV_{te}$ ) is an index of the ratio of the basolateral over the apical membrane resistances. For a single PCT, VDR was obtained in paired fashion, first during peritubular Ringer perfusion, then in the presence of ATP: a decrease in VDR indicates a decrease in the fractional resistance of the basolateral membrane, and conversely.

#### Solutions

The composition of the amphibian Ringer solution was (mmol l<sup>-1</sup>): 100 NaCl, 3 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, buffered at pH 7.6 with 5 Tes-NaOH. For the low Cl<sup>-</sup> solution, 100 mmol l<sup>-1</sup> sodium gluconate was substituted for NaCl, and CaCl<sub>2</sub> was increased to 5.6 mmol l<sup>-1</sup> to compensate for Ca<sup>2+</sup> chelation by gluconate<sup>-</sup>.

#### Chemicals

ATP, ADP, 2',3'-O-(4-benzoylbenzoyl) ATP (BzATP),  $\alpha,\beta$ -methylene ATP (AMPCPP), uridine 5'-triphosphate (UTP), adenosine, staurosporin, 8-4-chlorophenylthio-cAMP (8-4-CPT cAMP) and Reactive Blue 2 (RB2) were obtained from Sigma, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) and 2-methylthioATP (2MeSATP) from RBL. Suramin and thapsigargin (TG) were from Calbiochem and Alexis Corp. (San Diego, CA, USA), respectively. Fura-2, fura-2 AM, Pluronic acid F127 and BAPTA AM were from Molecular Probes. Diphenylamine 2-carboxylic acid (DPC) was purchased from Merck and SITS from Aldrich or Research Organic, Inc. (Cleveland, OH, USA). Calix-4-arene was obtained from Acros Organics (Pittsburg, PA, USA) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was a generous gift from Dr H. J. Lang (Hoechst Laboratory). In solutions containing one of the following compounds: ATP, ADP, BzATP, AMPCPP, UTP, adenosine and 2MeSATP, total CaCl<sub>2</sub> and MgCl<sub>2</sub> concentrations were calculated according to Fabiato (1981) to obtain 1.8 and 1 mmol l<sup>-1</sup>, respectively, of free salts.

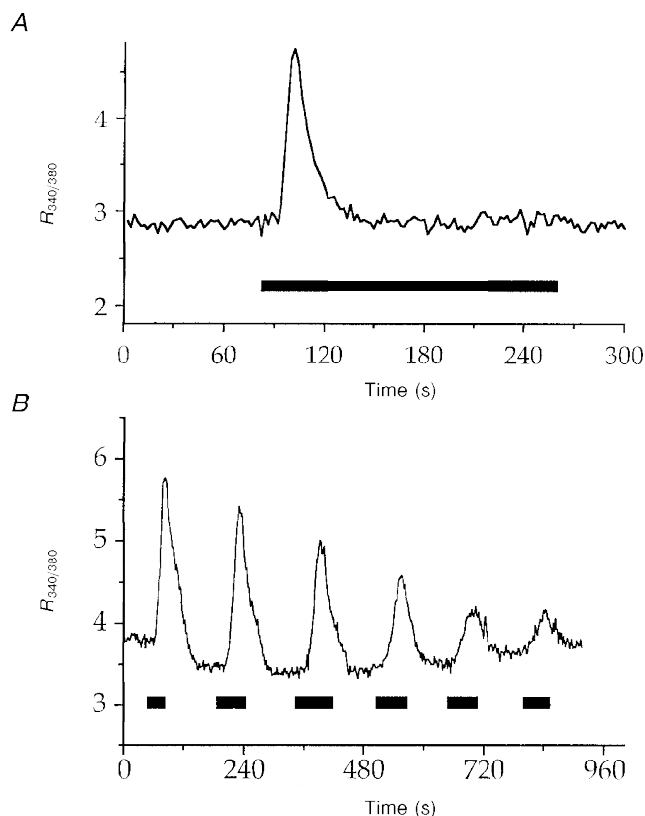
#### Data analysis

Unless stated in the text, results are expressed as means  $\pm$  s.e.m., with  $n$  the number of observations, and statistical significance was assessed by Student's paired  $t$  test.

## RESULTS

### Effects of external ATP on $[Ca^{2+}]_i$ in *Necturus* PCT

The effects of ATP addition to the superfusate on the 340/380 fluorescence ratio,  $R$ , in fura-2-loaded PCTs are illustrated in Fig. 1A. ATP (100  $\mu\text{mol l}^{-1}$ ) systematically induced a rapid increase in  $[Ca^{2+}]_i$ , followed by a slower decline. In this experimental series, the mean basal  $R$  value was  $3.3 \pm 0.3$  ( $n = 28$ ). The maximal amplitude of the change in  $R$ ,  $\Delta R$ , induced by 100  $\mu\text{mol l}^{-1}$  ATP varied from tubule to tubule, the mean  $\Delta R$  being  $1.8 \pm 0.2$  ( $n = 28$ ). When ATP exposure was limited to 1 min ( $n = 20$ ),  $[Ca^{2+}]_i$  in most tubules was clearly still declining ( $n = 12$ ), whereas it appeared to stabilize for eight tubules. In all twenty of these tubules,  $[Ca^{2+}]_i$  slowly recovered its resting level upon ATP withdrawal. To test whether  $[Ca^{2+}]_i$  could recover its basal level in the continuous presence of ATP, eight tubules were exposed to ATP over 3 min (Fig. 1A). Under this experimental condition, a transient initial increase in  $[Ca^{2+}]_i$  was observed, then  $[Ca^{2+}]_i$  reached a level not statistically



**Figure 1. ATP increases cytosolic free calcium concentration in isolated fura-2-loaded *Necturus* PCT**

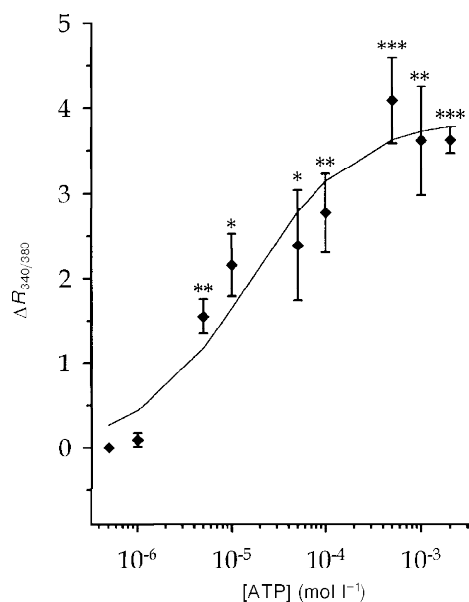
Fura-2 fluorescence was monitored and converted to  $R_{340/380}$  values as described in Methods. *A*, a PCT was continuously superfused with Ringer medium and exposed to  $100 \mu\text{mol l}^{-1}$  ATP for the period indicated by the horizontal bar. *B*, effects of repeated exposures to ATP on  $[\text{Ca}^{2+}]_i$  of *Necturus* PCT. A fura-2-loaded PCT bathed in Ringer medium was exposed to  $100 \mu\text{mol l}^{-1}$  ATP for the periods indicated by the horizontal bars. The trace shown is representative of 4 similar experiments.

different ( $P = 0.4$ ,  $n = 8$ ) from the resting level, and ATP withdrawal induced no further change in  $[\text{Ca}^{2+}]_i$ .

As shown in Fig. 1*B*, repeated short ( $\sim 1$  min) exposures of PCTs to  $100 \mu\text{mol l}^{-1}$  ATP caused a progressive reduction in the amplitude of the  $[\text{Ca}^{2+}]_i$  response, until it almost vanished. We determined that under this experimental condition a washing-out period of at least 10 min with Ringer solution between two consecutive short ATP exposures was necessary to overcome this phenomenon (not

shown). When appropriate, longer ATP exposures were separated by a washing-out period of at least 25 min.

The effects of ATP on  $[\text{Ca}^{2+}]_i$  were concentration dependent. In a separate series of experiments ( $n = 27$ ), responses to various ATP concentrations (ranging from  $500 \text{ nmol l}^{-1}$  to  $2 \text{ mmol l}^{-1}$ ) were observed and quantified by measuring  $\Delta R$ . Figure 2 summarizes these results. For each ATP concentration inducing  $[\text{Ca}^{2+}]_i$  changes, the response was characterized by a transient peak, the amplitude of which



**Figure 2. Relationship between extracellular ATP concentration and changes in  $R_{340/380}$  in *Necturus* PCT**

Experimental procedures were as described for Fig. 1.  $\Delta R_{340/380}$  represents the maximal change in  $R_{340/380}$  above baseline induced by each ATP concentration. Results are shown as means, and s.e.m. are represented by vertical bars ( $n \geq 3$  for all conditions). Data were fitted by the equation  $\Delta R = \Delta R_{\text{max}} / (1 + ([\text{ATP}] / K)^n)$ , where  $\Delta R_{\text{max}}$  (the maximum  $\Delta R$ ) was  $3.9 \pm 0.4$ ,  $K$  (the ATP concentration yielding the half-maximal effect) was  $15 \pm 8 \mu\text{mol l}^{-1}$  and  $n$  (the Hill coefficient) was  $0.8 \pm 0.2$  (mean  $\pm$  s.d.). Average resting  $R_{340/380}$  was  $3.38 \pm 0.2$  for this series of experiments.

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  vs. resting level.



increased with ATP concentration. From these results, the ATP concentration inducing a half-maximal response was about  $15 \mu\text{mol l}^{-1}$  (see legend to Fig. 2).

### Changes in $[\text{Ca}^{2+}]_i$ upon PCT exposure to various ATP analogues and effects of antagonists

The above results suggested that extracellular ATP raised  $[\text{Ca}^{2+}]_i$  through its binding to a membrane receptor. To establish its pharmacological profile, the effects of various nucleotides and adenosine on  $[\text{Ca}^{2+}]_i$  were studied. Due to the variability of the ATP-induced transient  $[\text{Ca}^{2+}]_i$  peak from tubule to tubule (see above), the effect of  $100 \mu\text{mol l}^{-1}$  of each compound was compared with that of  $100 \mu\text{mol l}^{-1}$  ATP on the same tubule in paired fashion. Results are summarized in Fig. 3. ADP (an agonist of P2x, P2y and P2t receptors) and 2MeSATP (a P2y receptor agonist) elicited transient  $[\text{Ca}^{2+}]_i$  responses qualitatively similar to those obtained with ATP. The maxima of the ADP effect ( $\Delta R = 2.3 \pm 0.6$ ,  $n = 8$ ) or the 2MeSATP effect ( $\Delta R = 2.9 \pm 0.4$ ,  $n = 7$ ) were not statistically different from the paired changes under ATP ( $P = 0.5$  and  $P = 0.2$ , respectively). By contrast, as shown in Fig. 3, UTP (a P2u receptor agonist) and BzATP (a P2z receptor agonist) elicited much smaller transients in  $[\text{Ca}^{2+}]_i$  as compared with the paired ATP effect ( $\Delta R = 0.2 \pm 0.1$  vs.  $1.4 \pm 0.4$  for UTP vs. ATP,  $n = 5$ , and  $\Delta R = 0.6 \pm 0.2$  vs.  $3.5 \pm 0.7$  for BzATP vs. ATP,  $n = 6$ ). Figure 3 also shows the lack of effect of AMPCPP (a P2x receptor agonist) and adenosine (a P1 receptor agonist) on  $[\text{Ca}^{2+}]_i$ , yielding the following potency sequence: 2MeSATP = ADP = ATP  $\gg$  UTP, BzATP, AMPCPP, adenosine.

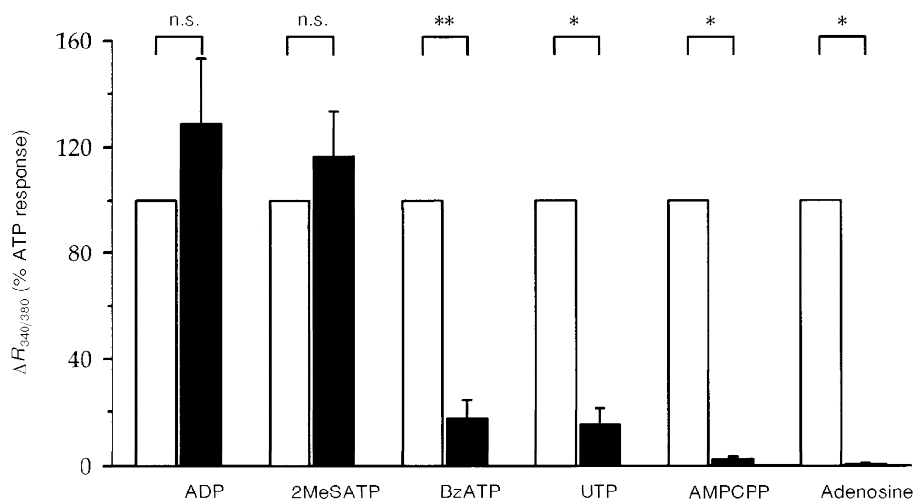
Next, we investigated the effect of various P2 receptor antagonists on the ATP-induced  $[\text{Ca}^{2+}]_i$  response; as in the previous series, experiments were conducted in a paired fashion. As shown in Fig. 4, the response to  $100 \mu\text{mol l}^{-1}$

ATP was dramatically reduced by  $300 \mu\text{mol l}^{-1}$  suramin, a non-specific P2 receptor antagonist ( $\Delta R = 0.2 \pm 0.1$  vs.  $\Delta R = 1.2 \pm 0.2$ ,  $P < 0.01$ ,  $n = 8$ ). By contrast, neither  $10 \mu\text{mol l}^{-1}$  RB2, nor up to  $100 \mu\text{mol l}^{-1}$  PPADS had an inhibitory action on the ATP-induced  $[\text{Ca}^{2+}]_i$  effect.

### Origin of the increase in $[\text{Ca}^{2+}]_i$ on PCT exposure to extracellular ATP

The binding of ATP to the P2y-type receptor generally leads to a  $[\text{Ca}^{2+}]_i$  increase due to mobilization of  $\text{Ca}^{2+}$  contained in intracellular stores. We therefore investigated whether thapsigargin (TG), an inhibitor of the non-mitochondrial  $\text{Ca}^{2+}$ -ATPase which causes depletion of intracellular  $\text{Ca}^{2+}$  stores, could influence the response to ATP. Addition of  $2 \mu\text{mol l}^{-1}$  TG to the superfusate for 10 min induced a slow increase in  $R$  from  $4.7 \pm 0.4$  to  $7.4 \pm 1.3$ . After this treatment, addition of  $100 \mu\text{mol l}^{-1}$  ATP had no significant effect on  $[\text{Ca}^{2+}]_i$  ( $R = 7.9 \pm 1.6$ ,  $P = 0.3$ ,  $n = 5$ ). These results suggest that  $\text{Ca}^{2+}$  mobilization from intracellular pools is a major component of the ATP-induced response.

Next, we investigated whether extracellular  $\text{Ca}^{2+}$  also participates in the ATP-induced  $[\text{Ca}^{2+}]_i$  transient. We observed that the presence of the  $\text{Ca}^{2+}$  channel blockers  $\text{La}^{3+}$  ( $50 \mu\text{mol l}^{-1}$ ), nifedipine ( $50 \mu\text{mol l}^{-1}$ ) and verapamil ( $100 \mu\text{mol l}^{-1}$ ) did not affect the ATP-induced  $[\text{Ca}^{2+}]_i$  changes (Table 1). Then we measured the ATP-induced  $[\text{Ca}^{2+}]_i$  transients upon adding ATP to a  $\text{Ca}^{2+}$ -free medium. We exposed five PCTs simultaneously to  $1 \text{ mmol l}^{-1}$  ATP (a concentration which gave near-maximal changes in  $[\text{Ca}^{2+}]_i$ , see Fig. 2) and to a  $\text{Ca}^{2+}$ -free medium supplemented with  $100 \mu\text{mol l}^{-1}$  EGTA. When compared with the effect of  $1 \text{ mmol l}^{-1}$  ATP in  $\text{Ca}^{2+}$ -containing medium, we did not observe any quantitative or qualitative modification of the  $[\text{Ca}^{2+}]_i$  changes (not shown). Then, we measured in paired



**Figure 3.** Relative potency of various nucleotides and adenosine on  $[\text{Ca}^{2+}]_i$

Experimental procedures were as described for Fig. 1. The maximal change in  $R_{340/380}$  above baseline ( $\Delta R_{340/380}$ ) induced by  $100 \mu\text{mol l}^{-1}$  of the indicated compound was normalized to that of  $100 \mu\text{mol l}^{-1}$  ATP (open bars) on the same tubule. Results are given as means and s.e.m. are represented by vertical bars ( $n \geq 5$  for all conditions). n.s., not significantly different from ATP. \*  $P < 0.05$  and \*\*  $P < 0.01$  vs. ATP.

Table 1. Changes in  $[Ca^{2+}]_i$  ( $\Delta R$ ) during ATP exposure

Superfusate	n	ATP ( $100 \mu\text{mol l}^{-1}$ )		
		$\Delta R$ , peak	$\Delta R$ , 1 min 30 s	$\Delta R$ , 3 min
Control	3	$1.09 \pm 0.23$	$0.12 \pm 0.07$	$0.02 \pm 0.01$
La <sup>3+</sup> ( $50 \mu\text{mol l}^{-1}$ )		$0.95 \pm 0.21$ n.s.	$0.06 \pm 0.08$ n.s.	$-0.04 \pm 0.04$ n.s.
Control	5	$1.49 \pm 0.29$	$0.19 \pm 0.10$	$-0.09 \pm 0.05$
Verapamil ( $100 \mu\text{mol l}^{-1}$ )		$1.01 \pm 0.29$ n.s.	$-0.19 \pm 0.13$ n.s.	$0.01 \pm 0.03$ n.s.
Control	6	$1.24 \pm 0.30$	$0.13 \pm 0.04$	$0.01 \pm 0.02$
Nifedipine ( $50 \mu\text{mol l}^{-1}$ )		$1.12 \pm 0.41$ n.s.	$0.14 \pm 0.05$ n.s.	$0.05 \pm 0.01$ n.s.
Control	14	$1.13 \pm 0.01$	$0.31 \pm 0.05$	$0.09 \pm 0.03$
Ca <sup>2+</sup> -free + EGTA		$1.18 \pm 0.15$ n.s.	$0.20 \pm 0.03$ *	$0.03 \pm 0.01$ n.s.

Changes in  $[Ca^{2+}]_i$  (expressed as the variation in the  $R_{340/380}$  ratio compared with its basal value ( $\Delta R$ ) induced by adding  $100 \mu\text{mol l}^{-1}$  ATP in a Ringer superfusate (control) or in a modified superfusate (Ca<sup>2+</sup>-free or supplemented with a Ca<sup>2+</sup> channel blocker).  $\Delta R$  was measured at its maximal change ('peak'), then after 1 min 30 s or 3 min of ATP exposure. \*  $P < 0.05$ ; n.s., not significant.

fashion and in a random sequence the changes in  $[Ca^{2+}]_i$  upon adding ATP to a Ca<sup>2+</sup>-containing medium or in a Ca<sup>2+</sup>-free medium (no CaCl<sub>2</sub> added plus  $100 \mu\text{mol l}^{-1}$  EGTA). PCTs were exposed for 3 min successively to  $100 \mu\text{mol l}^{-1}$  ATP; a 25 min period of washing separated the two ATP challenges. In a first series ( $n = 7$ ), preincubation in Ca<sup>2+</sup>-free medium was limited to 30 s before adding ATP, to prevent possible depletion of Ca<sup>2+</sup> stores (Yamada *et al.* 1996). For four out of the seven PCTs, the withdrawal of extracellular Ca<sup>2+</sup> decreased basal  $[Ca^{2+}]_i$ , but this fall did not reach statistical significance ( $\Delta R = -0.2 \pm 0.1$ ,  $n = 4$ ,  $P = 0.1$ ), and in the three other PCTs, this manoeuvre had

no visible effect on  $[Ca^{2+}]_i$ ; thus removal of external Ca<sup>2+</sup> for 30 s had no significant effect on basal  $[Ca^{2+}]_i$  ( $R = 3.4 \pm 0.7$  vs.  $3.3 \pm 0.7$ ,  $n = 7$ ,  $P = 0.2$ ). Addition of ATP to the Ca<sup>2+</sup>-free medium led to a transient increase in  $[Ca^{2+}]_i$  similar to that obtained in the presence of external Ca<sup>2+</sup> ( $\Delta R = 1.63 \pm 0.45$  vs.  $1.25 \pm 0.25$ ,  $n = 7$ ,  $P = 0.4$ ). However, it can be observed from the representative tracing from this series shown in Fig. 5 that the readmission of external Ca<sup>2+</sup> induced an increase in  $[Ca^{2+}]_i$ ; this observation prompted us to lengthen the preincubation in Ca<sup>2+</sup>-free medium before checking the effect of ATP addition. Thus, in a second paired experimental series ( $n = 14$ ), we measured the ATP-

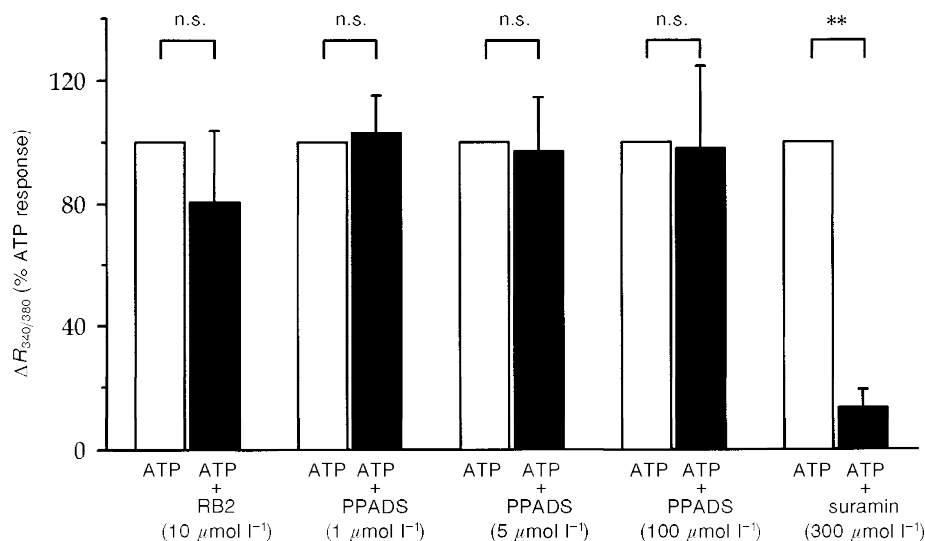


Figure 4. Effects of various P2 receptor antagonists on the response to ATP

The effect of each indicated compound on the response to  $100 \mu\text{mol l}^{-1}$  ATP was observed and quantified as for Fig. 3. Results are given as means and s.e.m. are represented by vertical bars ( $n \geq 4$  for all conditions). n.s., not significantly different from ATP. \*\*  $P < 0.01$  vs. ATP.

Table 2. Effect of ATP on basolateral membrane potential

Experimental series	<i>n</i>	$V_m$ (Ringer)	$V_m$ (Ringer + ATP)	( $\Delta V_m$ )
ATP (50 $\mu\text{mol l}^{-1}$ )	3	$-71.7 \pm 4.2$	$-65.0 \pm 4.3^*$	$(6.6 \pm 1.6)$
ATP (100 $\mu\text{mol l}^{-1}$ )	16	$-63.4 \pm 2.5$	$-54.7 \pm 2.4^{***}$	$(9.3 \pm 0.8)$
ATP (1 $\text{mmol l}^{-1}$ )	8	$-54.1 \pm 3.7$	$-39.7 \pm 4.0^{***}$	$(14.6 \pm 1.1)$

Basolateral membrane potential values ( $V_m$ , mV) measured in proximal tubular cells during a peritubular Ringer perfusion and upon addition of extracellular ATP at various concentrations, as indicated by the experimental series. The amplitudes of changes in  $V_m$  ( $\Delta V_m$ ) induced by the presence of ATP are indicated in parentheses. \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ .

induced  $[\text{Ca}^{2+}]_i$  change in the presence of external  $\text{Ca}^{2+}$ , or after 5 or 10 min ( $n = 6$  and 8, respectively) exposure to the  $\text{Ca}^{2+}$ -free solution. In all but one tubule, ATP still induced a transient change in  $[\text{Ca}^{2+}]_i$ . Table 1 shows that after this long  $\text{Ca}^{2+}$ -free exposure, the maximal ATP-induced  $[\text{Ca}^{2+}]_i$  increase was unchanged; however, when measured at the mid-time of ATP exposure,  $\Delta R$  was significantly lower than the paired value measured in the presence of external  $\text{Ca}^{2+}$  ( $P < 0.05$ ,  $n = 14$ ). Taken together, these results suggest that  $\text{Ca}^{2+}$  entry from extracellular medium may transiently participate in the ATP-induced  $[\text{Ca}^{2+}]_i$  changes.

#### Effects of extracellular ATP on cell membrane potential and conductance

As indicated in Table 2, addition of ATP (100  $\mu\text{mol l}^{-1}$ ) to the peritubular Ringer perfusate significantly changed  $V_m$  by  $+9.3 \pm 0.8$  mV ( $n = 16$ ,  $P < 0.001$ ). During ATP exposure, this depolarization was either sustained (as shown in Fig. 6) or followed by a partial recovery;  $V_m$  change was usually reversible upon ATP withdrawal. As also shown in Table 2, 50  $\mu\text{mol l}^{-1}$  ATP induced a smaller change in  $V_m$  (transient

spike depolarization), while 1  $\text{mmol l}^{-1}$  ATP induced a larger membrane depolarization (sustained and poorly reversible upon ATP withdrawal).

Addition of 100  $\mu\text{mol l}^{-1}$  ATP to the peritubular Ringer solution had no influence on  $V_{te}$  or  $dV_{te}$  ( $P > 0.9$ ,  $n = 6$ ). In contrast, this experimental manoeuvre induced, concomitantly with the above-mentioned  $V_m$  change, a biphasic change in the cell membrane input conductance:  $dV_{m,ATP}/dV_{m,Ringer}$  immediately decreased to  $0.74 \pm 0.03$  ( $n = 13$ ,  $P < 0.001$ ), then rapidly rose to  $1.29 \pm 0.11$ , a value significantly higher than the resting level ( $P < 0.01$ ); this secondary rise dissipated slowly upon ATP removal.

To better ascertain whether these changes reflected changes in cell membrane properties, VDR measurements were performed. The initial effect of adding 100  $\mu\text{mol l}^{-1}$  ATP to the basolateral Ringer perfusate was a significant decrease in VDR, from  $0.41 \pm 0.01$  to  $0.28 \pm 0.03$  ( $n = 8$ ,  $P < 0.001$ ). During ATP exposure, VDR stabilized ( $n = 3$ ), or returned (partly,  $n = 3$  or fully,  $n = 2$ ) towards its initial value; a VDR increase over the control level was not observed.

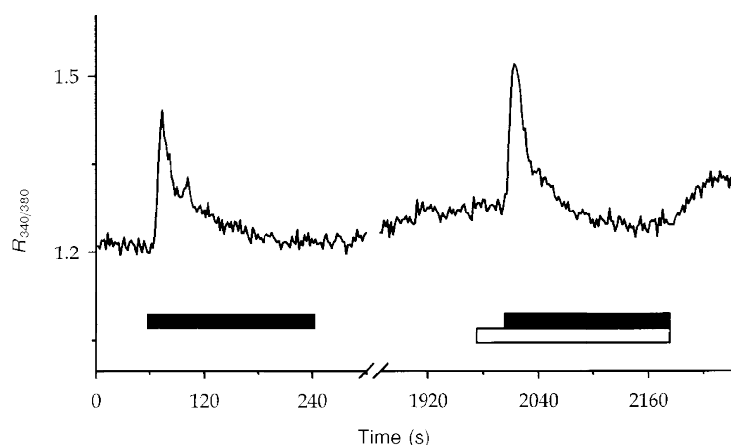


Figure 5. Effect of ATP in the presence and absence of extracellular  $\text{Ca}^{2+}$

Experimental procedures were as described for Fig. 1. A PCT was exposed to 100  $\mu\text{mol l}^{-1}$  ATP for the periods indicated by the horizontal filled bars successively in the presence (1.8  $\text{mmol l}^{-1}$   $\text{CaCl}_2$ ) and absence (100  $\mu\text{mol l}^{-1}$  EGTA, open bar) of external  $\text{Ca}^{2+}$ . The break in the trace represents a 25 min period of superfusion with Ringer solution.

Table 3. Effect of ATP on basolateral membrane potential in the presence of various inhibitors

Experimental series	<i>n</i>	Control $V_m$	+ATP (100 $\mu\text{mol l}^{-1}$ )			
			Initial effect		Final effect	
			$V_m$	( $\Delta V_m$ )	$V_m$	( $\Delta V_m$ )
Ba <sup>2+</sup> (2 mmol l <sup>-1</sup> )	9	-29.9 ± 1.6	-36.7 ± 1.1 ***	(-7.4 ± 0.7)	-30.0 ± 1.8 <sup>n.s.</sup>	(0.5 ± 1.3)
DPC (500 $\mu\text{mol l}^{-1}$ )	6	-59.5 ± 3.8	-52.0 ± 4.2 **	(7.5 ± 1.8)	-53.5 ± 4.1 *	(6.0 ± 1.8)
SITS (1 mmol l <sup>-1</sup> )	16	-67.5 ± 2.6	-66.0 ± 2.7 ***	(1.4 ± 0.3)	-68.4 ± 2.6 ***	(0.9 ± 0.2)

Basolateral membrane potential values ( $V_m$ , mV) of proximal tubular cells measured in a control condition (peritubular Ringer solution perfusate, supplemented with an inhibitor as indicated by the experimental series) and in the presence of ATP (100  $\mu\text{mol l}^{-1}$ ) added to the control solution. The changes in  $V_m$  ( $\Delta V_m$ ) induced by the presence of ATP are in parentheses. \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; n.s., not significant, compared with control  $V_m$  values.

### Basolateral membrane mechanism underlying the electrophysiological effects of ATP

We used a K<sup>+</sup> conductance inhibitor to depolarize the basolateral membrane: BaCl<sub>2</sub> (2 mmol l<sup>-1</sup>) was added both to the control and the ATP-containing solutions. Under this condition, 100  $\mu\text{mol l}^{-1}$  ATP induced a transient membrane hyperpolarization (Table 3), associated with a large increase in the cell membrane input conductance ( $dV_{m,\text{ATP}}/dV_{m,\text{Ba}} = 0.42 \pm 0.08$ ,  $n = 6$ ,  $P < 0.001$ ). During ATP exposure, the initial membrane hyperpolarization was followed by its repolarization (Table 3), and  $dV_m$  returned towards its control value ( $0.91 \pm 0.04$ ,  $P = 0.2$ ).

Next, we examined the effect of ATP on  $V_m$  in the presence of SITS and DPC, these inhibitors of anionic transport systems being added both to the Ringer and to the ATP-containing solutions. Table 3 shows the  $V_m$  response on adding 100  $\mu\text{mol l}^{-1}$  ATP in the presence of these compounds: 500  $\mu\text{mol l}^{-1}$  DPC did not significantly modify ( $P = 0.3$ , Student's unpaired *t* test) the amplitude of the previously reported ATP-induced depolarization ( $9.3 \pm 0.8$  mV,  $n = 16$ ), whereas in the presence of 1 mmol l<sup>-1</sup> SITS, ATP induced only a slight membrane

depolarization ( $1.4 \pm 0.3$  mV,  $n = 16$ ), significantly smaller than that observed in the absence of inhibitor ( $P < 0.001$ , unpaired *t* test). In the presence of SITS, this tiny  $V_m$  change was not accompanied by an initial change in the  $dV_m$  value ( $dV_{m,\text{ATP}}/dV_{m,\text{SITS}} = 0.95 \pm 0.03$ ,  $n = 8$ ,  $P = 0.14$ ), whereas its secondary increase was still observed ( $dV_{m,\text{ATP}}/dV_{m,\text{SITS}} = 1.17 \pm 0.06$ ,  $n = 8$ ,  $P < 0.05$ ).

These observations raise the possibility that the early effect of ATP is an increase in the basolateral Cl<sup>-</sup> conductance,  $G_{\text{Cl}}$ . To ascertain this point, a different protocol of peritubular perfusion was introduced: a triple-barrelled micropipette allowed alternative delivery of a Ringer solution, a low Cl<sup>-</sup> solution, or a low Cl<sup>-</sup> solution supplemented with 100  $\mu\text{mol l}^{-1}$  ATP. This protocol allowed an evaluation of  $G_{\text{Cl}}$  in the control condition and in the presence of ATP. Table 4 shows the hyperpolarizing effect of a low Cl<sup>-</sup> solution and the large  $V_m$  change ( $+17.4 \pm 1.7$  mV,  $n = 14$ ) induced by the addition of ATP to this solution. These results are consistent with a low basal  $G_{\text{Cl}}$ , largely increased by ATP. A typical experiment from this series is illustrated by Fig. 7. It can also be noticed from this figure that ATP withdrawal did not totally repolarize the membrane to the



Figure 6. Effect on basolateral membrane potential of ATP (100  $\mu\text{mol l}^{-1}$ ), added to a basolateral Ringer perfusion

Tracing obtained with an intracellular microelectrode. Timing of perfusion with experimental solutions is indicated by horizontal bars below the graph: filled bar, 100  $\mu\text{mol l}^{-1}$  ATP; open bar, Ringer solution. Before and after these experimental solutions, perfusion was with blood from normal circulation. In this experiment, ATP induced a sustained membrane depolarization.



Table 4. Effect of various inhibitors on the ATP-induced change in basolateral membrane potential

Experimental series	n	Control $V_m$	+ATP (100 $\mu\text{mol l}^{-1}$ )		
			Low $\text{Cl}^-$ condition		
			$V_m$	$V_m$	( $\Delta V_m$ )
No inhibitor	14	$-67.5 \pm 1.9$	$-74.3 \pm 2.1$	$-57.3 \pm 3.0^{***}$	( $17.4 \pm 1.7$ )
DPC (500 $\mu\text{mol l}^{-1}$ )	11	$-59.1 \pm 2.3$	$-63.8 \pm 3.5$	$-48.4 \pm 3.8^{**}$	( $12.7 \pm 2.1$ )
Niflumic acid (100 $\mu\text{mol l}^{-1}$ )	4	$-62.5 \pm 3.4$	$-70.0 \pm 3.8$	$-46.5 \pm 3.3^{**}$	( $21.0 \pm 4.1$ )
NPPB (100 $\mu\text{mol l}^{-1}$ )	5	$-57.8 \pm 2.5$	$-61.8 \pm 3.1$	$-44.2 \pm 3.4^{***}$	( $17.2 \pm 1.1$ )
Calix-4-arene (100 nmol $\text{l}^{-1}$ )	4	$-63.7 \pm 3.8$	$-70.7 \pm 3.4$	$-42.5 \pm 2.6^{**}$	( $26.5 \pm 4.1$ )
SITS (1 mmol $\text{l}^{-1}$ )	7	$-70.7 \pm 3.0$	$-79.3 \pm 3.4$	$-79.0 \pm 3.3^{\text{n.s.}}$	( $-0.3 \pm 0.5$ )

Basolateral membrane potential values ( $V_m$ , mV) of proximal tubular cells measured in a control condition (peritubular perfusate with normal  $\text{Cl}^-$  concentration), in a low  $\text{Cl}^-$  condition (peritubular perfusate in which sodium gluconate replaces NaCl) and in the low  $\text{Cl}^-$  condition supplemented with 100  $\mu\text{mol l}^{-1}$  ATP. The changes in  $V_m$  ( $\Delta V_m$ ) induced by the presence of ATP to the low  $\text{Cl}^-$  solution are indicated in parentheses. The experimental series indicates when an inhibitor was present in the perfusates. \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; n.s., not significant compared with the low  $\text{Cl}^-$  condition.

previous  $V_m$  value, suggesting that  $G_{\text{Cl}}$  did not immediately return to its basal state. Similar results were obtained in luminal oil-blocked tubules: adding 100  $\mu\text{mol l}^{-1}$  ATP to the low  $\text{Cl}^-$  solution depolarized the basolateral membrane by  $22.8 \pm 2.9$  mV ( $n = 6$ ), a value not different from that obtained in free-flow conditions ( $P = 0.12$ , unpaired  $t$  test). This latter observation confirmed that ATP acts on the basolateral cell membrane.

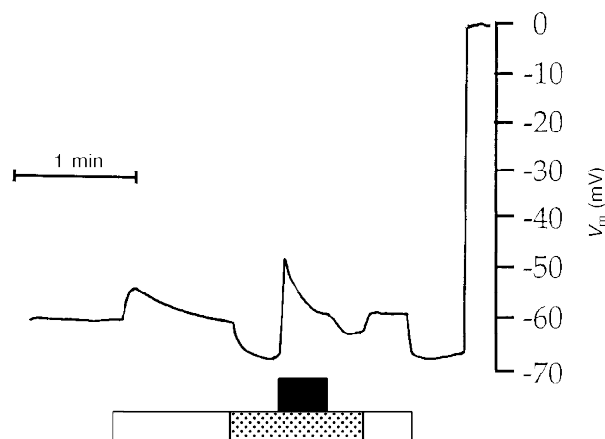
Using a peritubular triple-barrelled micropipette, we investigated the effects of several  $\text{Cl}^-$  conductance inhibitors on the ATP-induced  $V_m$  depolarization. Inhibitors were added to the Ringer, the low  $\text{Cl}^-$  and the ATP-containing low  $\text{Cl}^-$  solutions. Results are summarized in Table 4. In the presence of 1 mmol  $\text{l}^{-1}$  SITS, the ATP-containing low  $\text{Cl}^-$  solution did not affect the  $V_m$  value observed with the low  $\text{Cl}^-$  perfusate. In contrast, 500  $\mu\text{mol l}^{-1}$  DPC, 100  $\mu\text{mol l}^{-1}$  NPPB, 100  $\mu\text{mol l}^{-1}$  niflumic acid and 100 nmol  $\text{l}^{-1}$  calix-4-arene did not prevent the ATP membrane effect.

#### The $\text{Cl}^-$ conductance activation is related to the purinergic receptor, but not to the $[\text{Ca}^{2+}]_i$ increase

We next investigated a possible link between P2y receptor activation and  $G_{\text{Cl}}$  increase. We first observed that repeated exposures to 100  $\mu\text{mol l}^{-1}$  ATP caused a progressive reduction of the ATP-induced membrane hyperpolarization in the presence of  $\text{BaCl}_2$  and a progressive reduction of the ATP-induced membrane depolarization in a low  $\text{Cl}^-$  medium (Fig. 8). Second, adding 100  $\mu\text{mol l}^{-1}$  ADP to the Ringer perfusate elicited a  $V_m$  change similar to that observed with 100  $\mu\text{mol l}^{-1}$  ATP ( $9.0 \pm 0.7$  mV,  $n = 7$  vs.  $9.3 \pm 0.8$  mV,  $n = 16$ ,  $P = 0.8$ , unpaired  $t$  test), while  $V_m$  was stable upon adding 100  $\mu\text{mol l}^{-1}$  adenosine ( $-62.5 \pm 2.5$  vs.  $-62.6 \pm 2.7$  mV,  $n = 11$ ), as well as on adding 100  $\mu\text{mol l}^{-1}$  UTP ( $-61.5 \pm 2.6$  vs.  $-61.9 \pm 2.9$  mV,  $n = 8$ ). Third, we performed an experimental series ( $n = 9$ ) in the continuous presence of 300  $\mu\text{mol l}^{-1}$  suramin: measured  $V_m$  values were  $-65.5 \pm 2.5$  and  $-72.4 \pm 2.5$  mV during Ringer and

Figure 7. Effect on basolateral membrane potential of ATP (100  $\mu\text{mol l}^{-1}$ ) added to a basolateral low  $\text{Cl}^-$  perfusion

Tracing obtained with an intracellular microelectrode. Timing of perfusion with experimental solutions is indicated by the horizontal bars: filled bar, 100  $\mu\text{mol l}^{-1}$  ATP; open bar, Ringer solution; stippled bar, low  $\text{Cl}^-$  solution. Before adding ATP, the effect of reducing basolateral  $\text{Cl}^-$  content was tested. Before and after these experimental solutions, perfusion was with blood from normal circulation.

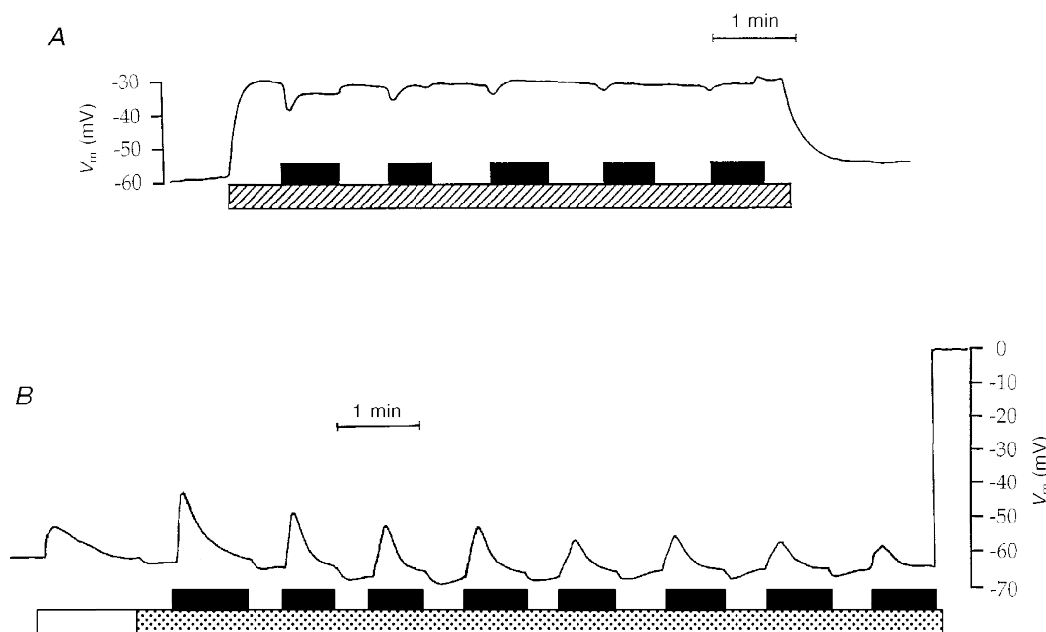


low  $\text{Cl}^-$  perfusion, respectively, and the  $100 \mu\text{mol l}^{-1}$  ATP-containing low  $\text{Cl}^-$  solution induced an insignificant  $2.5 \pm 1.2$  mV change in  $V_m$  ( $P = 0.06$ ). These observations suggest that the activation of  $G_{\text{Cl}}$  and the activation of P2y receptor were linked.

We then looked for a relationship between the rise in  $[\text{Ca}^{2+}]_i$  and the increase of  $G_{\text{Cl}}$ . Adding  $1 \text{ mmol l}^{-1}$  ATP to a peritubular  $\text{Ca}^{2+}$ -free Ringer solution induced a membrane depolarization which was not different to that measured in the presence of external  $\text{Ca}^{2+}$  ( $13.4 \pm 2.0$  mV,  $n = 5$  vs.  $14.6 \pm 1.1$  mV,  $n = 8$ ,  $P = 0.6$ , unpaired  $t$  test). We also tested whether an increase in  $[\text{Ca}^{2+}]_i$  could increase  $G_{\text{Cl}}$ .  $[\text{Ca}^{2+}]_i$  increase was achieved by using  $2.5 \mu\text{mol l}^{-1}$  ionomycin-supplemented peritubular perfusates (a condition which increased  $[\text{Ca}^{2+}]_i$  in less than 1 min,  $n = 2$ , data not shown), while monitoring  $V_m$ . Under this experimental condition, switching from Ringer to low  $\text{Cl}^-$  solution changed  $V_m$  from  $-56.7 \pm 2.4$  to  $-62.3 \pm 2.5$  mV ( $n = 9$ ), whereas a membrane depolarization would be expected if the induced rise in  $[\text{Ca}^{2+}]_i$  had an activating effect on  $G_{\text{Cl}}$ . We also performed peritubular perfusion of six PCTs with thapsigargin ( $2 \mu\text{mol l}^{-1}$ )-supplemented solutions for up to 30 min (which totally inhibited the ATP-induced  $[\text{Ca}^{2+}]_i$  transients): measured  $V_m$  values were  $-59.3 \pm 2.7$  mV under Ringer solution perfusion and  $-63.3 \pm 3.7$  mV upon switching to the low  $\text{Cl}^-$  solution; the addition of  $100 \mu\text{mol l}^{-1}$  ATP to the low  $\text{Cl}^-$  solution still induced a large membrane depolarization ( $\Delta V_m = 28.1 \pm 2.6$  mV,  $P < 0.001$ ). Similarly, basolateral addition of  $20 \mu\text{mol l}^{-1}$  of BAPTA AM

in the perfusate for up to 30 min (a condition preventing the  $[\text{Ca}^{2+}]_i$  transients elicited by  $100 \mu\text{mol l}^{-1}$  ATP,  $n = 4$ , data not shown), did not reduce the  $V_m$  change induced by adding ATP to the low  $\text{Cl}^-$  solution ( $\Delta V_m = 27.0 \pm 3.5$  mV,  $n = 4$ ,  $P < 0.001$ ).

Finally, we investigated whether protein kinase C (PKC) or intracellular cAMP may induce an increase of basolateral  $\text{Cl}^-$  conductance in PCT, thus being the link between a G protein-coupled P2 receptor and the  $G_{\text{Cl}}$  activation. Possible PKC activation was prevented by the basolateral perfusion (for up to 30 min) of a Ringer solution containing  $1 \mu\text{mol l}^{-1}$  staurosporin; in the continuous presence of staurosporin, a low  $\text{Cl}^-$  and an ATP-containing low  $\text{Cl}^-$  solution were then substituted. Under this experimental condition ( $n = 4$ ),  $V_m$  was  $-58.5 \pm 3.1$  and  $-63.5 \pm 3.0$  mV during Ringer and low  $\text{Cl}^-$  perfusion, respectively. Addition of  $100 \mu\text{mol l}^{-1}$  ATP to the low  $\text{Cl}^-$  solution still changed  $V_m$  by  $+26.2 \pm 3.1$  mV ( $P < 0.001$ ). Next, to test whether an increase in intracellular cAMP may activate  $G_{\text{Cl}}$ ,  $50 \mu\text{mol l}^{-1}$  of the membrane permeant cAMP analogue 8-4 CPT-cAMP was added to a standard Ringer and to a low  $\text{Cl}^-$  solution. Upon adding 8-4 CPT-cAMP to the Ringer perfusate,  $V_m$  significantly increased from  $-59.0 \pm 3.9$  to  $-65.7 \pm 2.7$  mV,  $n = 8$ ,  $P < 0.001$ ). In the presence of 8-4 CPT-cAMP, the low  $\text{Cl}^-$  solution induced a tiny membrane depolarization in two out of eight tubules (+1 and +4 mV), whereas in the six other PCTs, it hyperpolarized the membrane in a way similar to that observed in cAMP-free low  $\text{Cl}^-$  condition ( $P = 0.12$ , unpaired  $t$  test).



**Figure 8.** Effect on basolateral membrane potential of repeated ATP ( $100 \mu\text{mol l}^{-1}$ ) exposure

Tracings obtained with intracellular microelectrodes. Timing of perfusion with experimental solutions is indicated by the horizontal bars. In *A*, ATP was added to a  $\text{BaCl}_2$ -containing Ringer solution. Hatched bar, Ringer solution +  $2 \text{ mmol l}^{-1}$   $\text{BaCl}_2$ ; filled bar,  $100 \mu\text{mol l}^{-1}$  ATP. In *B*, ATP was added to a low  $\text{Cl}^-$  solution. Open bar, Ringer solution; stippled bar, low  $\text{Cl}^-$  solution; filled bar,  $100 \mu\text{mol l}^{-1}$  ATP. Before and after these experimental solutions, perfusion was with blood from normal circulation.

## DISCUSSION

### Extracellular ATP raises $[Ca^{2+}]_i$ through P2y receptor activation

Exposure of isolated fura-2-loaded *Necturus* PCTs to ATP led to a concentration-dependent increase in  $[Ca^{2+}]_i$  with an apparent half-maximal effect at about  $15 \mu\text{mol l}^{-1}$  ATP. Comparison of the effects of ATP with the effects of other nucleotides and adenosine yielded the sequence  $2\text{MeSATP} = \text{ADP} = \text{ATP} \gg \text{UTP}$ ,  $\text{BzATP}$ ,  $\text{AMPCPP}$ , adenosine, which corresponds to conventional P2y-type receptor pharmacology (Dubyak & El-Moatassim, 1993). One may question the exact validity of such a sequence, since it was established in the absence of ectonucleotidase inhibitors, a condition which did not prevent a possible breakdown of unstable compounds and could alter their potency (Kennedy & Leff, 1995). In particular, the relative effectiveness of 2 MeSATP and AMPCPP can be notably changed by ectonucleotidase inhibition (Kennedy & Leff, 1995), hampering discrimination between P2x and P2y receptors. However, AMPCPP had no significant effect on  $[Ca^{2+}]_i$  ( $\Delta R = 0.1 \pm 0.1$ ,  $n = 5$ ), whereas at least a moderate  $\Delta[Ca^{2+}]_i$  would be expected in the presence of a P2x receptor. In addition, the P2x receptor antagonist PPADS (Zinganshin *et al.* 1994) had no inhibitory effect on the ATP-induced  $[Ca^{2+}]_i$  transients. Furthermore, the P2x receptor functionally acts as a ligand-gated ion channel, leading to  $Ca^{2+}$  influx, while the P2y receptors essentially mobilize  $Ca^{2+}$  contained in intracellular stores (Dubyak & El-Moatassim, 1993). Our results demonstrating that the latter mechanism accounts for the ATP-induced  $[Ca^{2+}]_i$  transients further support the hypothesis that ATP acts via a P2y receptor in *Necturus* PCTs, and are consistent with results in PCTs of other species (Cejka *et al.* 1994; Yamada *et al.* 1996).

As reported in rabbit PCT (Yamada *et al.* 1996), ATP binding to a P2y receptor induced a transient increase in  $[Ca^{2+}]_i$  in *Necturus* PCT and no secondary sustained phase in  $[Ca^{2+}]_i$  rise. Experiments performed in the presence of thapsigargin showed that the ATP-induced  $[Ca^{2+}]_i$  transients in rabbit (Yamada *et al.* 1996) and *Necturus* (this study) PCTs were dependent on the release of  $Ca^{2+}$  from intracellular stores. Our results did not show clearly an ATP-induced  $Ca^{2+}$  influx through  $Ca^{2+}$  channels in *Necturus* PCTs. On the one hand, part of the results argue against the participation of extracellular  $Ca^{2+}$  in the ATP-induced  $[Ca^{2+}]_i$  change: the removal of extracellular  $Ca^{2+}$  for 30 s, as well as the addition of verapamil and nifedipine, known to be dihydropyridine-sensitive  $Ca^{2+}$  channel inhibitors (Speeding & Paoletti, 1992) or of  $La^{3+}$ , had no detectable effect on the response to ATP. On the other hand, the effects of long-term removal of external  $Ca^{2+}$  argue for the participation of extracellular  $Ca^{2+}$  in ATP-induced  $[Ca^{2+}]_i$  change: this experimental manoeuvre totally abolished the ATP-induced response in one out of fourteen tested tubules and slightly decreased the response when measured after 1.5 min of nucleotide exposure. From these results, it is safe to conclude that the ATP-induced  $[Ca^{2+}]_i$  increase is mainly due to  $Ca^{2+}$  release

from intracellular pools, although we emphasize that we do not exclude the participation of transient influx of extracellular  $Ca^{2+}$ . Obviously, our results contrast with those obtained in rabbit proximal tubular cells in primary culture (Cejka *et al.* 1993, 1994), where an ATP-induced sustained secondary increase in  $[Ca^{2+}]_i$  was abolished by removal of extracellular  $Ca^{2+}$  and blocked by verapamil and  $La^{3+}$ . They also contrast with observations on rabbit PCTs (Yamada *et al.* 1996): in this tissue, the ATP binding to a basolateral P2y receptor induced  $[Ca^{2+}]_i$  transients which were markedly blunted by the removal of external  $Ca^{2+}$  over 30 s and vanished after a 3 min incubation with the  $Ca^{2+}$ -free solution. It should also be emphasized that the membrane localization (apical *vs.* basolateral) of the P2y receptor inducing  $[Ca^{2+}]_i$  rise in *Necturus* PCT cannot be firmly established from our  $[Ca^{2+}]_i$  measurements, which were performed on superfused tubules.

### An early effect of extracellular ATP is the activation of a basolateral $Cl^-$ conductance

In a recent study performed on isolated rabbit PCTs (Yamada *et al.* 1996) it was reported that peritubular ATP induced an occasional basolateral membrane depolarization ( $< 4$  mV up to  $100 \mu\text{mol l}^{-1}$  ATP, and from 4 to 6 mV with  $300 \mu\text{mol l}^{-1}$  ATP), but its origin was not clarified inasmuch as statistical significance was not reached. In experiments on *Necturus* PCTs *in vivo*, addition of ATP (from  $50 \mu\text{mol l}^{-1}$  to  $1 \text{ mmol l}^{-1}$ ) to a peritubular Ringer perfusate consistently induced membrane depolarization. To better understand the discrepancies between these studies, we performed a few experiments on isolated *Necturus* PCTs under experimental conditions similar to those in the fura experiments. We observed (results not shown) that  $100 \mu\text{mol l}^{-1}$  ATP added to a Ringer solution induced a constant but slight membrane depolarization ( $4.0 \pm 0.7$  mV,  $n = 6$ ,  $P < 0.01$ ), whereas *in vivo* a larger  $\Delta V_m$  was measured (see Results). Thus, it could be that species differences explain the differences between the results of Yamada *et al.* (1996) and us, but it could also be the case that the *in vitro* condition impairs electrophysiological results, as recently reported by Müller-Berger, Coppola, Samarzija, Seki & Frömter (1997).

The ATP-induced membrane depolarization is consistent with either inhibition of a  $K^+$  conductance or with activation of other conductances, such as those of  $Cl^-$  or  $Na^+$ . However, since the membrane depolarization was associated with an initial drop in VDR, one can exclude the former hypothesis as a major effect of ATP and conclude that the early effect of extracellular ATP is the activation of a basolateral rheogenic pathway.

Several arguments prompted us to test the hypothesis of an increase in the basolateral  $Cl^-$  conductance. In the presence of  $BaCl_2$ , a condition used to depolarize  $V_m$  (Planelles, Teulon & Anagnostopoulos, 1981), the addition of ATP hyperpolarized the basolateral membrane. Thus, the equilibrium potential of the ionic species whose conductance is increased by the presence of ATP is less negative than the

basal  $V_m$  value (measured during a peritubular Ringer perfusion), but more negative than the  $V_m$  value measured in the presence of  $\text{BaCl}_2$ . In the case of the activation of a diffusive pathway (the simplest hypothesis), transported ions might be  $\text{Cl}^-$ : in *Necturus* PCT cells, across the basolateral membrane the  $\text{Cl}^-$  equilibrium potential is near to  $-36$  mV (Edelman, Bouthier & Anagnostopoulos, 1981), whereas the  $\text{Ca}^{2+}$  equilibrium potential is highly positive, the equilibrium potential for non-selective cation channels is nil, and the  $\text{Na}^+$  equilibrium potential, calculated from our previous measurements (Planelles, Thomas & Anagnostopoulos, 1993), is  $+56$  mV. Moreover, SITS, an inhibitor of anionic pathways (Aronson, 1989), prevented the ATP-induced changes in  $V_m$  and cell membrane input conductance. Taken together, the results strongly suggested that extracellular ATP activated basolateral  $G_{\text{Cl}}$ , which was previously shown to be low in *Necturus* PCT (Anagnostopoulos & Planelles, 1979). Indeed, the sudden reduction of peritubular  $\text{Cl}^-$  concentration (a manoeuvre displacing the  $\text{Cl}^-$  equilibrium potential towards more positive values) induced a slight apparent hyperpolarization (related to the gluconate $^-$  liquid junction potential), confirming that under control conditions,  $G_{\text{Cl}}$  is low. The large  $V_m$  depolarization observed upon adding  $100 \mu\text{mol l}^{-1}$  ATP to the low  $\text{Cl}^-$  solution is consistent with a  $G_{\text{Cl}}$  increase. Again we checked this protocol on microdissected tubules (results not shown); we observed that in the *in vitro* condition, adding  $100 \mu\text{mol l}^{-1}$  ATP to a low  $\text{Cl}^-$  solution induced a membrane depolarization of  $8.8 \pm 1.3$  mV ( $n = 11$ ,  $P < 0.001$ ), consistent with  $G_{\text{Cl}}$  activation. Another possibility could be that ATP activates the  $\text{Na}^+-(\text{HCO}_3^-)_n$  symport, which is present on the basolateral membrane of PCT cells and is sensitive to SITS (Boron & Boulpaep, 1983), leading to a SITS-sensitive membrane depolarization and an increase in cell conductance. However, this latter hypothesis is neither supported by our experimental protocol (nominally  $\text{CO}_2/\text{HCO}_3^-$  free) nor by results obtained with ATP-containing low  $\text{Cl}^-$  solutions: due to the  $\text{Cl}^-$  independence of the  $\text{Na}^+-(\text{HCO}_3^-)_n$  symport (Boron & Boulpaep, 1983) the perfusion of a low  $\text{Cl}^-$  solution would not enhance the ATP-induced membrane depolarization compared with that observed with ATP-containing Ringer solution. We conclude that in the presence of extracellular ATP a basolateral electrogenic,  $\text{Cl}^-$ -dependent, SITS-sensitive pathway is activated; unless some yet unknown transport system is involved, our results are consistent with  $G_{\text{Cl}}$  activation.

Secondary effects of ATP on electrophysiological parameters were not investigated in detail, but several observations can be discussed. During ATP exposure and after its removal, the initial increase in cell membrane input conductance was followed by a secondary decrease. This secondary rise in  $dV_m$  overwhelmed the control cell membrane input conductance value, except in the presence of  $\text{BaCl}_2$ . On the other hand, VDR first decreased, then tended to return towards its control value; this latter observation is consistent with a

transient activation of  $G_{\text{Cl}}$  and/or with a sustained activation of  $G_{\text{Cl}}$  counterbalanced with the delayed inhibition of another membrane conductance. In fact, a transient activation of  $G_{\text{Cl}}$  was not supported by the delayed return of the  $V_m$  value upon ATP withdrawal (see Fig. 7), consistent with a sustained activation of  $G_{\text{Cl}}$ . Recalling that with an intracellular current source,  $dV_m$  results from current flow along the epithelial cable, and that with an intraluminal current source,  $dV_m$  arises from current flow along the transcellular route, the ratio  $dV_{m,\text{exptl}}/dV_{m,\text{ctrl}}$  estimates the change in cell conductance and cable core conductance (including junctional conductance), whereas the voltage divider ratio  $dV_m/dV_{te}$  (both values being recorded at the same distance from the source) estimates the ratio of the membrane resistances. As a consequence, the above results suggest that, as a secondary effect, ATP induces a slight inhibition of membrane conductance (probably a  $\text{Ba}^{2+}$ -sensitive  $\text{K}^+$  conductance; Tsuchiya, Wang, Giebisch & Welling, 1992), and an increase in cell-to-cell coupling junctional resistance (possibly induced by  $[\text{Ca}^{2+}]_i$  changes; Jojov, Lewis, Crowe, Berg & Wills, 1994). Further experiments, including cable analysis, would be needed to support this interpretation.

#### Further characterization of the activated $\text{Cl}^-$ conductance

It has been shown in various epithelia that P2 receptors may activate different  $\text{Cl}^-$  channels (Guo *et al.* 1995; Nilius, Seherer, Heinke & Droogmans, 1995; Hwang *et al.* 1996; Yamaya, Sekizawa, Kakuta, Ohru, Sawai & Sasaki, 1996). We attempted to better define the channel(s) underlying the macroscopic  $G_{\text{Cl}}$  activated in the presence of ATP by using several  $\text{Cl}^-$  channel inhibitors and by searching for the intracellular signal leading to  $G_{\text{Cl}}$  activation.

We observed that SITS had a powerful inhibitory effect on the ATP-induced activated  $G_{\text{Cl}}$ , consistent with the inhibition of an outwardly rectifying  $\text{Cl}^-$  channel (ORCC) (Hwang *et al.* 1996) or of a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel. The hypothesis of ORCC activation was contradicted by the lack of effect of calix-4-arene to reduce the ATP-induced membrane depolarization, since a calix-4-arene derivative is a very potent inhibitor of ORCC in epithelial airway cells (Schwiebert, Flotte, Cutting & Guggino, 1994). Thus, we turned to the hypothesis that ATP activates a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channel, inasmuch as ATP induces a  $[\text{Ca}^{2+}]_i$  increase: from this hypothesis, it follows that a  $[\text{Ca}^{2+}]_i$  increase would reproduce the ATP membrane effect, and that preventing ATP-induced  $[\text{Ca}^{2+}]_i$  increase would suppress it. To induce a rise in basal  $[\text{Ca}^{2+}]_i$ , a kidney region was perfused with ionomycin-supplemented solutions, but no significant increase in basolateral  $G_{\text{Cl}}$  was observed. We also observed that TG raised basal  $[\text{Ca}^{2+}]_i$ , but in its presence the reduction of extracellular  $\text{Cl}^-$  concentration induced a  $V_m$  change similar to that observed under control conditions; moreover, ATP still induced a large membrane



depolarization, whereas  $\text{Ca}^{2+}$  release from intracellular stocks was expected to be inhibited. In addition, ATP was introduced in a low  $\text{Cl}^-$  solution in the continuous presence of BAPTA AM, a cell-permeant  $\text{Ca}^{2+}$  chelator; under this experimental condition, ATP-containing low  $\text{Cl}^-$  solution induced a large membrane depolarization, comparable to that observed in the absence of BAPTA AM. A final argument suggesting that we were not dealing with a  $\text{Ca}^{2+}$ -activated  $G_{\text{Cl}}$  was the lack of inhibitory effect of niflumic acid, considered an inhibitor of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Pacaud, Loirand, Lavie, Mironneau & Mironneau, 1989). Another possibility would be that ATP binding to a P2y receptor induced a rise in  $\text{cAMP}_i$ , leading to the activation of a SITS-sensitive  $\text{Cl}^-$  channel. It should be noted that the inhibition of ATP-induced  $V_m$  changes by this stilbene derivative, as well as the lack of inhibitory effect of DPC and NPPB, did not support the hypothesis of the activation of the cystic fibrosis transmembrane regulator (CFTR) (Hwang *et al.* 1996), although a  $\text{Cl}^-$  channel presenting CFTR properties has been described on the basolateral membrane of amphibian PCT cells (Segal & Boulpaep, 1992). In the presence of a permeant  $\text{cAMP}$  compound, the inconstant and tiny membrane depolarization observed upon reduction of bath  $\text{Cl}^-$  content might reflect activation of  $G_{\text{Cl}}$  by  $\text{cAMP}_i$ , but the amplitude of this  $V_m$  change was certainly not comparable with the large ATP-induced membrane depolarization. Moreover, the main effect of adding 8-4 CPT-cAMP to a Ringer solution was a basolateral membrane hyperpolarization, clearly showing that the major effect of increasing  $\text{cAMP}$  content is not a  $G_{\text{Cl}}$  activation. The last hypothesis investigated in this study was that PKC may be involved in the ATP-induced  $G_{\text{Cl}}$  activation, but when preventing PKC activation by staurosporin, the effect of extracellular ATP on  $V_m$  was unchanged.

In summary, our results indicate that amphibian PCT cells are endowed with P2y receptors. ATP binding to these receptors induces (i) an increase in  $[\text{Ca}^{2+}]_i$  and (ii) an increase in basolateral  $G_{\text{Cl}}$  which does not seem to be related to PKC activation nor to  $[\text{Ca}^{2+}]_i$  or  $\text{cAMP}_i$  increases. Molecular identification of the  $\text{Cl}^-$  channel(s) underlying the ATP-induced macroscopic  $G_{\text{Cl}}$ , as well as the complete exploration of the signal transduction system possibly involved in  $G_{\text{Cl}}$  activation, require further investigation. The activation of a basolateral  $G_{\text{Cl}}$  may have a physiological role in  $\text{Cl}^-$  reabsorption by the proximal tubule. In this tubular segment, transepithelial  $\text{Cl}^-$  reabsorption is considered to be mostly paracellular. However, part of it seems to be transcellular: since  $\text{Cl}^-$  uptake by apical membrane transport systems has been reported in various species, including amphibians (Seifter & Aronson, 1984; Wang, Giebisch & Aronson, 1992), a basolateral  $G_{\text{Cl}}$  activated under certain circumstances would represent a basolateral step for transcellular  $\text{Cl}^-$  reabsorption by proximal tubules.

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