

# IDENTIFICATION OF A PURINE (P<sub>2</sub>) RECEPTOR LINKED TO ION TRANSPORT IN A CULTURED RENAL (MDCK) EPITHELIUM

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**1** Exogenous adenosine triphosphate (ATP) stimulates the short circuit current (SCC) in a cultured renal-derived epithelium (MDCK). Half-maximal stimulation is achieved at  $1.91 \times 10^{-5}$  M ATP.

**2** It is suggested that ATP interacts with a P<sub>2</sub> purine receptor upon the basis of (a) agonist potency (ATP > adenosine diphosphate >> adenosine monophosphate, adenosine; ATP > uridine triphosphate > inosine triphosphate >> cytosine triphosphate, guanosine triphosphate); (b) the inhibition of the ATP response by quinidine ( $1 \times 10^{-3}$  M) but not by theophylline ( $1 \times 10^{-3}$  M).

**3** Indomethacin ( $1 \times 10^{-5}$  M) inhibits the response of the cultured epithelium to ATP.

**4** Prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) stimulates SCC but potentiates the effect of ATP on SCC. The divalent cationic ionophore A23187 ( $1 \times 10^{-6}$  M) transiently stimulates SCC itself and abolishes ATP-induced stimulation of the SCC.

## Introduction

Exogenous adenosine triphosphate (ATP) has potent effects upon cellular function in a wide variety of cell types (Aiton & Lamb, 1980) which may be important in terms of physiological regulatory mechanisms (see Burnstock, 1978). Such effects are mediated by two types of specific surface receptor (P<sub>1</sub> and P<sub>2</sub>) having different agonist and antagonist specificities (Burnstock, 1978).

Identification of purine effects in tissues of interest, is complicated by rapid hydrolysis, (Arch & Newsholme, 1978) or tissue uptake, of exogenously applied purine nucleotides (Burnstock, 1978). Hydrolysis of triphosphates can also provide equivocal results concerning receptor type (Burnstock, 1978).

In a preliminary paper (Simmons, 1979) exogenous ATP was found to stimulate Cl ion transport in a semi-artificial cultured epithelium derived from dog kidney (MDCK). Since the cultured epithelium consists of a single layer of cells grown on a permeable millipore filter (Misfeldt, Hamamoto & Pitelka, 1976) and since the Ussing chambers have large volumes in respect of the tissue mass, this seemed a good system in which to investigate some pharmacological aspects of the purine receptor present in this cell type. The results are discussed in relation to the known features of purine receptors and to possible regulation by purines of ion transport in kidney epithelium *in vivo*.

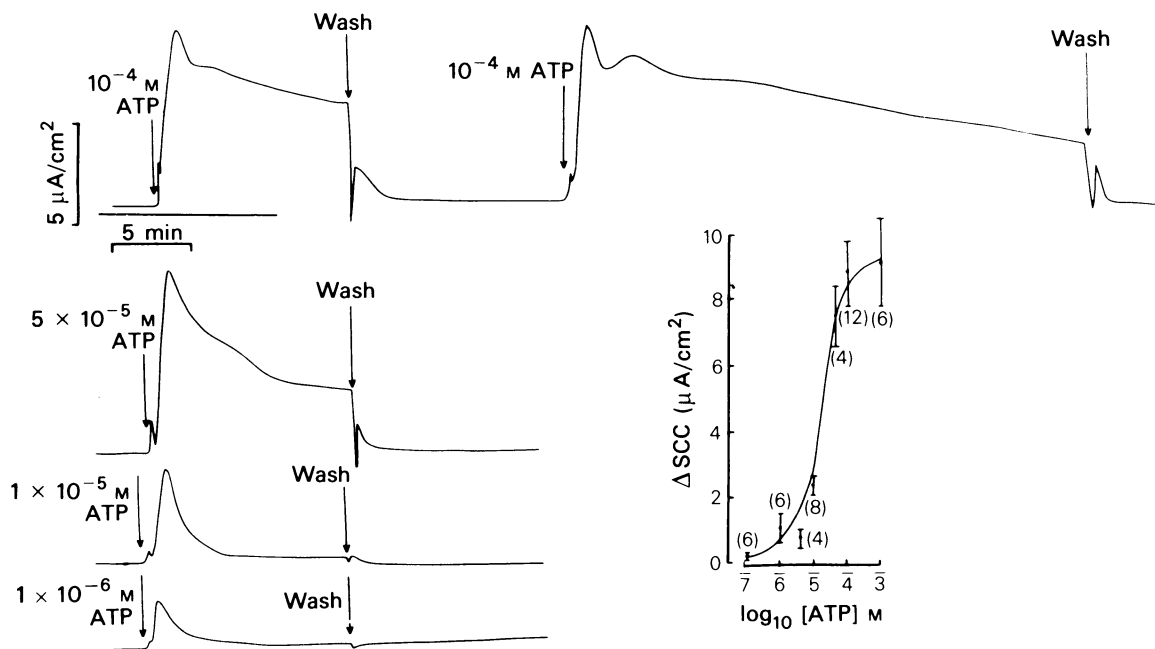
## Methods

### Cell culture

Conditions were essentially identical to those described by Misfeldt *et al.* (1976) and by Cereijido, Robbins, Dolan, Rotunno & Sabatini (1978). Cultures of MDCK cells were obtained at 60 serial passages from Flow Laboratories (Irvine, Scotland) and maintained in serial culture in Roux flasks in Minimum Essential Medium Eagles (M.E.M.E.) supplemented with non-essential amino acids (Flow), 2 mM glutamine, 1 unit/cm<sup>3</sup> gentamycin antibiotic and 10% v/v foetal bovine serum (Flow) at 37°C in an air/5% CO<sub>2</sub> atmosphere. Epithelial monolayers were prepared by seeding millipore filters (0.22 μm pore diameter) at high density ( $1 \times 10^6$  cells/cm<sup>3</sup>) followed by growth to confluence in complete M.E.M.E. supplemented with 1iu/cm<sup>3</sup> insulin (Boots).

### Ussing chambers and short-circuit current measurements

The apparatus consisted of temperature-regulated Ussing chambers with the capacity for 6 adjacent epithelial monolayers. The exposed epithelial area was 1.76 cm<sup>2</sup>. An automatic voltage-clamp (Sim-



**Figure 1** Tracings of original chart recordings showing the effect of various concentrations of adenosine triphosphate (ATP) upon the short circuit current (SCC). Inset shows a graph of the peak ATP-dependent increment in SCC ( $\Delta$ SCC) plotted against the  $\log_{10}$  ATP concentration. Figures in parentheses are the number of separate determinations. Error bars indicate s.e. mean.

mons & Naftalin, 1976) was connected to the Ussing chamber via matched calomel half cells (for potential measurement), silver/silver chloride half cells (for current passage) and saturated KCl-agar salt bridges. The use of saturated KCl-agar salt bridges minimized error due to liquid junction potentials. The short-circuit current (SCC) was recorded continuously on a Houston-Instruments flat bed chart recorder. Resistance determinations were made by occasionally passing  $2 \mu\text{A}/\text{cm}^2$  hyperpolarizing current pulses and monitoring the voltage deflection.

#### Experimental solutions

The experiments were carried out at pH 7.4 in modified Krebs solutions containing (mmol/l): NaCl 137, KCl 5.4,  $\text{CaCl}_2$  2.8,  $\text{MgSO}_4$  1.2,  $\text{NaH}_2\text{PO}_4$  0.3,  $\text{KH}_2\text{PO}_4$  0.4, HCl 12, Tris base 14, glucose 10, glutamine 2, Na pyruvate 2 and 2% v/v foetal bovine serum and amino acids for Eagles medium (Flow).

#### Chemicals

All chemicals used were of Analar grade. Adenosine

triphosphate was obtained from BDH chemicals (grade 1 synthetic Na salt). All other nucleotides and nucleosides were obtained from the Sigma Chemical Company. All nucleotides/nucleosides were made up as  $10^{-1}$ ,  $10^{-2}$  or  $10^{-3}\text{M}$  stock solutions in Krebs solution and the pH was adjusted where necessary with NaOH. All drug solutions were kept on ice before addition to the Ussing chambers. Indomethacin and quinidine were obtained from Sigma. Prostaglandin  $\text{E}_1$  was a gift from Dr Pike of Upjohn and A23187 was a gift from the Lilly Research Center. Indomethacin and A23187 were added to the Ussing chambers as ethanolic solutions. The carrier had no effect on SCC or monolayer resistance in separate experiments.

#### Statistical methods

Variation in results is routinely expressed as the standard error of the mean (s.e. mean). Significance of differences between mean values was tested by Student's *t* tests. Probit analysis was performed by a best-fit iterative method written in BASIC (Davies, 1971) and run on an Olivetti P6060 minicomputer.

**Table 1** The nucleotide/nucleoside-dependent peak increment in the short circuit ( $\Delta$ SCC peak)

<i>Nucleotide/ nucleoside</i>	$\Delta$ SCC peak ( $\mu$ A/cm <sup>2</sup> ) at $1 \times 10^{-4}$ M	<i>Maximum <math>\Delta</math>SCC peak with dose (<math>\mu</math>A/cm<sup>2</sup>)</i>	<i>K<sub>50</sub>(M)</i>
ATP	8.75 $\pm$ 2.96 (11)	9.18 $\pm$ 2.18 (6) $10^{-3}$ M	1.91 $\pm$ 0.06 $\times 10^{-5}$ (37)
ADP	12.50 $\pm$ 3.10 (4)	13.10 $\pm$ 3.92 (3) $10^{-3}$ M	6.91 $\pm$ 0.05 $\times 10^{-5}$ (20)
AMP	No response (4)	No response (4) $10^{-3}$ M	—
Adenosine	No response (4)	No response (4) $5 \times 10^{-3}$ M	—
AMP-PNP	5.33 $\pm$ 2.06 (4)*	—	—
UTP	12.33 $\pm$ 2.60 (3)	23.00 $\pm$ 2.80 (3) $4 \times 10^{-4}$ M	1.42 $\pm$ 0.03 $\times 10^{-4}$ (27)
ITP	2.2 $\pm$ 0.28 (3)	11.40 $\pm$ 1.97 (3)* $5 \times 10^{-4}$ M	—
CTP	No response (4)	No response (4)* $4 \times 10^{-4}$ M	—
GTP	No response (4)	0.46 $\pm$ 0.13 (3)* $3 \times 10^{-4}$ M	—

All nucleotides/nucleosides were dissolved in Krebs solution added to the solution bathing the basal surface. The concentration giving 50% of the maximal response ( $K_{50}$ ) was obtained by probit analysis. All values are  $\pm$  s.e. Numbers in parentheses are the number of separate determinations, or in the case of  $K_{50}$  values, the degrees of freedom of the best-fit probit line. Asterisks (\*) denote that higher concentrations than indicated were not tested. AMP-PNP = adenelyl imidioldiphosphate; UTP = uridine triphosphate; ITP = inosine triphosphate; CTP = cytosine triphosphate; GTP = guanyl triphosphate.

## Results

### *The effect of exogenous adenosine triphosphate*

When epithelial monolayers of MDCK cells formed from cell stocks of 60 serial passages are clamped into standard Ussing chambers, a mean transepithelial resistance of 4.1 k $\Omega$ /cm<sup>2</sup> is recorded together with a small basal surface positive transepithelial p.d. of 2 to 3 mV (Barker & Simmons, 1979). The spontaneous short circuit current (SCC) is therefore small ( $\sim 0.5 \mu$ A/cm<sup>2</sup>). Net ion transport in these epithelial monolayers as measured by isotopes is also of a small magnitude (Simmons, 1979). Addition of  $1 \times 10^{-4}$ M ATP to the basal bathing solution results in a prompt stimulation of the SCC from resting values to a peak increment of 8.3  $\mu$ A/cm<sup>2</sup> (Figure 1). The increased SCC is primarily a consequence of Cl movement from basal to apical surfaces of the epithelium (Simmons, 1979). The short latency, (15 s) is consistent with the interaction of ATP with the plasma membrane of the

MDCK cells following solution mixing. The time course of the increased SCC following ATP addition is complex; a small shoulder precedes the maximum response, thereafter there is a gradual decline in the SCC. Removal of ATP from the basal bathing solution with repeated washing with Krebs solution results in a rapid decline of the SCC; prestimulation levels being attained after 2.5 min. Reapplication of an equivalent concentration of ATP results in an undiminished response (Figure 1); the increased SCC in this response is still observed 30 min after the initial addition of ATP, though there is a progressive decline throughout this period. The response of the cultured epithelium is dose-dependent, smaller molar additions resulting in a diminished peak response and an accelerated decline of the SCC to prestimulation levels (Figure 1). If maximum peak increments of SCC are plotted against ATP concentration, a sigmoidal curve is obtained (mean data from 12 separate epithelial monolayers in which ATP concentration was varied) with a 50% maximum

stimulation at  $1.91 \pm 0.06$  (s.e.)  $\times 10^{-5}$  M (Figure 1, Table 1).

The possibility that the decay of the elevated SCC at all concentrations of ATP was a consequence of hydrolysis due to exo-ATPases present in the MDCK cells was tested by repeated application of the original ATP solution ( $1 \times 10^{-4}$  M) to six consecutive cell monolayers. A marked, undiminished, stimulation was observed in all consecutive monolayers, each individual response showing progressive reduction with time, indicating that the progressive decline in SCC resulted from alternative (perhaps intrinsic) causes.

**Sidedness of the response** All results so far described relate to basal application of ATP, that is application to the basal-lateral surfaces of the epithelial monolayers. Addition of equimolar doses of ATP to the apical bathing solution resulted in an identical response to that observed for basal addition except that the small initial shoulder to the SCC trace was considerably elevated. This situation contrasts with that seen with prostaglandin ( $\text{PGE}_1$ ) which was only maximally effective when applied to the basal bathing solution (unpublished observations).

**Specificity of the response** Adenosine diphosphate (ADP) has a similar effect on the SCC to ATP at an equimolar dose (Table 1). The concentration giving 50% stimulation is  $6.91 \pm 0.05$  (s.e.)  $\times 10^{-5}$  M. Adenosine monophosphate (AMP) and adenosine (AD) are without effect upon the SCC. The order of potency of adenosine nucleosides/nucleotides is therefore  $\text{ATP} > \text{ADP} \gg \text{AMP}$ , AD, similar to that found for  $\text{P}_2$  purinoceptors (Burnstock, 1978). Of the other triphosphates tested to stimulate the

SCC, uridine triphosphate (UTP) was effective at  $1 \times 10^{-4}$  M, though the 50% response was obtained at  $1.42 \pm 10^{-4}$  M (Table 1). Inosine triphosphate and guanyl triphosphate were only partially effective whilst cytosine triphosphate was without effect (Table 1). The non-hydrolysable derivative of ATP, adenelyl imidiodiphosphate was a partially effective substitute for ATP at an equimolar concentration (Table 1). All compounds which stimulated the SCC had a similar time course to the ATP-stimulated SCC, and a similar ionic basis as judged by ion-substitutions (Cl by isethionate, Na by choline) of the bathing Krebs solution.

*Action of possible antagonists/interaction with intracellular cyclic adenosine 3', 5'-monophosphate (cyclic AMP) and divalent cations*

Theophylline added to both bathing solutions had no inhibitory effect upon the ATP-mediated SCC, indeed this treatment augmented the response (Table 2). Quinidine blocked the response to ATP when applied in the same bathing solution as ATP. The blocking action of quinidine was seen only at high concentrations, lower concentrations ( $1 \times 10^{-4}$  M) having no effect. The action of quinidine was readily reversed (Table 3). Indomethacin ( $1 \times 10^{-5}$  M) was a potent inhibitor of the ATP response when applied together with ATP in the same bathing solution; an effect of indomethacin was also observed with apical addition although transepithelial diffusion of indomethacin cannot be excluded. The effect of indomethacin ( $1 \times 10^{-5}$  M) was not readily reversed (Table 3).

**Table 2** The effect of a 10 min preincubation of prostaglandin  $\text{E}_1$ , Ca-Mg ionophore A23187 or theophylline upon the ATP ( $10^{-4}$  M)-mediated increment in SCC (net peak increment)

Pretreatment	SCC due to pretreatment ( $\mu\text{A}/\text{cm}^2$ )	SCC (peak) due to $1 \times 10^{-4}$ M ATP ( $\mu\text{A}/\text{cm}^2$ )
None	—	$9.75 \pm 2.99$ (12)
Prostaglandin $\text{E}_1$ $1 \times 10^{-6}$ M basal addition	$1.18 \pm 0.31$ (4) <sup>a</sup>	$19.80 \pm 3.81$ (4)**
A23817 $1 \times 10^{-6}$ M (basal and apical additions)	$8.80 \pm 2.06$ (4) <sup>b</sup>	No response (4)**
Theophylline $1 \times 10^{-3}$ M (basal and apical additions)	No response (4) <sup>c</sup>	$31.80 \pm 1.45$ (4)**

Numbers in parentheses indicate the number of separate determinations. Results obtained from three separate batches of epithelial monolayers.

\*\* $P < 0.01$ .

<sup>a</sup> $\text{PGE}_1$  gives a maintained increase in SCC.

<sup>b</sup>A peak value is reached in 2 min, followed by a rapid decline in SCC, prestimulation levels being attained at  $\sim 5$  min.

<sup>c</sup>Theophylline gives a response similar to A23187 but this is seen only at 5 and 10 mM. No direct effect on SCC is observed at 1 mM.

**Table 3** Effect of (A) quinidine ( $1 \times 10^{-3}$ M) and (B) indomethacin ( $1 \times 10^{-5}$ M) upon the ATP-dependent increment in SCC

	A	B
Control ATP response ( $1 \times 10^{-4}$ M) basal addition $\mu\text{A}/\text{cm}^2$	$14.88 \pm 1.98$ (8)	$8.20 \pm 2.30$ (6)
ATP response (basal addition) with basal drug addition $\mu\text{A}/\text{cm}^2$	$2.30 \pm 0.98$ (4)	$0.22 \pm 0.10$ (3)
ATP response (basal addition) with apical drug addition $\mu\text{A}/\text{cm}^2$	$13.98 \pm 0.9$ (4)	$1.36 \pm 0.32$ (3)
ATP response after repeated washing ( $10 \times 5$ ml Krebs over 15 min) $\mu\text{A}/\text{cm}^2$	$15.62 \pm 1.00$ (8)	$1.73 \pm 0.42$ (6)

For each batch of epithelial monolayers a control response for ATP was obtained (basal addition); quinidine or indomethacin was then added to either the apical or basal bathing solution for a pre-incubation period of 3 min and the ATP addition was repeated. Finally the reversibility of drug effects was tested by repeated washing followed by ATP. Figures in parentheses are the number of separate determinations made.

PGE<sub>1</sub> ( $1 \times 10^{-6}$ M) added to the basal bathing solution stimulated SCC, a maintained SCC being observed. Addition of ATP in the presence of PGE<sub>1</sub> augmented the ATP effect (Table 2).

A23187 ( $1 \times 10^{-6}$ M) stimulated the SCC, but only transiently; addition of ATP following decay of this transient effect had no effect on SCC (Table 2).

## Discussion

The results presented in this paper show that exogenous ATP stimulates ion transport in the cultured MDCK epithelium. That this stimulation is due to interaction with a specific surface receptor as envisaged by Burnstock (1978) is suggested by: (1) the short latency of the onset of increased transepithelial ion transport upon addition of ATP to the bathing solution, (2) the specificity of the response; the potency series for adenosine nucleotides and nucleosides is ATP > ADP >> AMP, AD and that for purine and pyrimidine trinucleotides is ATP > UTP > ITP >> CTP, GTP, (3) the action of quinidine, although being of low affinity, is consistent with a specific reversible antagonism, (4) the non-hydrolysable derivative of ATP, adenyl imidodiphosphate (Young, Babcock, Ballantyne & Ojala, 1971), has a similar action to ATP, obviating the possibility that intracellular metabolism of ATP stimulates ion transport (Westcott, Engelhard & Storm, 1979).

A diffusion barrier across the epithelial monolayer

is indicated by the high transepithelial resistance. Application of ATP to either bathing solution elicits similar responses, indicating that receptors for ATP are present at each of the cellular membranes of the epithelium in contrast to the usual distribution of humoral receptors and adenylyl cyclase in epithelial cells to the basal-lateral membranes (Liang & Sacktor, 1977). PGE<sub>1</sub> stimulation of SCC is effective from only the basal surfaces in MDCK epithelia; that ATP stimulates SCC from either bathing solution suggests that ATP-dependent increases in SCC do not result directly from activation of adenylyl cyclase.

This discussion is invalid if ATP has significant transcellular permeability. Boyd & Forrester (1968) have observed release of ATP from frog skeletal muscle *in vitro* dependent upon contractile activity, suggesting transmembrane flux of ATP. Clearly more data on the transfer of ATP across cellular plasma membranes are needed.

Is ATP stimulation of prostaglandin production a likely mechanism for the ATP stimulation of the SCC? PGE<sub>1</sub> ( $1 \times 10^{-6}$ M) stimulates SCC (Table 2) and MDCK cells are able to produce prostaglandins (Levine, 1977). Indomethacin is a potent inhibitor of prostaglandin production (Vane, 1971) and indomethacin ( $1 \times 10^{-5}$ M) is an effective inhibitor of the ATP-mediated increase in SCC whether applied from the apical or basal bathing solutions. However, applied PGE<sub>1</sub>, in addition to stimulating the SCC, facilitates the response to ATP; this result implies that ATP stimulation of prostaglandin production cannot be the sole mediator of the increased SCC

observed in MDCK apithelia with exogenous ATP. Intracellular cyclic AMP or cytoplasmic  $\text{Ca}^{2+}$  levels are important regulatory cytoplasmic signals in epithelia (Ilundain & Naftalin, 1979). In the present experiments both theophylline and  $\text{PGE}_1$  augment the effect of exogenous ATP upon ion transport, whereas A23187, the  $\text{Ca}^{2+}$  ionophore, though stimulating ion transport in a transient fashion, inhibits the effect of exogenous ATP. The time-dependent decay of the ATP increase in SCC resembles the inactivation seen with A23187 and it is possible that these phenomena and the inhibition of the ATP response in the presence of A23187 have a similar cellular basis. Taken together, these results suggest an important role for both intracellular cyclic AMP and  $\text{Ca}^{2+}$  as intracellular mediators of the increased SCC observed with exogenous ATP.

$\text{P}_2$  purine receptors predominate in the gastrointestinal and urinogenital tracts (Burnstock, 1978) and affect the vasculature and smooth muscle. Rorive & Kleinzeller (1974) and Kohn, Newey & Smyth (1970) have shown effects of exogenous ATP upon renal cellular ion transport and intestinal trans-epithelial ion transport respectively, raising the possibility of purinergic regulation of transepithelial ion transport in these tissues. The results presented here are consistent with this idea for renal tissue. Intratubular concentration of adenine nucleotides may be important in this respect, since MDCK monolayers respond to ATP from either epithelial surface.

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