Evidence for P₂-purinoceptor-mediated uptake of Ca²⁺ across a fish (*Oreochromis mossambicus*) intestinal brush border membrane

Peter H. M. KLAREN*, Sjoerd E. WENDELAAR BONGA and Gert FLIK†

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

We have studied the effect of ATP on Ca²⁺ uptake in intestinal brush border membrane vesicles (BBMVs) of the teleost tilapia (*Oreochromis mossambicus*). ATP stimulated Ca²⁺ uptake 12-fold over the control, with a linear time course. Ionomycin and detergent treatment did not reduce BBMVs' Ca²⁺ content, indicating the binding of Ca²⁺ to a membrane component. A rank order of ATP > ADP > AMP was established for the stimulation of Ca²⁺ uptake. Adenosine, vanadate, adenosine 5'-[α,β -methylene]triphosphate (a P_{2X} purinoceptor agonist) and adenosine 5'-[γ -thio]triphosphate (a P-type ATPase inhibitor)

were without effect. 2-Methylthioadenosine 5'-triphosphate, a P_{2Y} purinoceptor agonist, mimicked the stimulation by ATP. As judged from a kinetic comparison, ATP hydrolysis and the stimulation by ATP of Ca²⁺ uptake were not compatible. The P₂ purinoceptor antagonist suramin and the P_{2Y} purinoceptor antagonist Reactive Blue-2 inhibited the Ca²⁺ uptake stimulated by 1 mM ATP (IC₅₀ 0.17 mM and 58 μ M respectively). We conclude that ATP-stimulated Ca²⁺ uptake in tilapia intestine is dissociated from ATPase activity and is mediated through a P₂ purinoceptor.

INTRODUCTION

Extracellular ATP-stimulated Ca²⁺ influx has been demonstrated in many different cell types, including ovarian tumour cells, smooth-muscle cells, cells from HL-60, HeLa and NG108-15 lines, J774 mouse macrophages, rat thyroid cells and atrial myocytes [1–8]. The literature on ATP-stimulated Ca²⁺ uptake in intestinal cells is scarce; stimulatory effects of ATP in isolated enterocytes from human colon and from rat and chicken intestine have been reported [9–11]. A P₂ purinoceptor [10], an ATP-activated, protein kinase-regulated ion channel [11] and a Ca²⁺ uptake system sensitive to prenylamine (an antagonist for calmodulin) [9] have been advanced to explain the stimulation by ATP of Ca²⁺ uptake in these cells. Unfortunately, observations on isolated cells do not allow discrimination between the apical and basolateral membrane domains as the site where ATP interacts with the Ca²⁺ transport system. Proceeding from the stimulatory effect of extracellular ATP on Ca²⁺ uptake in isolated enterocytes [9–11], and from the observation that millimolar concentrations of a nucleoside triphosphate occur in tilapia intestinal mucosal fluid, we here focus on the hypothesis that ATP regulates Ca²⁺ entry across the apical membrane of the enterocyte. Purified brush border membrane vesicles (BBMVs) were used as a tool. We provide evidence for an ATPase-independent, ATP-stimulated Ca²⁺ uptake in intestinal BBMVs and conclude from pharmacological studies that a subtype of a P₂ purinoceptor is present in the apical membrane of the enterocyte and is involved in Ca²⁺ uptake.

MATERIALS AND METHODS

Animals

Sexually mature tilapia (*Oreochromis mossambicus*) of both sexes, weighing 250–450 g, were obtained from laboratory stock. Fish were kept in 100-litre aquaria, supplied with running Nijmegen tap water ([Ca²⁺] 0.8 mM, at 25 °C) under a photoperiod of 16 h

of light alternating with 8 h of darkness. The animals were fed with Trouvit® commercial fish food (Trouw, Putten, The Netherlands), at a ration of 1.5 % (w/w) of their body weight per day.

Materials

⁴⁵CaCl₂ was purchased from Amersham International (Aylesbury, Bucks., U.K.). Scintillator 299® was from Packard Instrument Co. (Meriden, CT, U.S.A.). ATP (Tris salt), adenosine 5'-[α,β -methylene]triphosphate (pp[CH₂]pA) and ADP [di(monocyclohexylammonium) salt] were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). AMP (free acid) and adenosine 5'-[γ -thio]triphosphate (ATP[S]) were from Boehringer (Mannheim, Germany). Reactive Blue-2 and the tetrasodium salt of 2-methylthioadenosine 5'-triphosphate (2-MeSATP) were from Research Biochemicals International (Natick, MA, U.S.A.). SK&F 96365 (dissolved in DMSO) was kindly provided by Dr. R. J. M. Bindels (Department of Physiology, Faculty of Medicine, University of Nijmegen, Nijmegen, The Netherlands). Suramin was a gift from Bayer AG (Leverkusen, Germany). Solutions of suramin were made in ultrapure water and were prepared freshly before experimentation. All chemicals were of analytical grade and obtained from commercial suppliers. Membrane protein was determined with a Coomassie Brilliant Blue kit (Bio-Rad, München, Germany), with BSA as a reference.

Isolation of intestinal brush border membranes

Fish were killed by spinal transection, and intestinal brush border membranes were isolated by using a magnesium aggregation technique and differential centrifugation as described in detail previously [12]. Brush border membranes were collected in 150 mM KCl/0.8 mM MgCl₂/20 mM Hepes/Tris (pH 7.4). The final membrane preparation was enriched 17-fold in alkaline phosphatase (EC 3.1.3.1). We previously determined that almost 100 % of the vesicles are oriented right-side-out [12].

Abbreviations used: BBMV, brush border membrane vesicle; pp[CH₂]pA, adenosine 5'-[α,β -methylene]triphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; 2-MeSATP, 2-methylthioadenosine 5'-triphosphate.

* Present address: Department of Biomedical Science, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K.

† To whom correspondence should be addressed.

Ca²⁺ transport assays

Zero *trans* Ca²⁺ uptake

The assay medium was identical with the buffer in which the membranes were suspended and contained calculated free concentrations of 5 mM Ca²⁺ and 0.8 mM Mg²⁺, and graded amounts of total nucleotide. The Ca²⁺ concentration used in our assays was in the range of the ambient intestinal luminal concentration, which we measured to range from 2 to 27 mM. Ca²⁺ uptake in the presence of a fixed nucleotide concentration was measured at 1 mM nucleotide. This concentration represents $V_{\max}$ conditions with respect to ATP, as measured by ATP hydrolysis in a 5 min assay. The ⁴⁵CaCl₂ radioactive concentration was 2.5 MBq/ml. Assays were performed at 37 °C; membranes and assay media were prewarmed before incubation. The incubation temperature chosen was the optimum for another important Ca²⁺ transporting protein in fish, namely the high-affinity Ca²⁺-ATPase [13]. We calculate that in the presence of 1 mM total ATP, 96 % of the ATP is complexed with Ca (0.72 mM CaATP²⁻) or Mg (0.24 mM MgATP²⁻), leaving a free ATP⁴⁻ concentration of 35 μM. Incubation was initiated by mixing 10 μl of a preparation of BBMV with 70 μl of the assay medium. Zero *trans* Ca²⁺ uptake was measured as the difference between uptake for 5 min at 37 °C and at 0 °C. SK&F 96365 was tested at a concentration of 15 μM, which is in the range of the IC₅₀ values reported for different preparations [7,14,15]. Suramin is generally employed at concentrations equal to or higher than the agonist concentration [2,16,17]. We added suramin at 0.1 and 1.0 mM to the assay medium. Reactive Blue-2 was added at concentrations ranging from 30 to 500 μM. All antagonists were tested with 1 mM ATP present in the incubation medium. The reaction was quenched by the addition of 1 ml of ice-cold isotonic stop buffer containing 150 mM KCl, 20 mM Trizma-7.0® (pH 7.4 at 0 °C) and 10 mM LaCl₃. The quenched sample was immediately filtered over an 80 kPa vacuum, using ME25 nitrocellulose filters with a pore size of 0.45 μm (Schleicher & Schuell, Dassel, Germany). Filters were rinsed three times with 2 ml of the ice-cold stop buffer and dissolved in 4 ml Scintillator 299® scintillation cocktail. The radioactivity retained on the filters was measured in a Wallac 1410 (Pharmacia, Turku, Finland) liquid-scintillation counter. Uptake values are expressed as nmol of Ca²⁺ per 5 min per mg of protein.

Isotope equilibrium exchange

In isotope equilibrium exchange experiments ⁴⁵Ca²⁺ movements were measured in the absence of an electrochemical driving force for Ca²⁺. Membrane vesicles were effectively loaded with Ca²⁺ as described earlier [12]. Incubations were performed in the same assay buffer and at the same temperature as was used in zero *trans* experiments. Intravesicular (*trans*) and extravesicular (*cis*) free Ca²⁺ concentrations were 5 mM. Quenching and filtering were done as described for the zero *trans* experiments. Blank values were determined in quadruplicate. The tracer content of the BBMV was divided by the specific activity of the assay medium to obtain the amount of Ca²⁺ exchanged across the membrane.

Enzyme assays

Adenine nucleotidase activity was measured by incubating 10 μl of membrane suspension (protein concentration 0.3–0.7 mg/ml) for 5 min at 37 °C in 500 μl of medium including 150 mM KCl, a calculated 5 mM free Ca²⁺ (added as CaCl₂), 0.8 mM free Mg²⁺ (added as MgCl₂), 20 mM Hepes/Tris (pH 7.4) and graded

amounts of ATP or ADP. Ouabain, theophylline and oligomycin-B were added at concentrations of 1 mg/ml, 2 mM and 5 μg/ml respectively. The reaction was quenched by the addition of 1 ml ice-cold 8.6 % (w/v) trichloroacetic acid to the mixture. Liberated P_i was colorimetrically measured at a wavelength of 700 nm with the iron molybdate reagent described by Fiske and SubbaRow [18]. An inorganic phosphate solution (Sigma Chemical Co.) served as a P_i standard.

ATP detection in intestinal luminal contents

An ATP diagnostic kit (Sigma Chemical Co.) was used. Luminal contents were sampled immediately after the fish was killed. To 1 vol. of a sample (approx. 10 μl) was added 1 vol. of 8.6 % trichloroacetic acid. Samples were mixed and left on ice for 5 min, and then centrifuged at 4500 *g* for 10 min in a Microfuge Centaur centrifuge to obtain a clear supernatant on which the assay was performed. The assay is based on the phosphorylation of 3-phosphoglycerate by ATP and the subsequent conversion of 1,3-diphosphoglycerate to glyceraldehyde 3-phosphate, during which reaction NADH is oxidized to NAD⁺. The decrease in NADH content of the reaction mixture is then a measure of the initial ATP content of the sample. A solution containing 1 mM ATP was run in parallel with the intestinal samples and served as a standard. According to the manufacturer's information, the ATP-diagnostic kit is equally sensitive to the nucleoside triphosphates GTP, ITP and UTP. We therefore refer to the results of this assay as nucleoside triphosphate levels. A method in which a firefly luciferase–luciferin reagent was employed proved unsuitable for the detection of ATP in luminal samples: because of the turbidity of the samples, luminescence could not be detected even after the addition of an internal ATP standard.

Calculations and statistics

To calculate free and complexed Ca, Mg and ATP concentrations in our assay media we used the computer program Chelator [19]. Stability constants were from Sillén and Martell [20]. Data points from inhibition studies were fitted to the equation:

$$v = v_0 \{1 - [I]/([I]K_i)\}$$

where v_0 is the uptake rate in the absence of the inhibitor, $[I]$ is the inhibitor concentration and K_i is the value of $[I]$ at which inhibition is half-maximal under the specific incubation conditions. Data were analysed with a nonlinear regression data analysis program [21]. Results are presented as means ± S.E.M. unless stated otherwise. Student's *t* test for unpaired data and Welch's approximate, and Kruskal-Wallis' non-parametric, analysis of variance followed by Dunn's multiple comparison test were used for statistical evaluation as appropriate. Significance was accepted at $P < 0.05$. Asterisks indicate a significant difference with respect to the control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

RESULTS

The average nucleoside triphosphate concentration in the luminal fluid of tilapia was 0.9 ± 0.3 mM ($n = 9$).

Figure 1 shows a linear time course of the ATP-stimulated zero *trans* Ca²⁺ uptake. A slope of 55 nmol/min per mg is calculated. The tracer content of the BBMV was not affected by the addition of 10 μM ionomycin/1 % Triton X-100, indicating the binding of Ca²⁺ to membrane components. In contrast, in control incubations (i.e. in the absence of extracellular ATP) the addition of ionomycin resulted in a 2-fold increase in the

Figure 1 Time course of ATP-stimulated zero *trans* Ca²⁺ uptake in tilapia intestinal BBMVs (means $\pm$ S.E.M., $n = 5$)

Effects of 10 μ M ionomycin and 1% Triton X-100 in the extravesicular medium are shown. The free Ca²⁺ concentration was 5 mM. Symbols: $\circ, \triangle, \square$, uptake in the presence of 1 mM ATP *cis*; $\bullet, \blacktriangle, \blacksquare$, controls (i.e. uptake in the absence of ATP). Arrows indicate the addition of ionomycin ($\blacktriangle$) and Triton X-100 ($\blacksquare$) respectively. $\circ, \bullet$, Controls (untreated membranes). The slope of the straight line indicates an uptake rate of 55 nmol/min per mg.

Figure 2 ATP-stimulated ⁴⁵Ca²⁺ uptake, measured in a 5 min incubation interval, plotted as a function of protein concentration in the membrane vesicle preparation

The ⁴⁵Ca in the BBMVs is expressed as a fraction of the specific radioactivity of the tracer. The straight line is described by the function $y = 4.3 \times 10^{-2}x - 3 \times 10^{-3}$ ($r = 0.69$, $n = 23$, $P = 0.0003$). A runs test did not indicate a significant departure from linearity ($P = 0.36$).

tracer content in BBMVs. Here, Triton X-100 treatment, in disrupting the vesicular osmotic space, caused the tracer content to fall just below zero.

Figure 2 shows that the ATP-stimulated Ca²⁺ uptake is linearly correlated with the protein concentration in the preparation of BBMVs ($r = 0.69$, $P = 0.0003$), indicating a relation between the stimulated Ca²⁺ uptake and a component of the membrane vesicle preparation.

In our experimental set-up the ATP-stimulated Ca²⁺ uptake is measured as a temperature-sensitive uptake. This approach is common in many membrane transport studies and is used to distinguish non-specific binding of tracer to extravesicular sites from transmembrane translocation. Ca²⁺ uptake at 0 °C and in the presence of 1 mM ATP was $31 \pm 3\%$ ($n = 23$) of the total uptake measured at 37 °C; 70% of the ATP-stimulated Ca²⁺ uptake is thus temperature-dependent. Compared with control BBMVs, at 0 °C 1 mM ATP increased the Ca²⁺ content in

Figure 3 Effect of 1 mM ATP *cis* on isotope equilibrium exchange rate ($n = 4$)

Means $\pm$ S.E.M. are shown. Isotope exchange rate in the presence of 1 mM ATP *cis* can be described by a straight line with a slope of 68 nmol/mg. Symbols: $\circ$, uptake in the presence of 1 mM ATP *cis*; $\bullet$, controls.

Table 1 Effect of ATP, ADP, AMP, ATP[S], pp[CH₂]pA and 2-MeSATP (all 1 mM), 10 μ M vanadate, 15 μ M SK&F 96365 or 0.1 or 1 μ M suramin in the extravesicular medium on zero *trans* Ca²⁺ uptake (J^{Ca}) in tilapia intestinal BBMV

Uptake after 5 min was measured, [Ca²⁺]_{cis} = 5 mM. Controls represent uptake in the absence of ATP. Abbreviation: n , number of experiments.

Addition	J^{Ca} (nmol per 5 min per mg)	n
Control	25.2 ± 1.4	5
ATP	$372.4 \pm 150.1^{**}$	5
ADP	157.9 ± 26.5	5
AMP	38.9 ± 4.1	5
Adenosine	26.4 ± 3.3	5
ATP + 10 μ M vanadate	$333.3 \pm 101.2^{**}$	5
ATP, no SK&F 96365	291.7 ± 71.1	3
ATP + 15 μ M SK&F 96365	278.7 ± 54.5	3
Control	24.5 ± 9.5	4
ATP	$112.7 \pm 26.4^*$	4
ATP + 0.1 mM suramin	80.1 ± 23.1	4
ATP + 1 mM suramin	$37.8 \pm 11.0^\dagger$	4
Control	35.5 ± 5.2	13
ATP	$423.5 \pm 65.5^{***}$	14
ATP[S]	83.6 ± 12.6	4
pp[CH ₂]pA	43.2 ± 12.3	5
2-MeSATP	$352.1 \pm 72.5^{**}$	5

† Suramin (1 mM) significantly inhibits ATP-stimulated Ca²⁺ uptake ($P = 0.04$).

BBMVs by a factor of 1.9 ± 0.2 ($n = 21$). At 37 °C this increase was a factor of 11.8 ± 1.7 ($n = 23$), again showing temperature dependence. We further investigated extravesicular Ca²⁺ binding by using an isotope equilibrium exchange protocol. During the loading procedure, vesicles are not only loaded intravesicularly (as validated in a previous paper [12]), but it can also be assumed that extravesicular binding sites will be masked by Ca²⁺. Figure 3 shows that 1 mM ATP in the extravesicular medium produces a linear rate of tracer equilibrium exchange, compared with control incubations. From the knowledge that the specific vesicular volume of the BBMVs is 6.0 μ l/mg it can be calculated that from a vesicular Ca²⁺ content of 30 nmol/mg intravesicular loading takes place. These results are best explained by assuming

Figure 4 Initial rates of a nucleotidase activity in resealed tilapia intestinal BBMVs

Means $\pm$ S.E.M. are shown. Data points were fitted to a Michaelis–Menten equation. Symbols: $\bullet$, ATP ($n = 5$), $V_{\max} = 285 \mu\text{mol/h per mg}$, $K_m = 88 \mu\text{M ATP}$; $\circ$, ADP ($n = 4$), $V_{\max} = 233 \mu\text{mol/h per mg}$, $K_m = 230 \mu\text{M ADP}$.

the exposure of intravesicular Ca^{2+} -binding sites on incubation with ATP.

ATP, ADP and AMP (1 mM) stimulated Ca^{2+} uptake in BBMVs, albeit with a decreasing amplitude (Table 1). Adenosine was without effect. Vanadate (10 μM), an inhibitor of P-type ATPases, and SK&F 96365 (15 μM), an antagonist of receptor-mediated Ca^{2+} entry [15], did not inhibit ATP-stimulated Ca^{2+} uptake in BBMVs.

From the results obtained so far it seems that an adenosine phosphoesterase is important in the uptake of Ca^{2+} in BBMVs. Because ATPase activity has been localized to the apical membrane mammalian intestinal cells [22–27], an investigation of the involvement of an ATPase in the uptake of Ca^{2+} was warranted.

In the presence of inhibitors of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and alkaline phosphatase activity, BBMVs showed a nucleotidase activity, hydrolysing ATP and ADP (Figure 4). Apparent affinity

coefficients (K_m) were 88 μM ATP and 230 μM ADP respectively. Because AMP did not affect transport (Table 1) we did not assay AMP hydrolysis by BBMVs. When BBMVs were incubated at pH 10.2 and with a zinc supplementation, i.e. under optimal conditions for alkaline phosphatase, the potency rank order was reversed: AMP ADP ATP (results not shown). Because all of the BBMVs preparation is oriented right-side-out [12], the nucleotidase activity must be an ecto-enzymic activity in the enterocyte. These results could indicate that hydrolysis of a phosphate ester is necessary to cause the stimulatory effect of ATP. We therefore examined the kinetics of ATP hydrolysis and the ATP concentration dependence of Ca^{2+} uptake in BBMVs. Both experiments were performed under conditions that yielded initial velocities. Figures 5(A) and 5(B) show that the kinetics of these processes, measured in paired preparations, differed significantly. ATP hydrolysis was well described by single Michaelis–Menten kinetics (Hill coefficient 1.0). The kinetic parameters $V_{\max}$ and K_m were calculated to be 82 $\mu\text{mol of P}_i/\text{h per mg}$ and 64 $\mu\text{M ATP}$ respectively. In the same substrate range, the ATP-stimulated Ca^{2+} uptake was linear, having a slope of 60 $\mu\text{l/min per mg}$ and an offset representing ATP-independent uptake (as described previously [12]). These results show that ATP hydrolysis is not responsible for the ATP-stimulated Ca^{2+} uptake in BBMVs. The free ATP^{4-} concentration in our assay media can be calculated from the total ATP concentration. The inset in Figure 5(B) shows that, up to 180 μM free ATP^{4-} (which is equivalent to 5 mM total ATP in our assay medium), Ca^{2+} uptake is also a linear function of the ATP^{4-} concentration.

We finally tested the involvement of an ATP receptor. Incubations of BBMVs with 1 mM suramin, a P_2 purinoceptor antagonist, resulted in a significant decrease in the ATP-stimulated Ca^{2+} uptake (Table 1). From Table 1 a K_i of 0.17 mM suramin was calculated. An ATP analogue of which the adenine base is modified (2-MeSATP, a P_{2Y} agonist) mimicked the stimulatory effect of ATP (Table 1). Analogues with modifications in the triphosphate chain (ATP[S] and $\text{pp[CH}_2\text{]pA}$) were unable to stimulate Ca^{2+} uptake in BBMVs (Figure 6). The P_{2Y} antagonist Reactive Blue-2 dose-dependently inhibited the ATP-stimulated Ca^{2+} uptake (Figure 6). A K_i of 58 μM was calculated.

Figure 5 A comparison of the ATP concentration-dependence of ATPase activity and ATP-dependent Ca^{2+} uptake in resealed tilapia BBMVs

(A) ATPase activity in resealed tilapia intestinal BBMVs as a function of the extravesicular ATP concentration. Means $\pm$ S.E.M. ($n = 3$). The curve is described by a single Michaelis–Menten equation. The inset shows an Eadie–Hofstee transformation of the data. Kinetic parameters are: $V_{\max} = 82 \mu\text{mol of P}_i/\text{h per mg}$, $K_m = 64 \mu\text{M ATP}$, Hill coefficient 1.0. (B) ATP-dependent Ca^{2+} uptake in tilapia intestinal BBMVs. Means $\pm$ S.E.M. ($n = 3$). Slope and intercept of the straight line are 60 $\mu\text{l/min per mg}$ and 19 nmol of $\text{Ca}^{2+}/\text{min per mg}$ respectively. The inset shows Ca^{2+} uptake as a function of the calculated free ATP^{4-} concentration. Slope and intercept of the straight line in the inset are 2 $\mu\text{l/min per mg}$ and 19 nmol of $\text{Ca}^{2+}/\text{min per mg}$ respectively.

Figure 6 Dose-dependent inhibition by Reactive Blue-2 of ATP-stimulated Ca^{2+} uptake in intestinal brush border membranes

The extravesicular ATP concentration was 1 mM. K_i is calculated as 58 μ M Reactive Blue-2.

DISCUSSION

We conclude from this study that a P_2 purinoceptor located in the apical membrane of the enterocyte is involved in ATP-stimulated Ca^{2+} transport in tilapia intestinal BBMV. The kinetics of ATP-stimulated Ca^{2+} uptake and ATP hydrolysis by ecto-ATPase activity proved incongruent. Consequently we rejected an adenosine (ecto)nucleotidase activity as a mediator of the ATP-stimulated Ca^{2+} transport. For Ca^{2+} uptake in tilapia BBMV we established an agonist rank order of $ATP = 2\text{-MeSATP} > ADP > AMP$. Adenosine, ATP[S] and $pp[CH_2]pA$ were ineffective in stimulating Ca^{2+} uptake. Ca^{2+} uptake was a linear function of ATP^{4-} concentration, and this is indicative of a P_{2Z} subtype purinoceptor. However, it has been reported that ADP and AMP are ineffective in stimulating P_{2Z} receptors [28]. There is a consensus [28–30] on the non-subtype-specific inhibitory action of suramin on P_{2X} and P_{2Y} receptors, the insensitivity of P_{2Y} receptors for $pp[CH_2]pA$ and the sensitivity of the P_{2Y} subtype for 2-MeSATP and Reactive Blue-2. Our results therefore seem to be congruent with a P_{2Y} purinoceptor's [28–31] mediating Ca^{2+} transport in tilapia intestine. However, the pharmacological profile (i.e. inhibition by Reactive Blue-2 and stimulation by 2-MeSATP) of a recently cloned P_{2X} purinoceptor [32] is similar to that of a cloned P_{2Y} purinoceptor [29]. For this reason, and because of possible differences between mammalian and non-mammalian purinoceptors, further experiments are needed to obtain a definite subtyping of the P_2 purinoceptor. The compound SK&F 96365, an antagonist for receptor-mediated Ca^{2+} entry (RMCE), failed to block the ATP effect. SK&F 96365 blocks L-type voltage-operated Ca^{2+} channels in smooth-muscle cells and pituitary GH_3 cells [15] and an ATP-stimulated Ca^{2+} current in human neutrophils [7]. However, in a smooth-muscle preparation SK&F 96365 failed to inhibit ATP-gated Ca^{2+} channels [15]. It has been concluded that SK&F 96365 modulates post-receptor events, i.e. the Ca^{2+} entry step rather than receptor binding of the agonist [15].

ATP and ATP analogues have been reported to act on the smooth musculature of the gastrointestinal tract of several teleost species [33–36]. Knight and Burnstock [37] were the first to demonstrate the involvement of P_1 and P_2 purinoceptors in the actions of purines on the contraction of stomach and intestine in *Gasterosteus aculeatus*. Our results show that purinoceptors in the gastrointestinal tract of teleosts are involved not only in regulating muscular contractions but also in the absorption of Ca^{2+} .

ATP-stimulated Ca^{2+} influx has been demonstrated in a variety of cell types [1–6]. However, the literature on ATP-stimulated

Ca^{2+} uptake in intestinal cells is scarce. Kimmich and Randles [11] found an increase in cytosolic Ca^{2+} concentration in isolated chicken intestinal epithelial cells when 2 mM ATP was added. Chlorpromazine, a calmodulin antagonist, inhibited ATP-stimulated Ca^{2+} uptake in these cells. In cell lines of human colonic epithelial origin, 100 μ M ATP increased the cytosolic Ca^{2+} concentration 20-fold over the resting level [10]. An extracellular P_2 purinoceptor, regulating epithelial cell ion transport, was suggested on the basis of the potency of different agonists ($ATP > ADP \ll$ Nadenosine). Exogenous ATP increased Ca^{2+} uptake 2–6-fold in isolated rat intestinal epithelial cells [9]. A maximal effect was observed at 1 mM ATP. Adenosine, 5'-AMP, ADP and non-hydrolysable ATP analogues proved ineffective. However, the slowly hydrolysable ATP analogue ATP[S] fully mimicked the ATP-stimulated Ca^{2+} uptake. The calmodulin antagonist prenylamine inhibited the ATP effect, which was also observed by Popper and Batra [3] in a human ovarian cancer cell line. The inhibition by prenylamine is congruent with observations on chlorpromazine inhibition [11]. Richards et al. [9] suggest that hydrolysis of a terminal phosphate of ATP is required for the stimulated Ca^{2+} uptake, but they do not rule out the involvement of a nucleotide-specific receptor. To summarize, experimental results on ATP-stimulated Ca^{2+} uptake in (mammalian) enterocytes indicate the involvement of a calmodulin-sensitive P_2 purinoceptor. However, the membrane domain in which this putative purinoceptor resides remains unresolved.

A prerequisite for a functional purinergic receptor in the enterocyte's brush border membrane is the presence of the proper ligand, i.e. ATP, in the intestinal lumen. One point that needs to be addressed is the source of the ligand. A possible source for luminal ATP would be sloughed-off epithelial cells. In isolated enterocytes from rat and human, cellular values of ATP were measured to range from 5 to 6 nmol of ATP/mg of protein [38]. If we assume cellular ATP levels in tilapia enterocytes to be in the same range, we calculate that a luminal concentration of 0.9 mM ATP is equivalent to 150–180 mg of protein/ml of luminal fluid. Assuming a cytoplasmic ATP concentration of 5 mM [31], cellular dimensions of $5 \mu\text{m} \times 5 \mu\text{m} \times 20 \mu\text{m}$, and a cellular volume of 5×10^{-16} litre for an enterocyte, we calculate that a luminal ATP concentration of 0.9 mM is equivalent to 4×10^{11} cells per ml of luminal fluid. We therefore do not think that sloughed-off enterocytes are a plausible source for ATP. Ingested food could be another source for luminal ATP, but the industrially processed fish feed we provide our fish contains no detectable amounts of ATP. Ultrastructural studies on intestinal absorptive cells in tilapia (S. E. Wendelaar Bonga, unpublished work) and other teleost species [39–41] demonstrate the presence of numerous mitochondria and clear vesicles in the apical cytoplasm of the enterocyte. It is tempting to correlate these structural phenomena with the presence of a secretory pathway for ATP in the intestine. Every cell could potentially serve as a source of extracellular ATP [1,31], and nanomolar/micromolar pericellular ATP concentrations have been predicted [1]. These predicted ATP levels differ by at least one order of magnitude from the nucleotide concentrations measured in intestinal luminal samples. The regulation of intestinal transmembrane Ca^{2+} transport by extracellular ATP of enterocytic origin, via a P_2 purinoceptor, is an interesting working hypothesis for future research.

REFERENCES

- 1 El-Moatassim, C., Dornand, J. and Mani, J.-C. (1992) *Biochim. Biophys. Acta* **1134**, 31–45

- 2 Aloj, S. M., Liguoro, D., Kiang, J. G. and Smallridge, R. C. (1993) *Biochem. Biophys. Res. Commun.* **195**, 1–7
- 3 Popper, L. D. and Batra, S. (1993) *Cell Calcium* **14**, 209–218
- 4 Hirano, Y., Sawanobori, T. and Hiraoka, M. (1993) *Jpn. J. Physiol.* **43**, S101–S103
- 5 Smit, M. J., Leurs, R., Bloemers, S. M., Tertoolen, L. G. J., Bast, A., de Laat, S. W. and Timmerman, H. (1993) *Eur. J. Pharmacol.* **247**, 223–226
- 6 Chueh, S.-H. and Kao, L.-S. (1993) *J. Neurochem.* **61**, 1782–1788
- 7 Krautwurst, D., Seifert, R., Hescheler, J. and Schultz, G. (1992) *Biochem. J.* **288**, 1025–1035
- 8 Sung, S.-S., Young, J. D.-E., Origlio, A. M., Heiple, J. M., Kaback, H. R. and Silverstein, S. C. (1985) *J. Biol. Chem.* **260**, 13442–13449
- 9 Richards, N. W., Allbee, W. E., Gaginella, T. S. and Wallace, L. J. (1987) *Life Sci.* **40**, 1665–1672
- 10 Turner, J. T., Wu, H. and Camden, J. M. (1990) *FASEB J.* **4**, A447
- 11 Kimmich, G. A. and Randles, J. (1982) *Am. J. Physiol.* **243**, C116–C123
- 12 Klaren, P. H. M., Flik, G., Lock, R. A. C. and Wendelaar Bonga, S. E. (1993) *J. Membr. Biol.* **132**, 157–166
- 13 Flik, G., van Rijs, J. H. and Wendelaar Bonga, S. E. (1985) *J. Exp. Biol.* **119**, 335–347
- 14 Bo, X., Fischer, B., Maillard, M., Jacobson, K. A. and Burnstock, G. (1994) *Br. J. Pharmacol.* **112**, 1151–1159
- 15 Merritt, J. E., Armstrong, W. P., Benham, C. D., Hallam, T. J., Jacob, R., Jaxa-Chamiec, A., Leigh, B. K., McCarthy, S. A., Moores, K. E. and Rink, T. J. (1990) *Biochem. J.* **271**, 515–522
- 16 Nörenberg, W., Langosch, J. M., Gebicke-Haerter, P. J. and Illes, P. (1994) *Br. J. Pharmacol.* **111**, 942–950
- 17 Langosch, J. M., Gebicke-Haerter, P. J., Nörenberg, W. and Illes, P. (1994) *Br. J. Pharmacol.* **113**, 29–34
- 18 Fiske, C. H. and SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- 19 Schoenmakers, T. J. M., Visser, G. J., Flik, G. and Theuvsen, A. P. R. (1992) *Biotechniques* **12**, 870–879
- 20 Sillén, L. G. and Martell, A. E. (1964) *Stability Constants of Metal Ion Complexes*, Special Publication 17, The Chemical Society, London
- 21 Leatherbarrow, R. J. (1987) *Enzfitter: A Non-linear Regression Data Analysis Program for the IBM PC*, Elsevier Science Publishers, Amsterdam
- 22 Ghijsen, W. E. J. M., de Jong, M. D. and van Os, C. H. (1980) *Biochim. Biophys. Acta* **599**, 538–551
- 23 Ghijsen, W. E. J. M. and van Os, C. H. (1979) *Nature (London)* **279**, 802–803
- 24 Haussler, M. R., Nagode, L. A. and Rasmussen, H. (1970) *Nature (London)* **228**, 1199–1201
- 25 Krawitt, E. L., Stubbert, P. A. and Ennis, P. H. (1973) *Am. J. Physiol.* **224**, 548–551
- 26 Martin, D. L., Melancon, Jr., M. J. and DeLuca, H. F. (1969) *Biochem. Biophys. Res. Commun.* **35**, 819–823
- 27 Wang, H. and Gilles-Baillien, M. (1992) *Arch. Int. Physiol. Biochim. Biophys.* **100**, 289–294
- 28 Watson, S. P. and Girdlestone, D. (1994) *Trends Pharmacol. Sci.* **15**, (suppl.), 1–51
- 29 Webb, T. E., Simon, J., Krishek, B. J., Bateson, A. N., Smart, T. G., King, B. F., Burnstock, G. and Barnard, E. A. (1993) *FEBS Lett.* **324**, 219–225
- 30 Kebabian, J. W. and Neumeyer, J. L. (eds.), (1994) *The RBL Handbook of Receptor Classification*, Research Biochemicals International, Natick, NJ
- 31 Gordon, J. L. (1986) *Biochem. J.* **233**, 309–319
- 32 Brake, A. J., Wagenbach, M. J. and Julius, D. (1994) *Nature (London)* **371**, 519–523
- 33 Burnstock, G. (1972) *Pharmacol. Rev.* **24**, 509–560
- 34 Holmgren, S. (1983) *Comp. Biochem. Physiol.* **74C**, 229–238
- 35 Holmgren, S. and Jensen, J. (1985) *Comp. Biochem. Physiol.* **82C**, 81–89
- 36 Kitazawa, T., Hoshi, T. and Chugun, A. (1990) *Comp. Biochem. Physiol.* **97C**, 13–24
- 37 Knight, G. E. and Burnstock, G. (1993) *Comp. Biochem. Physiol.* **106C** (1), 71–78
- 38 Hitchin, B. W., Dobson, P. R. M., Brown, B. L., Hardcastle, J., Hardcastle, P. T. and Taylor, C. J. (1991) *Gut* **32**, 893–899
- 39 Kanyan, F. I. B., Maloiy, G. M. O. and Reite, O. B. (1975) *Anat. Anz.* **138**, 451–462
- 40 Kuperman, B. I. and Kuz'mina, V. V. (1994) *J. Fish Biol.* **44**, 181–193
- 41 Noaillic-Depeyre, J. and Gas, N. (1979) *Anat. Rec.* **195**, 621–640