Functional role of ecto-ATPase activity in goldfish hepatocytes

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Within the past years, the use of hepatocytes isolated from goldfish liver has evolved to a cellular model for studies of metabolic depression, in particular when these cells are challenged by anoxia (13, 26) or hypothermia (12). A number of mechanisms that allow viability to be maintained in the face of a severe reduction of intracellular ATP turnover were described. These mechanisms include the simultaneous inhibition of K+ influx and efflux across plasma membranes, the redirection of the available ATP production according to metabolic demands (15), and the prevention of anoxia-induced increase in cytosolic free Ca2+ (13). Among these and other adaptive responses that cells may employ, the common view is that intracellular ATP (ATP) should be guarded at all costs and that ATP does not permeate the plasma membrane of viable cells. However, evidence has accumulated that cellular ATP can indeed be released from viable cells through different mechanisms, such as exocytosis and transport through intrinsic plasma membrane proteins (for a review, see Refs. 8 and 24). Once released, ATP or its hydrolysis products can influence several biological processes, such as excitation, contraction, metabolism, and secretion. The extracellular metabolism of ATP is usually mediated by a family of enzymes called ecto-ATPases (24). These may act in a sequence together with ecto-ADPase (activity of which could be due to the same ecto-ATPase) and ecto-5′-nucleotidase to achieve complete dephosphorylation of extracellular ATP (ATP5′) to adenosine. The rate of nucleotide to nucleoside conversion is relevant, since it determines the effective time a given nucleotide is available to interact with surface receptors before being locally hydrolyzed by ectoenzymes. In mammalian liver, ecto-ATPase activity is largely restricted to the bile canalicular membrane (apical domain), where it is assumed to contribute to the degradation of nucleotides secreted into the canalicular lumen (22). Even though the source of ATP5′ in the liver remains controversial, if the environment of the hepatocytes were to accumulate ATP, regardless of its origin, then the complete dephosphorylation of the nucleotide may serve for adenosine production. Adenosine, in turn, if not degraded extracellularly, could interact with specific purinoceptors (such as subtype 1) and/or be taken up by specific transporters for the resynthesis of nucleotides (4, 22). This process has systemic consequences, since, at least in mammals, the liver acts as a purine source for tissues that lack biosynthesis of purines (4).

Given the action that ATP5′ and its hydrolysis products exert on cellular metabolism, the present study is aimed at 1) identifying ecto-ATPase activity in viable hepatocytes from goldfish and 2) studying the effects of ecto-ATPase activity on metabolism and vice versa, particularly under conditions of limited availability of metabolic energy. Goldfish hepatocytes are an adequate cellular system for this purpose, since they have shown great tolerance to pharmacological manipulation, including the use of metabolic inhibitors and toxic agents.

MATERIALS AND METHODS

Materials. [γ-32P]ATP (10 Ci/mmol), [α-32P]ATP (30 Ci/mmol), [2,8-3H]ATP (32 Ci/mmol), adenosine 5′-O-(1-thiotriphosphate), and [α-35S]ATP (1,250 Ci/mmol) were purchased from DuPont. Suramin was purchased from Calbiochem. All other chemicals were obtained from Sigma.

Animals. Goldfish (Carassius auratus L.) were obtained from commercial suppliers and were kept in 200-liter aquaria at 20°C for 3 wk before being used.
Isolation of hepatocytes. The fish were killed, and hepatocytes were isolated from freshly excised livers as described in detail elsewhere (14). The final cell pellet was resuspended in an assay medium containing (in mM) 135 NaCl, 5 KCl, 1.3 CaCl₂, 1.2 NaH₂PO₄, 1.2 MgSO₄, 10 NaHCO₃, and 10 HEPES (pH 7.5 at 20°C), including 2% BSA, and kept on ice. Cells were counted in a Neubauer cytometer. Cellular viability assessed by trypan blue exclusion was >97% and remained constant until the end of the incubation period. Sixty minutes before measurements, the cell suspension (0.3 x 10⁶ to 3 x 10⁶ cells/ml) was preincubated at 20°C. Throughout the text, n represents the number of independent preparations used.

Conversion factors in hepatocytes. The ratio of fresh weight to cell number was 4.52 ± 0.13 mg fresh wt/10⁶ cells (n = 9), and the ratio of total protein content to cell number was 0.68 ± 0.09 mg protein/10⁶ cells (n = 13). Cell diameter assayed by a videomicroscope system was 12.83 ± 0.26 μm (51 cells measured from 3 independent preparations), and the percentage of dry weight was 24.7 ± 0.9 (n = 19).

Isolation of erythrocytes. Goldfish were killed by a blow on the head. Blood was withdrawn by cardiac puncture using heparinized syringes and subsequently spun for 4 s at 10,000 g to remove the plasma and the buffy coat. Erythrocytes were then incubated as described for isolated hepatocytes.

Assay of ecto-ATPase activity. Ecto-ATPase activity was determined at 20°C by following the release of γ-32Pp from [γ-32P]ATP as described elsewhere (25). In brief, the reaction was started by adding [γ-32P]ATP to a hepatocyte suspension under continuous stirring. At different times, a 50- to 100-μl aliquot of the suspension was withdrawn and poured into 750 μl of a stop solution containing 4.05 mM MgO₃(NH₄)₂ and 0.83 mM HClO₄. The ammonium molybdate solution formed an assay medium containing (in mM) 135 NaCl, 5 KCl, 1.3CaCl₂, 1.2 NaH₂PO₄, 1.2 MgSO₄, 10 NaHCO₃, and 10 HEPES (pH 7.5 at 20°C), including 2% BSA, and placed in a thermostated water bath at 37°C. After loading, cell suspensions were washed and resuspended in BSA-free incubation medium and placed in a thermostated cuvette equipped with a stirring device. Fluorescence measurements were performed at 20°C with a Hitachi F-2000 Fluorospectrophotometer with excitation wavelength set to 340 nm and emittance wavelength set to 480 nm. Calibrations were performed on each sample applying the method described by Moon et al. (21) for a single excitation instrument. Cai⁺ concentrations were calculated according to the formula given by Grynkiewicz et al. (9) using the dissociation constant of 135 nM given therein.

Determination of ATP. ATP contents of the incubation medium were measured by incubating cells (70 x 10⁶ cells/ml) in the presence of 5 mM EGTA. After 0, 60, and 120 min of incubation, duplicate samples of the cell suspension were withdrawn and centrifuged in small reaction tubes (5 s at 6,000 g), and an aliquot of the cell-free supernatant was removed and frozen for later analysis. ATP was measured in a luminometer using the luciferin-luciferase method as described by Brown (2).

Protein determination. This was carried out by the method of Lowry et al. (17).

Mathematical analysis. Paired t-test (2 sided) was used to determine differences in mean ecto-ATPase activities and Ca²⁺ values among the different treatments used. For experiments with IAA plus CN , analysis of variance was used to test whether cellular viability and ecto-ATPase activity decreased with time. A P value of 0.05 was considered significant. Results of ecto-ATPase activity vs. [ATP] were analyzed by means of nonlinear regression. Each point of the curve represents the result of a single experiment in which the value of the slope (±SE) was obtained by linear regression of the time course of γ-32Pp release with at least 10 experimental points.

Concentrations of ATP, (when given in mM) were estimated by considering 75.3% of cellular water and the volume of the hepatocyte as that of a sphere of 13-μm diameter (see Isolation of hepatocytes).

RESULTS

Identification of ecto-ATPase activity in intact hepatocytes of goldfish. Figure 1 shows four experiments in which ecto-ATPase activity was measured as the rate of...
release of $\gamma$-32P from labeled ATP. The reaction was linear in time from 5 to 5,000 µM ATP. No further production of $\gamma$-32P was detectable after removal of the cells from the suspension. Stoichiometric chelation of both extracellular Mg$^{2+}$ and Ca$^{2+}$ with EDTA led to 100% inhibition of ecto-ATPase activity.

Ecto-ATPase activity was assessed at 5 µM ATP in the presence of 10 mM suramin (inhibitor of ecto-ATPases), 2.6 µM oligomycin (inhibitor of F-type ATPases), 10 µM metavanadate (inhibitor of P-type ATPases), 1 mM ouabain (inhibitor of Na$^{+}$-K$^{+}$-ATPase), 1 mM N-ethylmaleimide (inhibitor of V-type ATPases), or 1 mM p-nitrophenyl phosphate (PNPP, substrate of phosphatases). A low micromolar concentration of ATP was selected so as to reflect the physiological nucleotide concentration that can be built up in the extracellular medium (8).

It can be seen (Table 1) that although 10 mM suramin inhibited ecto-ATPase activity by ~70%, inhibitors of transport ATPases of the F, V, and P type and ouabain did not have any effect on enzyme activity. Because 1 mM PNPP did not affect the hydrolysis rate of 5 µM ATP, the presence of ectophosphatases was ruled out. Figure 2 shows a curve of ecto-ATPase activity vs. [ATP] (5–10,000 µM). Ecto-ATPase activity followed a single hyperbola with apparent maximal activity ($V_{\text{max}}$) of 8.3 ± 0.4 nmol Pi·(10$^6$ cells)$^{-1}$·min$^{-1}$ and $K_{\text{m}}$ of 667 ± 123 µM (n = 2).

Adenosine production and uptake. In experiments of Fig. 3, hepatocytes were incubated for up to 10 min with 5 µM of different labeled ATP compounds with the following results. Addition of [$\alpha$-32P]ATP resulted in the release of $\alpha$-32P and adenosine at 8.2 ± 0.3 pmol·(10$^6$ cells$^{-1}$)·min$^{-1}$. After incubation of cells with [2,8-3H]ATP, the time course of [2,8-3H]adenosine uptake showed a lag phase, after which it reached a rate of 7.9 ± 0.4 pmol·(10$^6$ cells$^{-1}$)·min$^{-1}$. This was fully inhibited by 0.5 mM formycin B plus 0.125 mM dipyridamole. On the other hand, exposure of cells to the ATP analog, [$\alpha$-35S]ATP, showed no significant uptake of

Table 1. Effects of inhibitors of ATPases and PNPP on ecto-ATPase activity of goldfish hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Experimental</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suramin (10 mM)</td>
<td>0.031 ± 0.010</td>
<td>0.009 ± 0.003*</td>
</tr>
<tr>
<td>Metavanadate (10 µM)</td>
<td>0.025 ± 0.009</td>
<td>0.029 ± 0.018</td>
</tr>
<tr>
<td>Oligomycin (2.6 µM)</td>
<td>0.027 ± 0.009</td>
<td>0.025 ± 0.008</td>
</tr>
<tr>
<td>Ouabain (1 mM)</td>
<td>0.026 ± 0.008</td>
<td>0.029 ± 0.012</td>
</tr>
<tr>
<td>NEM (1 mM)</td>
<td>0.020 ± 0.005</td>
<td>0.021 ± 0.006</td>
</tr>
<tr>
<td>PNPP (1 mM)</td>
<td>0.019 ± 0.006</td>
<td>0.021 ± 0.007</td>
</tr>
</tbody>
</table>

Values given in nmol 32P·(10$^6$ cells)$^{-1}$·min$^{-1}$ are means ± SE; n, no. of independent preparations. PNPP, p-nitrophenyl phosphate; NEM, N-ethylmaleimide. Ecto-ATPase activity was assayed at 5 µM ATP (20 °C) and 10$^6$ cells/ml in the presence of different compounds. Cell suspensions were preincubated 5 min with different agents before addition of [$\gamma$-32P]ATP. Cell counting was performed before and after each experiment. *P < 0.05.
were preincubated for up to 120 min in the presence of 0.5 mM IAA and 2 mM CN−, inhibitors of glycolysis and oxidative phosphorylation, respectively. After 120 min, ATP, decreased to 20% of the control value, whereas Ca2+ increased about six times. At the same time ecto-ATPase activity (at 5 µM ATP) and cellular viability remained constant. In Fig. 4, results are shown as percentage of the respective control value at time 0. Control values were as follows: ATPi 2.37 ± 0.25 nmol/10⁶ cells, Cai 134 ± 47 nM (n = 4), and ecto-ATPase activity 0.018 ± 0.002 nmol·(10⁶ cells·min⁻¹) (n = 4).

Effects of nucleosides on Ca2+. In the absence or presence of 1.3 mM extracellular Ca2+ (Cae), addition of 5 µM ATP to the cell suspension increased Cai, 2.5 times (Table 2). As illustrated in Fig. 5, in the presence of Cae, the effect of ATP on Ca2+ was dose dependent. ADP, AMP, and adenosine (5 µM), when added separately, had no significant effect on Cai levels, whereas 100 µM ADP and adenosine increased Cai 1.8 and 1.5 times, respectively. Experiments performed with ATP analogs showed that β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) had no effect on Cai, whereas 100 µM adenosine 5′-O-(3-thiotriphosphate) (ATPγS) increased Cai 1.9 times.

Leakage of ATP. Cell suspensions (70 ± 10⁶ cells/ml) were incubated for 120 min in the presence of 5 mM EGTA to inhibit ecto-ATPase activity. Throughout the experiment, ATPi remained constant at 12.22 ± 3.9 pmol·10⁶ cells⁻¹·min⁻¹ (n = 9), i.e., 0.85 µM.

Ecto-ATPase activity of goldfish erythrocytes. Ecto-ATPase activity was 1.8 ± 0.1 pmol·10⁶ cells⁻¹·min⁻¹ (n = 4) at 5 µM ATP and 10.3 ± 0.7 pmol·10⁶ cells⁻¹·min⁻¹ (n = 4) at 50 µM ATP.

Fig. 2. Ecto-ATPase activity vs. ATP (0.01–10 mM) in hepatocytes (0.8 × 10⁶ to 3 × 10⁶ cells/ml) of goldfish. Each point represents slope (±SE) obtained by linear regression of a time course of γ³²P𝑖 release with at least 10 experimental points. Line represents fit by nonlinear regression of a single hyperbola to data. Different symbols represent 2 independent preparations.

Fig. 3. Time course of α³²P𝑖 release (●) from 5 µM [α-³²P]ATP, uptake of [2,8-³H]adenosine from 5 µM [2,8-³H]ATP in presence (■) and absence (□) of both 0.5 mM formycin B + 0.125 mM dipyridamole, and uptake of 5 µM [α-³⁵S]ATP (▲). Inset: same data are shown (except ■), together with rate of γ³²P𝑖 production (▲) from 5 µM [γ-³²P]ATP. Results are means ± SE of 4 independent preparations.

Fig. 4. Intracellular ATP (ATPi; ○), ecto-ATPase activity (●), viability (▲), and concentration of free cytosolic Ca2+ (Ca2+; □) after incubating hepatocytes with 2 mM NaCN + 0.5 mM iodoacetic acid. Results are means ± SE of 4–6 independent preparations.
Table 2. Effects of different nucleosides on cytosolic free Ca"" of isolated hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5 µM</th>
<th>100 µM</th>
</tr>
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<tbody>
<tr>
<td>ATP</td>
<td>253.2 ± 38.6*</td>
<td>221.6 ± 26.1*</td>
</tr>
<tr>
<td>ATP (at 0 Ca&quot;&quot;&quot;)</td>
<td>102.7 ± 2.77</td>
<td>193.9 ± 21.2*</td>
</tr>
<tr>
<td>AMP-PCP</td>
<td>98.5 ± 3.6</td>
<td>95.3 ± 1.4</td>
</tr>
<tr>
<td>ADP</td>
<td>155.3 ± 36.9</td>
<td>178.4 ± 25.7*</td>
</tr>
<tr>
<td>AMP</td>
<td>100.6 ± 4.0</td>
<td>101.6 ± 3.9</td>
</tr>
<tr>
<td>Adenosine</td>
<td>113.5 ± 7.8</td>
<td>148.3 ± 15.6*</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of respective control value before addition of agent ± SE; n, no. of independent preparations. Agents were given at 5 and 100 µM. Effect of ATP was studied in presence and in absence of extracellular Ca"" (Ca"""). AMP-PCP, β,γ-methyleneadenosine 5'-triphosphate; ATPγS, adenosine 5'-O-(3-thiotriphosphate). *P < 0.05.

DISCUSSION

This paper reports unequivocal evidence for the presence of ecto-ATPase activity in suspensions of goldfish hepatocytes. The external localization of the nucleotide-hydrolyzing site is supported by the observation that ATP added to the extracellular medium was hydrolyzed in intact cells, whereas hydrolysis of ATP did not continue when cells were removed from the suspension (Fig. 1). Suramin, one of the few substances known to inhibit ecto-ATPase activity in many systems (27), inhibited 70% of ecto-ATPase activity. A distinctive characteristic of the enzyme is the lack of sensitivity to specific inhibitors of P-, F-, and V-type ATPases (Table 1) and the absolute requirement of divalent cations for activity (Fig. 1). The lack of effect of vanadate points to the absence of a phosphorylated intermediate. Ecto-ATPase activity was measured over a wide range of hepatocyte (0.3 × 10⁶ to 10 × 10⁶ cells/ml) and ATP (5–10,000 µM) concentrations. When millimolar concentrations of extracellular Ca"" and Mg²⁺ were used, the apparent Vₘₐₓ as well as the Kᵢ, were in the range of those reported in many other systems (see Ref. 24), including rat hepatocytes (16).

In addition to a characterization of enzyme activity as such, the present study tried to shed light on the potential cellular function of ecto-ATPase activity, in particular during situations of energy limitation. For this purpose we incubated hepatocytes with CN⁻ and IAA, inhibitors of oxidative phosphorylation and glycolysis, respectively. As shown in Fig. 4, this treatment decreased ATP, by ~80% and increased Ca²⁺ up to sixfold. However, ecto-ATPase activity as well as cellular viability remained unchanged. If it is assumed that ecto-ATPase in goldfish hepatocytes is an integral membrane protein, this would imply that cytosolic aspects of the enzyme do not sense changes in the ATPᵢ concentration of this magnitude and that the severe metabolic consequences of cutting the most important pathways of ATP production are not relevant for ecto-ATPase function. The question arises as to how ecto-ATPase activity can be included in a general metabolic scheme. Isolated goldfish hepatocytes at 20°C showed an ATPᵢ of ~3.5 mM (estimated from Refs. 6 and 11, as described in Mathematical analysis).

In these cells, one of the most important promoters of ATPᵢ consumption is the Na⁺-K⁺-ATPase, whose activity in intact cells at 20°C is ~0.4 nmol Pᵢ (10⁶ cells)⁻¹ min⁻¹ (11, 26). This is almost the same activity that ecto-ATPase activity displays at the same temperature in the presence of only 40 µM ATPe. The difference between both enzyme activities is even more pronounced under metabolic inhibition caused by chemical anoxia or hypothermia; 90-min incubation of hepatocytes with CN⁻ decreases Na⁺-K⁺-ATPase activity to ~50%, representing 50–90% of total ATPᵢ turnover (14). However, incubation of hepatocytes with 2 mM CN⁻ for up to 120 min had no influence on ecto-ATPase activity (unpublished results). During cooling Na⁺-K⁺-ATPase activity displayed an apparent Q₁₀ ranging from 2 [Q₁₀(15–25°C)] to 8 [Q₁₀(20–15°C)] (12), whereas for ecto-ATPase activity apparent Q₁₀ was ~1.2–2 (7). Thus, due to the high specific activity displayed by ecto-ATPase and its relative independence from environ-

![Fig. 5. Representative traces of Ca²⁺ illustrating effect of β,γ-methyleneadenosine 5'-triphosphate (AMP-PCP) and extracellular ATP (ATPₑ) in presence of Ca₂⁺ (A) and ATPᵢ in absence of Ca₂⁺ (B).](http://ajpregu.physiology.org/)

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mental and/or metabolic factors, a putative ATP liberated into the surroundings of liver cells will be rapidly hydrolyzed. Once ATP is hydrolyzed to ADP or even further to AMP (because some ecto-ATPases display ecto-ADPase activity as well), the ultimate fate of the nucleotide depends on the presence of 5'-nucleotidase.

Experiments of Fig. 3 were specifically designed to test for the existence of ectonucleotidases capable of completing the sequence leading to the total dephosphorylation of ATP. By incubation of cell suspensions with [α-32P]ATP, the liberation of α-32P may be expected only if ectoenzymes are present that liberate γ-, β-, and α-P, of ATP at the surface of the cell. This is indeed what we observed: the total hydrolysis of the nucleotide was determined through the liberation of α-32P, from [α-32P]ATP. Moreover, experiments using [2,8-3H]ATP showed that as soon as adenosine is released, it is taken up at almost the same rate (Fig. 3). That is, the rates of α-32P production and [3H]adenosine uptake were similar, supporting the idea of a mechanism for adenosine salvage. Because dipyridamole plus formycin B fully inhibited the uptake of [3H]adenosine, we postulate adenosine uptake to occur through facilitated diffusion.

Figure 3 also shows that the initial rate of γ-32P release was about four times higher than adenosine production. This agrees well with results of other systems showing a delay in adenosine production due to inhibition of 5'-nucleotidase (which promotes conversion of AMP to adenosine) by ADP.

Concerning the use of [3H]ATP, it could be argued that these experiments rely on the assumption that nucleotides are impermeable to the membrane. However, when adenosine uptake was inhibited, there was no uptake of label from [3H]ATP. Moreover, [α-35S]ATP, a nonhydrolyzable ATP analog, did not permeate the plasma membrane (Fig. 3).

In rat hepatocytes, ATP acts as a substrate or second messenger for P2Y receptors (10), causing a rapid mobilization of Ca2+ from intracellular Ca2+ stores via inositol trisphosphate (23). Our results with goldfish hepatocytes are in agreement with these studies. It can be seen (Table 2, Fig. 5) that low ATP, independently of the presence of Ca2+, strongly increased Ca2+. ATP (5 µM) is sufficient to increase Ca2+ more than two times without significantly changing the amount of Pi of the incubation medium. Thus the observed effect cannot be due to Pi activation of a megachannel at the mitochondrial membrane, as postulated for rat hepatocytes (28). An effect on Pi receptors by ATP degradation products was ruled out, since 100 µM adenosine has little and 100 µM AMP no effect on Ca2+ (Table 2). That AMP-PCP, a nonhydrolyzable ATP analog, has no effect on Ca2+ is consistent with its postulated action on P2X receptors, which are not linked to intracellular Ca2+ release. Typical P2Z receptors can also be excluded, since addition of 1 mM ATP in the presence of extracellular Ca2+ leads to a rapid and reversible increase of Ca2+ (Fig. 5). As reported for rat hepatocytes (29), P2Z receptors mediate a late and sustained increase of Ca2+ that causes irreversible hepatocellular injury. Finally, the fact that 100 µM ATPγS significantly increased Ca2+ confirms that ATP hydrolysis is not necessary and that P1 receptors are not involved.

These results, together with the experimental evidence showing that the increase of Ca2+ at low [ATP], is entirely due to intracellular stores (Table 2), support the hypothesis that the Ca2+ increase is exerted through binding of the nucleotide to specific P2Y receptors. The rank order for the effect of the different nucleosides on Ca2+ was ATP > ATPγS > ADP > adenosine.

According to the above-mentioned results, ectonucleotidases are expected to reduce the effective concentration of hydrolyzable purinoceptor agonists and thus restrain the magnitude and/or duration of cell responses. Coupling of ecto-ATPase and 5'-nucleotidase with a nucleoside transport system would then decrease the toxicity of ATP and promote adenosine conservation.

ATP has been identified in the fluid surrounding many cell types (24). However, in goldfish hepatocytes, as in many other systems, the source of ATP remains to be detected. As mentioned in the results, we were unable to detect a significant leakage of ATP from hepatocytes. The ultrastructure of fish liver differs from that of higher vertebrates in that there is a large blood-hepatocyte exchange area (3). This means that, in principle, a putative ATP being released from blood cells, endothelial cells, or any other cell type could easily interact with the basolateral membrane of goldfish hepatocytes. However, considering the high levels of ecto-ATPase activity of goldfish erythrocytes (as opposed to mammalian erythrocytes, see Ref. 5), our present data tend to rule out a significant role of these cells as a source of ATP. Alternatively, ATP could also arise as a consequence of the export from endothelial cells (27). However, as pointed out by Gordon (8), ATP could locally reach micromolar concentrations without being detectable. With respect to the goldfish, on the basis of the fact that this species is a good anaerobe whose cells may repeatedly experience prolonged periods of hypoxia and steep oxygen gradients, the following scenario may be proposed. If as a consequence of any conditions of extreme energy limitation a certain population of cells died, their nucleotides would be released into the interstitial space with the subsequent uptake of adenosine by the surviving cells. As seen in mammalian hepatocytes, this extra adenosine may improve the energetic state of cells by increasing the phosphorylation potential together with a decreased glycolytic flux (18, 19). In this way, dying cells would constitute part of a mechanism mediated by ectoenzymes that improve the survival chances of the rest of the cells.

When trying to generalize about the possible functions of ecto-ATPase, a last word of caution should be given with regard to the model of hepatocytes isolated by the use of collagenase, since their plasma membranes may have lost their functional heterogeneity. In the intact liver, ecto-ATPase activity may be localized in different membrane domains and probably have
different functions. For example, the function of ecto-ATPase acting near purinoceptors may be different from a canalicular ecto-ATPase promoting adenosine salvage.

Perspectives

The presence of millimolar concentrations of ATP in the cytosol and the rapid extracellular hydrolysis of ATP by ectoenzymes points to the idea that ecto-ATPase activity may contribute to the maintenance of a steep electrochemical gradient of ATP across the plasma membrane of goldfish hepatocytes. On the basis of these data, one interesting point for future research should be to clarify whether this gradient can be utilized by the cells. In 1993, Abraham et al. (1) identified a P-glycoprotein capable of stimulating the export of ATP. Using different cell lines, these authors showed that once a certain extracellular ATP concentration is built up, the continuous export of ATP and the subsequent dephosphorylation by ectoenzymes allowed ATP concentration to remain constant. In the present study, we were unable to measure any export of ATP, although it could be possible that this mechanism can only be turned on under special physiological conditions. The observations by Abraham et al. (1) raise the possibility (among others) that the electrochemical gradient of ATP is the driving force to extrude intracellular toxicants by cotransport, a mechanism that could be extremely useful for a typical anaerobe, such as goldfish.

Another point that deserves clarification is the role of the adenosine liberated as a consequence of the extracellular ATPase acting near purinoceptors. Similar to the adenosine liberated as a consequence of the extracellullar toxicants by cotransport, a mechanism that could be extremely useful for a typical anaerobe, such as goldfish.

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