Extracellular ATP4- Modulates Organic Anion Transport by Rat Hepatocytes*

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The hepatocyte has an organic anion transport system that recognizes compounds such as bilirubin and sulfobromophthalein. These anions circulate bound tightly to albumin from which they are extracted rapidly by hepatocytes by an electroneutral process that requires extracellular inorganic anions such as Cl- for activity. Transport activity is reduced by depletion of intracellular ATP, but whether ATP interacts directly with this transporter is not known. In this study, the influence of extracellular ATP on the hepatocyte organic anion transport mechanism has been characterized. In the presence of 2.5 mM Ca2+ and 2 mM Mg2+, initial uptake of [35S]sulfobromophthalein was reduced by 50% at 1 mM ATP. In the absence of divalent cations sensitivity to ATP was 10-fold greater. Other nucleotides including UTP, CTP, GTP, ADP, AMP, and AMP-PCP (adenosine 5'-($\beta$, $\gamma$-methylene)triphosphate) were inactive. Decreased transport activity was rapidly reversible, was non-competitive with respect to ATP, did not require ATP hydrolysis, and did not correlate with P2y purinergic receptor activity. Differential activity of ATP on sulfobromophthalein transport in the presence and absence of divalent cations was not due to ecto-ATPase activity but rather to alteration in [ATP4-]. Although an ATP4- receptor in macrophages mediates increased cellular permeability, reduced organic anion permeability is seen in hepatocytes. This effect is not seen in the hepatoma cell line HepG2. Modulation of activity of the organic anion transporter by extracellular ATP may have important pathophysiological consequences in conditions resulting in liver cell injury.

A role for extracellular ATP in modulation of various cellular processes has become increasingly apparent (1–3). Several types of cell surface receptors for ATP have been described, including a receptor on macrophages that recognizes ATP tetra-anion (ATP4-). Binding of ATP4- to this receptor permeabilizes reversibly the cell to organic anions such as lucifer yellow (4). This has permitted loading of macrophages with lucifer yellow in order to study the organic anion export system (5).

The hepatocyte also has an organic anion transport system that recognizes compounds such as bilirubin and sulfobromophthalein (BSP). These anions circulate bound tightly to albumin from which they are extracted rapidly by hepatocytes (6, 7). This high affinity hepatocyte organic anion transporter is electroneutral and requires extracellular inorganic anions such as Cl- for activity (8, 9). Transport activity is reduced by depletion of intracellular ATP (8). Whether ATP interacts directly with this transporter is not known. In the present study, the influence of extracellular ATP on the hepatocyte organic anion transport mechanism has been characterized. Extracellular ATP interacts with P2y purinergic receptors on hepatocytes resulting in events such as elevation of intracellular Ca2+, cellular depolarization, and elevated membrane permeability to small molecules (1–3). The results of this study suggest, however, a unique and specific modulatory effect of ATP4- on hepatocyte organic anion transport.

MATERIALS AND METHODS

Isolation and Short Term Culture of Rat Hepatocytes—Rat hepatocytes were isolated from 200–250-g male Sprague-Dawley rats (Taconic Farms, Germantown, NY) after perfusion of the liver with collageenate type IV (Sigma) and cultured as described previously (8, 9). In brief, cells were suspended in medium consisting of Waymouth’s 752/1 (Life Technologies, Inc.) containing 25 mM Hepes, pH 7.2, 5% heat-inactivated fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 1.7 mM additional CaCl2, 5 µg/ml bovine insulin (Sigma), 100 units/ml penicillin (Life Technologies), and 0.1 mg/ml streptomycin (Life Technologies). Approximately 1.5 x 106 cells in 3 ml were placed in 60-mm Lux culture dishes (Nunc, Inc., Naperville, IL), and cultured in 5% CO2 atmosphere at 37°C. After 1–2 h, medium was changed and cells were cultured for an additional 16–18 h. Cell viability in culture was >90% as judged by trypan blue exclusion. Total cell protein was determined by the method of Lowry using BSA as standard (10).

Chemicals—ATP (disodium salt), ADP (potassium salt), AMP (sodium salt), CTP (sodium salt), UTP (sodium salt), AMP-PCP (tritiated), and AMP-PCP (sodium salt) were obtained from Sigma. GDP (disodium salt) was obtained from Boehringer Mannheim. AMP-PNP was obtained from Pharmacia LKB Biotechnology Inc. as the sodium salt. Stock solutions (10–100 mM) were prepared fresh in water, pH-neutralized, and diluted appropriately into cell medium, as indicated below.

Uptake of [35S]BSP by Cultured Hepatocytes in the Presence and Absence of Various Nucleotides—Uptake of [35S]BSP by overnight cultured hepatocytes was determined as previously described (8, 9). In brief, cells were washed three times with 1.5 ml of modified serum-free medium (SFM), consisting of 135 mM NaCl, 1.2 mM MgCl2, 0.81 mM MgSO4, 27.8 mM glucose, 2.5 mM CaCl2, and 25 mM Hepes adjusted to pH 7.2 with NaOH. In some experiments, SFM was prepared in which no Ca2+ or Mg2+ salts were added (Ca2+/Mg2+-free SFM). 1 ml of a 1% (14.7 µM) BSA (fraction V, Sigma) in SFM was added to each plate, which was then incubated for 15 min at 4°C. In other studies, SFM in the absence of BSA was used.
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[<sup>[35S]</sup>BSP (2,500–4,000 mCi/mmol) was prepared as described previously (11) and was dissolved in distilled water. Sufficient unlabeled BSP was added to make an 80 μM BSP stock solution containing 10<sup>4</sup> dpm/ml. A 10-μl aliquot of this stock solution was added to 1 ml of medium on each plate, and incubation was continued at 4 °C or 37 °C for various periods. After incubation with [<sup>[35S]</sup>BSP, plates were washed twice at 4 °C with 1.5 ml of SFM and incubated for 5 min in 1.5 ml of 5% BSA in SFM at 4 °C to displace surface-bound radioactivity. Plates were then washed three times with 1.5 ml of SFM at 4 °C. Cells were harvested, and radioactivity was quantified in a RackBeta model 1217 liquid scintillation counter (LKB Instruments, Gaithersburg, MD), after addition of 10 ml of Hydrofluor (National Diagnostics, Inc., Somerville, NJ). Replicate plates were washed and harvested in phosphate-buffered saline for determination of cellular protein by the method of Lowry using BSA as standard. In some studies, at the time of incubation with [<sup>[35S]</sup>BSP, a neutralized stock solution of nucleotide was added to provide a final concentration of 0.05–5.0 mM. Some of these studies were performed in Ca<sup>2+ </sup>/Mg<sup>2+ </sup>-free SFM at pH 6 and 8 in addition to pH 7.

Reversibility of Inhibition of [<sup>[35S]</sup>BSP Uptake—As presented below, both 0.5 mM ATP and 9.5 mM ATP's inhibited uptake of [<sup>[35S]</sup>BSP in Ca<sup>2+ </sup>/Mg<sup>2+</sup>-free SFM. To determine whether this effect was reversible, cultured hepatocytes were incubated with nucleotide at 37 °C or 4 °C, 1 ml of 0.1% BSA was added, and plates were incubated for an additional 5 or 10 min at 37 °C or 4 °C. Uptake of [<sup>[35S]</sup>BSP was then determined as above over 5 min.

Effect of ATP on Efflux of [<sup>[35S]</sup>BSP from Cultured Hepatocytes—Hepatocytes were incubated with [<sup>[35S]</sup>BSP at 37 °C for 15 min as described above. They were then washed four times with 1.5 ml of SFM at 4 °C and incubated in 1 ml of 5% BSA in SFM for 15 min at 4 °C or 37 °C. Efflux was determined in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free SFM containing 0.5 mM ATP and was measured in 0.5 ml of medium. Efflux was quantified as the difference of [<sup>[35S]</sup>BSP in medium at 37 °C versus that at 4 °C (8).

Determination of Ecto-ATPase Activity of Cultured Hepatocytes—Cultured hepatocytes were incubated in SFM containing 0.1% BSA in the presence or absence of 2.5 mM Ca<sup>2+</sup> and 2 mM Mg<sup>2+</sup>. ATP was added to a final concentration of 0.5 mM and incubated at 37 °C. At various times through 10 min, ATP was determined in medium using a coupled enzyme assay as previously described (5).

Quantification of Free Cytosolic Ca<sup>2+</sup> by Fluorescence Imaging Dye Loading with Fura-2—These studies were performed essentially as described previously for cultured corporal smooth muscle cells (14). Dissociated hepatocytes were plated onto type III calf-skin collagen-coated (Sigma) 1 cm diameter glass coverslips (13) and loaded with the acetoxyethyl-mester form of Fura-2 (Fura-2 AM, Molecular Prober, Eugene, OR). Briefly, Fura-2 AM was dissolved in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free SFM containing 10% Me<sub>2</sub>SO (dimethyl sulfoxide) (i.e. 500 μM stock solution). Coverslips with the hepatocytes were placed in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free SFM containing 10 μM Fura-2 for 30 min at 37 °C. The final Me<sub>2</sub>SO concentration was less than 0.1%, which by itself did not affect cell shape or viability. The incubation was terminated by washing the cells several times with fresh Ca<sup>2+</sup>/Mg<sup>2+</sup>-free SFM, and the cells were placed in a temperature-regulated (37 °C) chamber on a Zeiss Axiopt microscope for measurement of intracellular calcium. Cells were continuously superfused with Ca<sup>2+</sup>/Mg<sup>2+</sup>-free SFM at 37 °C for nucleotide delivery, superfusion was stopped and nucleotide was added from freshly diluted stock solution.

Optical Measurement of Intracellular Ca<sup>2+</sup>—Images were sequentially obtained from a manual gain intensified CCD camera (Quantax, Inc.), digitized at excitation wavelengths of 330 and 380 nm using a filter wheel (Sutter Instruments) with emission set above 480 nm by a dichroic mirror, and the resulting images were stored on an IBM compatible computer (Dell Computer System) (14). Data were analyzed for intracellular Ca<sup>2+</sup> levels using the Image 1AT/FL software package (Media, PA) and calibration was performed as previously described (14).

Studies in HepG2 Cells—The relatively well differentiated human hepatoma cell line, HepG2, was cultured as we have previously described (15). Cultures were fed on the 2nd day after seeding and were studied when they reached confluence at day 5, with each dish containing approximately 1.5 × 10<sup>6</sup> cells. Indicated studies were performed identically to those performed in cultured rat hepatocytes.

Statistical Analysis of Data—Statistical significance was calculated by Student's t test (Sigma Plot version 5.0).

RESULTS

Influence of Extracellular ATP on Initial Uptake and Efflux of [<sup>[35S]</sup>BSP—As seen in Fig. 1, ATP, in the presence of 2.5 mM Ca<sup>2+</sup> and 2 mM Mg<sup>2+</sup>, reduced initial uptake of [<sup>[35S]</sup>BSP. Control initial uptake of [<sup>[35S]</sup>BSP was 1.41 ± 0.26 pmol/min/mg protein (mean ± S.E., n = 3) and was reduced by half at an ATP concentration of approximately 1 mM. In the absence of divalent cations, initial uptake of [<sup>[35S]</sup>BSP was 1.47 ± 0.11 pmol/min/mg protein (n = 18), and sensitivity to ATP was approximately 10-fold greater (Fig. 1). When hepatocytes were preloaded with [<sup>[35S]</sup>BSP in the absence of divalent cations and then put into ligand-free medium, BSP effluxed at 0.30 ± 0.05 (mean ± S.D.) pmol/min/mg protein (n = 5). There was no difference in the rate of efflux in the presence of 0.5 mM ATP (0.29 ± 0.06 pmol/min/mg protein, n = 3). Effect of Other Nucleotides on Initial Uptake of [<sup>[35S]</sup>BSP—As seen in Fig. 2A (divalent cations present) and Fig. 2B (divalent cations absent), there was little or no effect on ATP uptake of UTP, CTP, GTP, AMP, ADP, or AMP-PCP. The ATP analog ATP<sub>γ</sub>S behaved similarly to ATP. Determination of initial uptake of [<sup>[35S]</sup>BSP at various concentrations of ATP, ADP, or GTP emphasized the marked difference in response to these nucleotides (Fig. 3, A and B).

Reversibility and Kinetics of ATP Inhibition of [<sup>[35S]</sup>BSP Uptake—As seen in Fig. 4, initial [<sup>[35S]</sup>BSP uptake more than doubled and was close to 70% of normal within 5 min of removal of ATP or ATP<sub>γ</sub>S from medium. Analysis of [<sup>[35S]</sup>BSP uptake in the presence of various concentrations of ATP in the absence of divalent cations was consistent with a non-competitive type of inhibition (Fig. 5), in which the K<sub>i</sub> for ATP was 0.08 mM.

Role of the P<sub>2y</sub> Purinergic Receptor in ATP Inhibition of [<sup>[35S]</sup>BSP Initial Uptake—Previous investigations revealed the presence of a P<sub>2y</sub> class of purinergic receptors on the surface of rat hepatocytes (1–3, 16, 17). These receptors have

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**Fig. 1.** Extracellular ATP reduces [<sup>[35S]</sup>BSP uptake by hepatocytes. Initial uptake of [<sup>[35S]</sup>BSP (0.8 μM) in 0.1% BSA (14.7 μM) by overnight cultured rat hepatocytes (1.5 × 10<sup>6</sup> cells/plate) was determined over 5 min at 37 °C as previously described (9). Studies were performed at various concentrations of ATP in medium with or without 2.5 mM Ca<sup>2+</sup>/2.0 mM Mg<sup>2+</sup>. In both cases, ATP significantly reduced [<sup>[35S]</sup>BSP uptake. In the absence of divalent cations, however, there was an approximately 10-fold increased sensitivity of BSP transport to ATP. In this representative study, each point represents the mean of duplicate determinations; initial uptake of [<sup>[35S]</sup>BSP in the absence of ATP was 1.58 and 1.86 pmol/min/mg protein, in the presence and absence of divalent cations, respectively.
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**FIG. 2.** Nucleotide specificity of [35S]BSP transport inhibition. Initial uptake of [35S]BSP was determined in 5.0 μM nucleotide in medium containing divalent cations (2.5 mM Ca$^{2+}$/2.0 mM Mg$^{2+}$) (panel A) and in divalent cation-free medium (panel B). Data are expressed as mean ± S.E. Numbers of studies performed are in parentheses above bars. In control studies performed in the presence of divalent cations, initial uptake of [35S]BSP was 1.41 ± 0.26 pmol/min/mg protein (n = 3 studies in which protein was determined). In control studies performed in the absence of divalent cations, initial uptake of [35S]BSP was 1.47 ± 0.11 pmol/min/mg protein (n = 18).

* p < 0.005; ** p < 0.04.

approximately equal sensitivity to ADP and ATP (16, 17). To determine whether overnight cultured rat hepatocytes retain this receptor, the effect of various nucleotides on single cell levels of cytosolic-free calcium was determined by a fluorescence imaging technique in the absence of extracellular divalent cations. As seen in Fig. 6, ADP was as effective as ATP in increasing cytosolic free calcium levels. This was in contrast to the lack of effect of ADP on inhibition of BSP uptake (Fig. 3A).

**Stability of ATP in Cell Medium—Ecto-ATPase activity**

Ecto-ATPase activity has been described previously in rat hepatocytes (18). Optimal activity has been seen in the presence of divalent cations (19). It is possible that differential activity of ATP on BSP transport in the presence and absence of divalent cations was due to modulation of ecto-ATPase activity. This was tested directly by assaying residual ATP content in medium of cells incubated for varied times at 37°C in SFM in the presence and absence of divalent cations. As seen in Fig. 7, ATP hydrolysis in medium was relatively slow, and there was only a small effect of divalent cations.

**Correlation of [ATP$^{-}$] with Inhibition of BSP Uptake**

The presence of divalent cations did not appear to influence substantially the concentration of ATP in cell medium. However, ATP forms complexes with Ca$^{2+}$ and Mg$^{2+}$, reducing the concentration of ATP$^{-}$ (20). Whether [ATP$^{-}$] correlated with inhibition of BSP uptake was determined by performing studies at pH levels 6, 7, and 8 in the absence of divalent cations. The pK$_a$ of ATP$^{-}$ is approximately 7 (20). Thus, at pH 6, less than 10% exists as ATP$^{-}$, rising to 50% and 90% at pH levels 7 and 8, respectively. BSP uptake in the presence of nucleotide at a given pH was compared to base-line BSP uptake at that pH. As compared to base-line uptake at pH 7 and 8, there was a small (≈30%) reduction in base-line BSP uptake at pH 6 (Fig. 8). As shown in Fig. 8, at pH 6, there was considerably less effect of ATP on BSP uptake as compared to results at pH 7 and 8. Studies were also performed with the non-hydrolyzable analog AMP-PNP. This compound has a pK$_a$ of 7.7 (21). Thus, at pH 6, less than 2% exists as the tetra-anion, as compared to less than 20% at pH 7 and almost 70% at pH 8. As seen in Fig. 8, only at pH 8, where tetra-anionic AMP-PNP predominated, was significant inhibition of BSP uptake seen.

**Influence of ATP on BSP Transport by HepG2 Cells**

HepG2 cells have been shown to have a low affinity high capacity organic anion transporter (reviewed in Ref. 15). Unlike results in overnight cultured rat hepatocytes, there was no effect of ATP at concentrations as high as 5 mM in the absence of divalent cations on BSP transport by HepG2 cells (Fig. 9). In four studies, initial uptake of [35S]BSP was 3.0 ± 0.3 (mean ± S.E.) pmol/min/mg protein as compared...
These results indicate that upon removal of ATP or ATPyS, BSP uptake returns toward normal. Results are expressed as mean ± S.E. Numbers of studies performed in each group are in parentheses. Initial uptake of \([35S]BSP\) in control cells was 1.53 ± 0.14 pmol/min/mg protein. *, p < 0.01.

Fig. 4. Inhibition of \([35S]BSP\) transport by ATP is reversible. Uptake of \([35S]BSP\) over 5 min by short-term cultured rat hepatocytes was determined at 37 °C in divalent cation-free medium in the presence or absence of 0.5 mM ATP or 0.5 mM ATPyS. In replicate plates, cells were incubated with nucleotide alone for 5 min. These replicate plates were then washed, incubated for 5 min with nucleotide-free medium at 37 °C, and uptake of \([35S]BSP\) determined. These results indicate that upon removal of ATP or ATPyS, BSP uptake returns toward normal. Results are expressed as mean ± S.E. Numbers of studies performed in each group are in parentheses. Initial uptake of \([35S]BSP\) in control cells was 1.53 ± 0.14 pmol/min/mg protein. *, p < 0.01.

Fig. 5. ATP is a non-competitive inhibitor of BSP transport. Uptake by cultured hepatocytes of 0.4 μM \([35S]BSP\) (●) and 0.8 μM \([35S]BSP\) (○) was determined in divalent cation-free medium at various ATP concentrations. This Dixon plot of the data of this representative study reveals intersection of the lines on the abscissa, consistent with non-competitive inhibition. In this study, \(K_i\) for ATP was 0.08 mM.

Fig. 6. Effects of ATP and ADP on intracellular free \([Ca^{2+}]\). Rat hepatocytes were cultured overnight on type III collagen-coated glass coverslips. HepG2 cells were cultured on untreated glass coverslips. At the time of study, cells were incubated for 30 min at 37 °C in divalent cation-free serum-free medium containing 10 μM Fura-2. Cells were washed, incubated in divalent cation-free medium, and intracellular free calcium concentration, \([Ca^{2+}]\), was determined by a fluorescence imaging technique in individual cells before and after addition of 100 μM ATP or ADP. These studies reveal that hepatocytes and HepG2 cells respond to ATP and ADP by increasing \([Ca^{2+}]\). This effect has been attributed to purinergic receptor activity. This contrasts with differential effect of these nucleotides on organic anion transport. Data are expressed as mean ± S.E. Numbers of studies are in parentheses.

Fig. 7. Influence of divalent cations on ecto-ATPase activity. Hydrolysis over 10 min of 0.5 mM ATP added to overnight cultured rat hepatocytes was determined in medium containing 0.1% BSA with (●) or without (○) 2.5 mM Ca²⁺/2.0 mM Mg²⁺. In both conditions, ATP hydrolysis was relatively low, with over 60% of ATP remaining intact at 5 min. Although hydrolysis was reduced in the absence of divalent cations, this could not account for the marked difference in sensitivity of BSP transport to ATP described above. Results of this representative study are expressed as mean ± S.D. of triplicate determinations.

**DISCUSSION**

ATP is a widely distributed intracellular molecule with important roles as an energy source, as a substrate for cellular enzymes such as kinases, and as a cofactor for various transporters. Although the biological importance of intracellular ATP has been long known, a role for extracellular ATP in modulation of various cellular processes has been described more recently (2, 3, 22). In particular, several classes of cell surface receptors that recognize ATP and other nucleotides have been described (see Refs. 1-3 for reviews). These receptors are located on a wide variety of cell types.

In the hepatocyte, extracellular ATP has been shown to have a number of important effects. Charest et al. (16) described rapid mobilization of intracellular Ca²⁺ in isolated rat
Despite some heterogeneity in function, the hepatocyte P_{zz} purinergic receptor responds equally well to ATP and ADP, as quantified by increased cytosolic free calcium concentrations (16, 17). This has been confirmed in the present study. In addition, we demonstrated a similar response in the relatively well differentiated human hepatoma line, HepG2. In contrast, an effect of extracellular nucleotides on hepatocyte organic anion transport is limited to ATP and its congener, ATP_{7,8}, at neutral pH. These results indicate that the effect of ATP on organic anion transport does not result from its interaction with P_{zz} receptors. Previous evidence has also been presented for the presence of UTP receptors in rat liver (25, 26). However, as shown in Fig. 2B, UTP had little effect on BSP transport by hepatocytes.

These data suggest that the mechanism by which extracellular ATP modulates hepatocyte organic anion transport is novel. Aside from the lack of effect of ADP, an indication that the P_{zz} receptor system does not play a role in this process is seen from studies performed in the presence and absence of divalent cations. As seen in Fig. 1, in the absence of divalent cations, hepatocyte BSP transport is approximately 10-fold more sensitive to ATP than in their presence. An initial hypothesis was that this was due to inactivation of ecto-ATPase activity. Ecto-ATPase activity has been described on the surface of many cell types. It is activated by Ca^{2+} or Mg^{2+} and hydrolyzes ATP and other nucleotides. A hepatocyte plasma membrane ecto-ATPase has been purified and cloned (18, 27). This 105,000-dalton molecule has been found to be identical to a hepatocyte cell adhesion molecule, CAM105 (28) and is particularly enriched in the canalicular plasma membrane (19, 29, 30). As seen in Fig. 7, however, ecto-ATPase activity in overnight cultured rat hepatocytes is low. It is likely that this is due to sequestration of the canalicular lumen of these cells in culture. There is a small degree of activation of ecto-ATPase activity by divalent cations (Fig. 7). However, this is not sufficient to explain the large difference in ATP sensitivity of organic anion transport produced by divalent cations (Fig. 1).

Modulation of ATP inhibition of hepatocyte organic anion transport could be due to variation of [ATP^+] caused by chelation of divalent cations. It has been established that ATP binds avidly to Ca^{2+} and Mg^{2+} (20, 31). Other cell types have been shown to have surface receptors that interact specifically with ATP^+ (4, 31–36). To test the hypothesis that extracellular ATP^+ rather than total ATP regulates hepatocyte organic anion transport, uptake of [^{35}S]BSP was determined in the presence or absence of ATP at pH 6, 7, or 8. As previously shown (9), BSP uptake by overnight cultured rat hepatocytes is little affected when determined over this pH range, although a small reduction in uptake was seen at pH 6 in the present study. As the pK_{a} of ATP is 7.0 (20), it is highly (>90%) ionized at pH 8.0 and little (<10%) ionized at pH 6.0. As seen in Fig. 8, there was a strong correlation of the concentration of ATP^+ with reduction of [^{35}S]BSP uptake. At neutral pH, there was little effect of the non-hydrolyzable ATP derivative AMP-PNP on BSP transport (Fig. 8). However, the pK_{a} of this compound is 7.7 (21). Thus, at pH 7, only 17% is tetra-anionic. In contrast, at pH 8, over 65% of AMP-PNP is tetra-anionic, and [^{35}S]BSP uptake was inhibited by approximately 70% (Fig. 8). Increased pK_{a} cannot explain the lack of effect of other nucleotides such as ADP, which has a pK_{a} of 6.9 (20).

As noted above, receptors for ATP^+ are present on a number of different cell types. ATP^+ has been described as mediating histamine release from mast cells (32, 33). In results similar to those in the present study, this effect was signifi-

**Fig. 8.** Inhibition of [^{35}S]BSP transport correlates with nucleotide tetra-anion concentration. Uptake of [^{35}S]BSP was determined in divalent cation-free medium at pH 6, 7, or 8, containing no added nucleotide, 0.5 mM ATP, or 0.5 mM AMP-PNP. The pK_{a} of ATP is 7.0, and that of AMP-PNP is 7.7. There was considerably less effect of either nucleotide on BSP transport at pH below the pK_{a}. Inhibition of uptake correlated with the concentration of the tetravalent anion form of each nucleotide. Specifically, at pH 6, less than 10% of either compound is tetra-anionic. At pH 7, 50% of ATP is 7.0, and that of AMP-PNP is 7.7. There was considerably less effect of either nucleotide on BSP transport at pH below the pK_{a}. At pH 8, over 90% of ATP and over 65% of AMP-PNP is tetra-anionic. The mean ± S.D. of four studies is shown. Each study was performed in triplicate, and results are presented as a percentage of control uptake at each pH. Control uptake was 1.13 ± 0.33, 1.71 ± 0.74, and 1.70 ± 0.54 (mean ± S.D.) pmol/mg/min at pH 6, 7, and 8, respectively.

**Fig. 9.** ATP does not reduce BSP transport by HepG2 cells. HepG2 cells have a low affinity, high capacity organic anion transporter. As they are unable to extract BSP from BSA (15), initial uptake of [^{35}S]BSP was determined in divalent cation-free medium in the absence of BSA at various concentrations of ATP. Unlike results in overnight cultured rat hepatocytes, there was no effect of ATP at concentrations as high as 5 mM on BSP transport by HepG2 cells. Results of this representative study are expressed as mean ± S.D. of triplicate determinations. In the study, in the absence of ATP, initial uptake of [^{35}S]BSP by cultured hepatocytes was 21.9 ± 0.68 pmol/min/mg protein, whereas that of HepG2 cells was 2.45 ± 0.14 pmol/min/mg protein.

hepatocytes exposed to 1 μM concentrations of ATP and ADP. They described increased inositol 1,4,5-trisphosphate levels preceding the rise in intracellular Ca^{2+}, and also activation of phospholipase a. This hepatocyte ATP receptor has been characterized as belonging to the P_{zz} class of purinergic receptors. That there is some heterogeneity of P_{zz} receptors in hepatocytes has been suggested based upon differential activation of pertussis toxin sensitive and insensitive G-proteins (3, 23). Heterogeneity has also been described as determined by differential Ca^{2+} transients in response to ATP and ADP (24).
cantly reduced in the presence of Ca\(^{2+}\) and Mg\(^{2+}\). Evidence has been presented that the plasma membrane of these cells becomes permeabilized to normally impermeant molecules. A similar non-selective permeabilization of transformed mouse fibroblasts (34, 37) and macrophages (4, 31, 36) produced by ATP\(^{4-}\) has also been described. Studies performed in the mouse macrophage cell line J774 incubated with ATP have revealed plasma membrane depolarization resulting from Na\(^{+}\) influx and K\(^{+}\) efflux (31, 38), attributed to an ATP\(^{4-}\) receptor (31, 36). These cells were found to be permeabilized to various organic molecules, including lucifer yellow and Fura-2. This effect was not seen with larger molecules such as trypan blue and Evans blue and was reversed rapidly following removal of ATP\(^{4-}\) from the medium (4).

The results of the present study suggest that rat hepatocytes possess a surface receptor that recognizes ATP\(^{4-}\). As distinct from macrophages and other cell types that increase their permeability in response to this nucleotide, hepatocyte permeability for BSP is markedly reduced. This is seen whether BSP is presented as a complex with albumin (Fig. 2) or as the free molecule of 794 daltons (Fig. 6). Although this inhibition of transport is reversible rapidly, its kinetics suggest inactivation of the organic anion transporter (Fig. 5). It is unlikely that this is due to alterations in cytosolic free [Ca\(^{2+}\)] or in altered cellular permeability to Ca\(^{2+}\), as these changes are seen with both ADP and ATP. Membrane depolarization and increased Na\(^{+}\) and K\(^{+}\) permeabilities have also been described in cells exposed to external ATP. However, in previous studies, we have found that uptake of \(^{35}\)S\)BSP was unaffected by cell depolarization (8, 9). As seen in this and previous studies (8), BSP transport by hepatocytes is bidirectional. Of interest is the fact that influx of BSP was inhibited by extracellular ATP while efflux of BSP was unaffected. The reason for this asymmetry is not known. Previous studies have suggested that, in the presence of albumin, high affinity low capacity organic anion influx is measured (8, 9, 15). Once inside the cell, however, it is possible that there are a number of potential efflux pathways as well as ATP-dependent excretion across the canalicular (apical) membrane (39-41). The relative importance and ATP sensitivity of these pathways in net BSP efflux as determined in the present study is unknown.

The present results are in contrast to several studies in which intracellular ATP was shown to activate canalicular organic anion transport (39-41). This may be accompanied by ATP hydrolysis (41). As indicated in the present study, extracellular non-hydrolyzable AMP-PNP at a pH above its pK\(_{a}\) inhibits organic anion transport. It may be speculated that, in conditions in which intracellular ATP is low (e.g. hypoxia), extracellular ATP may be high due to cellular injury. This may result in markedly reduced organic anion uptake and excretion. Whether, in the normal state, a balance between appearance of extracellular ATP and its hydrolysis modulates activity of the organic anion transporter remains to be determined.

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