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Inhibition by extracellular ATP of organic anion transport in the perfused rat liver

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Abstract

The action of extracellular ATP on organic anion transport in the bivascularly perfused rat liver was investigated, using bromosulfophthalein as a model substance. Transport was measured by means of the multiple-indicator dilution technique. The action of portal 100 μM ATP presented the following characteristics: (a) inhibition of bromosulfophthalein single pass extraction; the inhibition degree decreased with increasing bromosulfophthalein doses; (b) diminution of the influx rate coefficients; (c) 86.7% decrease of the maximal activity of the saturable component for bromosulfophthalein transport, but 100% increase of the non-saturable component; (d) diminution of the bromosulfophthalein flow-limited distribution space; (e) no significant alteration of the rate coefficients for metabolic sequestration. The action of ATP on organic anion transport in the intact liver occurred at much lower concentrations (10 ×) than those previously reported for isolated hepatocytes. This reinforces the suggestion that inhibition of organic anion transport could be a physiologically relevant effect of extracellular ATP.

Keywords: ATP; Organic anion transport; Perfused rat liver; Indicator-dilution experiment

1. Introduction

Several organic anions are transported across the hepatocyte membrane, a phenomenon which seems to be an important function of the liver (Wolkoff, 1996). The list of organic anions that are transported includes bile acids, bilirubin, indocyanine-green, bromosulfophthalein and cyclic nucleotides (Wolkoff, 1996; Vicentini et al., 2000). Responsible for organic anion transport are proteins of the oatp family (organic anion transport proteins) which differ from each other in substrate specificities and tissue distributions (Meier et al., 1997; Reichel et al., 1999; Glavy et al., 2000; Konig et al., 2000).

Bromosulfophthalein has been frequently used as a model substance for studying organic anion transport. Using bromosulfophthalein, Campbell et al. (1993) have found that extracellular ATP is able to inhibit organic anion transport in isolated hepatocytes. Initial transport rates are diminished 50% by 1 mM ATP, whereas other nucleotides, such as ADP, UTP, etc., are inactive. Glavy et al. (2000) have more recently shown that inhibition of organic anion transport by ATP in isolated hepatocytes is accompanied by serine phosphorylation of the organic anion transport protein 1 (oatp1). At a concentration of 5 mM, ATP inhibits bromosulfophthalein transport by 77% (Glavy et al., 2000). Protein phosphorylation was found with the same concentration.

Since extracellular ATP is an important agent due to its purinergic action (Buxton et al., 1986; Häussinger et al., 1987; Minguetti-Câmara et al., 1998), its effect on the transport of organic anions may be equally important. However, both kinds of effects of ATP seem to occur at quite different concentration ranges: the metabolic effects occur at concentrations up to 100 or 200 μM (Buxton et al., 1986; Häussinger et al., 1987; Minguetti-Câmara et al., 1998), whereas inhibition of organic anion transport in isolated hepatocytes has been reported to occur at much higher ones, namely, 1–5 mM (Campbell et al., 1993; Glavy et al., 2000). It is highly improbable that the extracellular ATP concentration may reach these high values. At least such extracellular concentrations have never been measured, a fact that raises the question about the physiological significance of the effects of ATP on transport in the

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hepatocyte membrane. There are indications, however, that in the intact liver, ATP may be acting on transport phenomena at low concentrations. This comes from a study in which the hemodynamic effects of ATP were investigated by means of the multiple-indicator dilution technique (Fernandes et al., 2002). Normally, the distribution of tritiated water in the liver is flow limited (i.e., very fast; Goresky, 1963), but under the influence of ATP, it becomes partly barrier limited (Fernandes et al., 2002). This means that ATP induces the formation of permeability barriers in the intact liver, an effect which occurs within the same concentration range (up to 100 μM) for which the purinergic actions have been reported (Buxton et al., 1986; Minguetti-Câmara et al., 1998). It is likely that these effects are elicited by interaction of non-parenchymal and parenchymal cells in the liver. Based on those observations, we decided to test the organic anion transport in the perfused liver for its sensitivity to ATP in the range up to 100 μM. Bromosulfophthalein was used as the model substance. The multiple-indicator dilution technique provides a suitable method for investigating transport in the intact liver. Preservation of microcirculation and cell-to-cell interactions is essential for the purposes of the present investigation.

2. Materials and methods

2.1. Materials

The liver perfusion apparatus and the rapid sampling apparatus for multiple-indicator dilution experiments were built in the University of Maringá. [U−^{14}C]Sucrose (612 mCi/mmol) and [^{3}H]water were purchased from Amersham International. ATP, bromosulfophthalein and fatty acid-free bovine serum albumin were purchased from Sigma.

2.2. Liver perfusion

Male albino rats (Wistar), weighing 180–220 g, were fed ad libitum with a standard laboratory diet. For the surgical procedure, the rats were anesthetized with sodium pentobarbital (50 mg/kg). Hemoglobin-free, non-recirculating bivascular liver perfusion was done at constant flow, as described elsewhere (Suzuki-Kemmelmeier et al., 1992). The perfusion fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4, 37 °C), saturated with a mixture of oxygen and carbon dioxide (95:5). Portal and arterial flows were 28–32 and 2–3 ml/min, respectively. All experiments were done in accordance with the recommendations of the Ethics Committee of the University of Maringá.

2.3. Multiple-indicator dilution experiments

A mixture (25 μl) containing [^{14}C]sucrose (5 μCi), [^{3}H]water (20 μCi) and variable amounts of bromosulfophthalein (~ 0.35 to ~ 3.2 μmol) was injected into the portal vein. The effluent perfusate was collected in 0.5–2.0-s fractions over a period of 90 s. Radioactivity was measured by liquid scintillation. The scintillation solution contained toluol/ethanol (10:2) and 5.0 g/l of 1,5-diphenyloxazole plus 0.2 g/l of 2,2-*p*-phenyl-bis-5-phenyleneoxazole. Bromosulfophthalein was measured spectrophotometrically at 578 nm after alkalinization (pH 11) of the appropriately diluted perfusate samples (Goresky, 1964). Standard curves were prepared for the different albumin concentrations. The indicator dilution curves were normalized as (amount in the effluent sample) × (number of seconds)$^{-1}$ × (total amount injected)$^{-1}$.

2.4. Data analysis and calculations

The outflow profiles of bromosulfophthalein were analyzed according to the model of Goresky (1963, 1964; Goresky et al., 1970). This model produces the following equation for a substance ($Q(t)$) that is injected as a bolus and that during its passage through the liver microcirculation has reversible access to the cellular space into which it can be irreversibly transformed (Goresky, 1964; Bracht et al., 1980):

$$Q(t) = Q_{\mathrm{ref}}(t)\mathrm{e}^{-k_{\mathrm{in}}(t-t_0)} + \int_0^{t-t_0} \mathrm{e}^{-k_{\mathrm{in}}\tau}\mathrm{e}^{-(k_{\mathrm{ef}}+k_{\mathrm{m}})(t-t_0-\tau)}Q_{\mathrm{ref}}(\tau+t_0) \times \sum_{n=1}^{\infty}\frac{(k_{\mathrm{in}}k_{\mathrm{ef}}\tau)^n(t-t_0-\tau)^{n-1}}{n!(n-1)!}\mathrm{d}\tau \quad (1)$$

In Eq. (1), t represents time, τ the variable sinusoidal transit times and t_0 the uniform transit time of the large non-exchanging vessels. The parameters k_{in} and k_{ef} represent, respectively, the transfer coefficients for influx into the cell space and for efflux from the cellular space; k_{m} represents the irreversible sequestration coefficient. The dimensions of the rate coefficients are inverse time (e.g., s^{-1}) and they are referred to the corresponding distribution spaces. The coefficient for influx (k_{in}) is referred to the whole extracellular space into which bromosulfophthalein undergoes flow-limited distribution, whereas k_{ef} and k_{m} are referred to the intracellular distribution space. $Q_{\mathrm{ref}}(t)$ is a reference curve for bromosulfophthalein, i.e., the outflow profile of a hypothetical substance that occupies the same, real or apparent, extracellular space as bromosulfophthalein. This extracellular space includes the adsorption phase to the cellular surface found by Schwenk et al. (1976). The reference, $Q_{\mathrm{ref}}(t)$, can be generated from the outflow profile of labeled sucrose under the assumption that it differs from the latter because it distributes in a flow-limited manner over a space which is in excess to that of labeled sucrose ($Q_{\mathrm{suc}}(t)$). If β is the ratio of excess extracellular space occupied by bromosulfophthalein to the sucrose space, the following linear transformation can be used (Goresky, 1963; Vicentini et al., 2000):

$$Q_{\mathrm{ref}}(t) = \frac{1}{1+\beta}Q_{\mathrm{suc}}\left(\frac{t-t_0}{1+\beta}+t_0\right) \quad (2)$$

Eq. (2) means that $Q_{ref}(t)$ equals the sucrose curve at time $[(t-t_0)/(1+\beta)+t_0]$, divided by the factor $(1+\beta)$. The latter corresponds to the bromosulfophthalein space into which this substance undergoes flow-limited distribution divided by the sucrose space.

Eqs. (1) and (2) were fitted simultaneously to the experimental outflow profiles of bromosulfophthalein. A nonlinear iterative least-squares procedure was used (Björck and Dahlquist, 1972). Preliminary estimates of β, k_{in}, k_{ef} and k_m were introduced as well as values for t_0, the transit time in the large non-exchanging vessels. Values for this parameter were obtained from the optimized linear superimposition of the [^{3}H]water and [^{14}C]sucrose curves, according to Goresky (1963):

$$Q_{water}(t) = \left[\frac{1}{1+\theta}\right] Q_{suc}\left(\frac{t-t_0}{1+\theta}+t_0\right) \quad (3)$$

$Q_{water}(t)$ represents the impulse response of the liver to [^{3}H]water, $Q_{suc}([t-t_0]/[1+\theta]+t_0)$ corresponds to the [^{14}C]sucrose curve at time $([t-t_0]/[1+\theta]+t_0)$ and θ the ratio of cellular to extracellular water spaces. Optimization of the superimposition was accomplished by means of a nonlinear iterative least-squares procedure. Alternatively, for those experiments that were done in the presence of ATP, t_0 was evaluated as the appearance time, as described previously (Fernandes et al., 2002). This was necessary because in the presence of ATP, the distribution of labeled water is only partially flow limited, a fact that invalidates Eq. (3).

Integrals and derivatives of the experimental [^{14}C]sucrose and [^{3}H]water curves were calculated analytically after approximating the curves by cubic splines, with monoexponential extrapolation to infinite time from the last experimental point (90 s). Before proceeding with the least-squares fits, the experimental sucrose, water and bromosulfophthalein curves were corrected for catheter delay and distortion (Goresky and Silverman, 1963). Interpolation between experimental points was accomplished by means of a spline-function (Björck and Dahlquist, 1972).

3. Results

3.1. Experimental protocols and outflow profiles of bromosulfophthalein plus indicators

The hemodynamic effects of portally infused ATP are exerted not only in the portal but also in the arterial bed, a phenomenon known as the "transhepatic action of ATP" (Browse et al., 1994; Fernandes et al., 2002). For preserving all possible cell-to-cell interactions, the multiple-indicator dilution experiments were done in the bivascularly perfused rat liver, i.e., both entry vessels, portal vein and hepatic artery were cannulated and perfused. Bromosulfophthalein plus indicators ([^{3}H]water and [^{14}C]sucrose) were simultaneously injected into the portal vein and the effluent perfusate was fractionated by means of a rapid sampling apparatus. In the hemoglobin-free perfused rat liver, the distribution space of [^{14}C]sucrose does not differ significantly from the distribution spaces of high-molecular-weight substances such as inulin, dextran and albumin (Bracht et al., 1980; Kelmer-Bracht et al., 1984). For this reason, [^{14}C]sucrose was used as the extracellular indicator even in the presence of high albumin concentrations. Bromosulfophthalein was measured spectrophotometrically as described in Materials and methods. In the cellular space, bromosulfophthalein can be conjugated to glutathione (Snel et al., 1993). Either non-transformed bromosulfophthalein or its conjugated form are rapidly excreted into bile. In the isolated perfused liver, the conjugated form was found in bile but not in the effluent perfusate (Snel et al., 1993). Consequently, bromosulfophthalein can be measured spectrophotometrically almost without interference.

Fig. 1 shows typical results of two indicator dilution experiments that were done with a low albumin concentra-

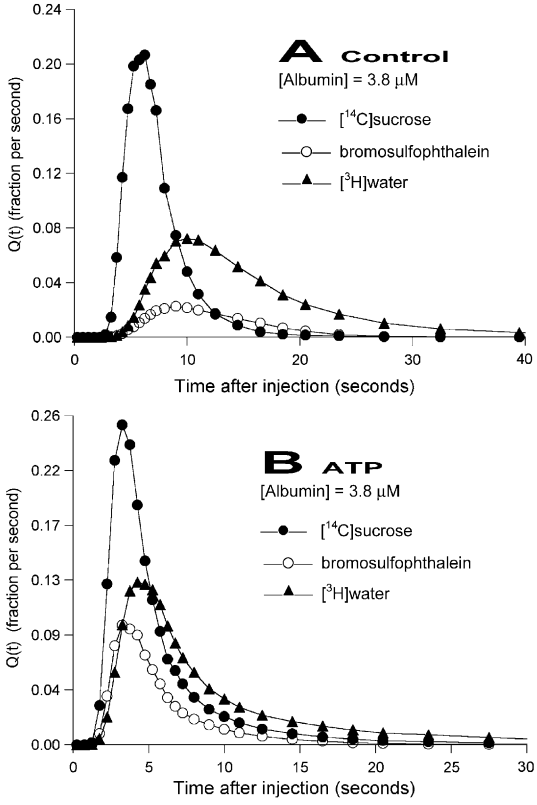

Fig. 1. Typical outflow profiles of bromosulfophthalein and indicators before and during ATP infusion at a low albumin concentration. Livers were perfused bivascularly with perfusion fluid containing 3.8 μM bovine serum albumin as described in Materials and methods. A mixture of bromosulfophthalein (0.8 μmol), [^{14}C]sucrose and [^{3}H]water was injected into the portal vein (first injection) and the effluent perfusate was sampled by means of a rapid sampling device. Approximately 10 min later, portal ATP infusion (100 μM) was initiated. At 1.5 min after the onset of the infusion, a second injection was done and the perfusate was again fractionated by means of a rapid sampling device. Bromosulfophthalein, [^{14}C]sucrose and [^{3}H]water were measured and the recovered fractions per second were represented against the time after injection.

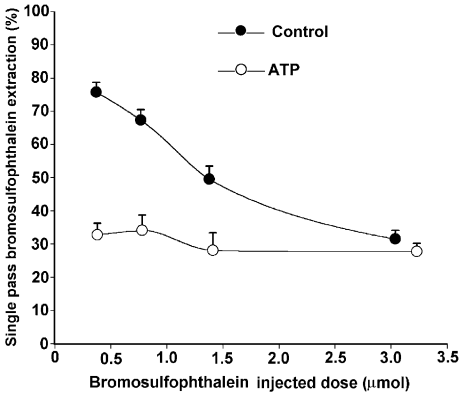

Fig. 2. Single pass extraction of bromosulfophthalein as a function of the injected dose before and after ATP infusion. Experiments were done and analyzed as described in Materials and methods and in the legends to Fig. 1. The bovine serum albumin concentration in the perfusate was 3.8 μM. Each data point corresponds to a minimum of five and a maximum of eight multiple-indicator dilution experiments. Bars are standard errors of the mean.

tion. A control injection was done first (panel A). After 10 min, ATP infusion was initiated, and after 1.5 min, a second injection was performed. The concentration of ATP was 100 μM, one order of magnitude below the concentrations used in studies with isolated hepatocytes (Campbell et al., 1993), but still producing well-defined purinergic effects in the perfused liver (Häussinger et al., 1987; Minguetti-Câmara et al., 1998). The time of 1.5 min was chosen because this is the time at which metabolic and hemodynamic effects are usually maximal (Häussinger et al., 1987; Minguetti-Câmara et al., 1998). In Fig. 1, the fractions of the injected amounts appearing in the effluent perfusate per second were represented against the time after injection (in seconds). When normalized in this way, the areas under the curves of non-transformed tracers (as [^{14}C]sucrose and [^{3}H]water) are equal to unity. For the bromosulfophthalein dose that was injected in the experiments shown in Fig. 1 (~ 0.8 μmol), the effects of ATP on the outflow profiles are quite pronounced. In the absence of ATP, the bromosulfophthalein curve was considerably delayed and compressed. The peak time was near to that of the [^{3}H]water curve. In the presence of ATP, however, the peak time of the bromosulfophthalein curve was practically the same as that of the [^{14}C]sucrose curve. The peak times of the indicator curves, especially [^{3}H]water, were also changed by ATP, a phenomenon that was described in previous work (Fernandes et al., 2002). The changes caused by ATP on the bromosulfophthalein outflow profiles, however, were much more pronounced. In the examples shown in Fig. 1A and B, whereas the peak value of the [^{3}H]water curve was increased by a factor of 1.5, the bromosulfophthalein peak value was raised by a factor of 3.6. When the injected dose was increased (up to ~ 3 μmol), the control bromosulfophthalein outflow profiles were also changed. Basically, the peak times were shifted to the left in the direction of the peak time of the sucrose curve and the peak values were increased (not shown). The latter phenomenon was also apparent when ATP was present and was partly caused by an increase in recovery.

The area under the bromosulfophthalein curve represents the recovered fraction, from which the percent single pass extraction can be calculated. This variable is represented graphically in Fig. 2 as a function of the injected bromosulfophthalein dose. Fig. 2 shows the mean values obtained in experiments of the same kind as those shown in Fig. 1. As expected for a saturable phenomenon, the extraction of bromosulfophthalein decreased as the dose was increased. In the presence of 100 μM ATP, the single pass extraction was considerably lower at low bromosulfophthalein doses (43.3% of the control at the lowest dose). The difference decreased as the dose was increased, however, because saturation was less pronounced in the presence of ATP. At the highest dose, almost no difference in single pass extraction was found.

The control outflow profiles of bromosulfophthalein obtained in the present work in the experimental series

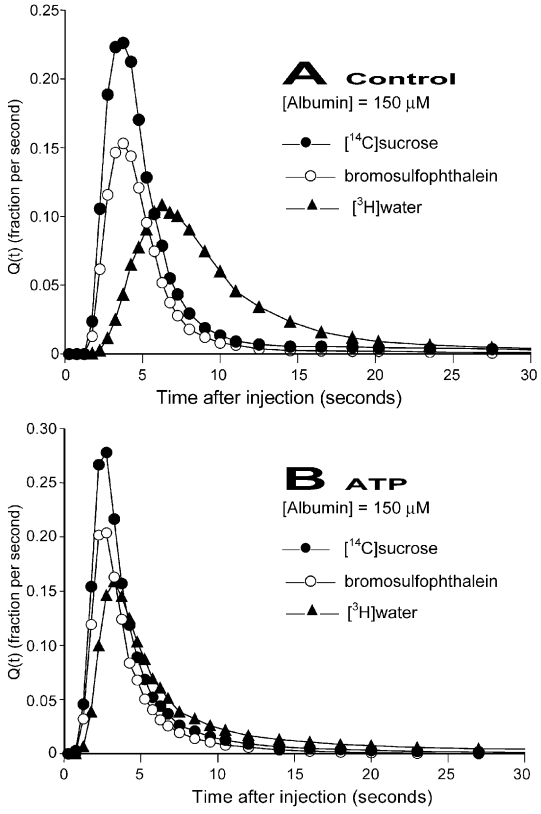

Fig. 3. Typical outflow profiles of bromosulfophthalein and indicators before and during ATP infusion at a high albumin concentration. Livers were perfused bivascularly with perfusion fluid containing 150 μM bovine serum albumin as described in Materials and methods. A mixture of bromosulfophthalein (0.37 μmol), [^{14}C]sucrose and [^{3}H]water was injected into the portal vein (first injection) and the effluent perfusate was sampled by means of a rapid sampling device. Approximately 10 min later, portal ATP infusion (100 μM) was initiated. At 1.5 min after the onset of the infusion, a second injection was done and the perfusate was again fractionated by means of a rapid sampling device. Bromosulfophthalein, [^{14}C]sucrose and [^{3}H]water were measured and the recovered fractions per second were represented against the time after injection.

represented by the graph in Fig. 1A differ from those obtained by Goresky (1963) with the dog liver under in vivo conditions. In the experiments of Goresky (1964), the outflow profiles of bromosulfophthalein were always contained within the envelope of the experimental extracellular reference curve (labeled albumin). The different serum albumin concentrations could be the cause for this difference. For this reason, we have also done several experiments in which the albumin concentration in the perfusate was increased to 150 μM. Typical outflow profiles are shown in Fig. 3. Comparison of Figs. 1A and 3A reveals that the outflow profile of bromosulfophthalein was markedly changed when the albumin concentration was increased from 3.8 to 150 μM (1 g%). At the latter albumin concentration, the whole bromosulfophthalein curve was contained within the envelope of the [^{14}C]sucrose outflow profile, which in the hemoglobin-free perfused rat liver has been shown to be practically equal to the [^{131}I]albumin outflow profile (Kelmer-Bracht et al., 1984). At the same time, the single pass extractions of similar bromosulfophthalein doses were substantially reduced when the albumin concentration was increased, as revealed by Table 1, in which the mean values obtained in several experiments are listed. At high albumin concentrations, ATP also changed the outflow profiles of indicators and bromosulfophthalein. The changes in the [^{14}C]sucrose and [^{3}H]water outflow profiles were similar to those found with low albumin concentrations. In relative terms, the bromosulfophthalein outflow profiles were less modified by ATP, but the single pass extraction was additionally decreased by ATP, as revealed by Table 1.

3.2. Model analysis of the bromosulfophthalein outflow profiles

Quantitative information about distribution spaces and influx and sequestration rates of bromosulfophthalein can be obtained by fitting Eqs. (1) and (2) to the experimental data. Eq. (2) allows to find the adequate reference curve for bromosulfophthalein, i.e., the curve of a hypothetical substance which undergoes flow-limited distribution into the same, real or apparent, space as bromosulfophthalein without suffering transformation. It should be recalled that Eq. (2) contains the parameter β, the ratio of the extra space into which bromosulfophthalein undergoes flow-limited distribution, divided by the sucrose space. Eq. (1) contains the influx (k_{in}), efflux (k_{ef}) and intracellular sequestration rate (k_m) coefficients. Both equations were fitted simultaneously to all sets of bromosulfophthalein plus indicators outflow profiles obtained in the present work. Two representative examples of experiments that were done with a low albumin concentration in the perfusate are shown in Fig. 4: a control curve (Fig. 4A) and the corresponding curve obtained during ATP infusion in the same liver perfusion experiment (Fig. 4B). In the graphs of Fig. 4A and B, the experimental bromosulfophthalein data points are shown in addition to the calculated curve (according to Eq. (1)) and the calculated reference curve (according to Eq. (2)). The optimized parameters are given in the legend. The graphs in Fig. 4 reveal that agreement between theory and experiment is good in spite of the pronounced changes caused by ATP in the outflow profiles. These changes evidently reflect in the values of the parameters (see legend of Fig. 4).

For the experiments that were done at low albumin concentrations, the fitting procedure allowed to obtain all rate coefficients and the parameter β. For the experiments that were done at high albumin concentrations, however, convergence during the successive iterations of the fitting procedure was poor, yielding either negative or infinite values for k_{ef} and k_m. However, fitting of Eq. (1) without the second term was always successful. Furthermore, when Eq. (1) without the second term was fitted to the data obtained at low albumin concentrations, the deviations between calculated and experimental curves were generally quite pronounced. Clearly, the bromosulfophthalein curves obtained

Table 1
Influence of albumin and ATP on bromosulfophthalein transport and transformation in the perfused liver

Bromosulfophthalein-injected dose (μmol)	ATP concentration (μM)	Albumin concentration (μM)	Bromosulfophthalein single pass extraction (%)	β	$k_{in}(1+\beta)$ (ml s^{-1} (ml extracellular space)$^{-1}$)	k_{ef} (ml s^{-1} (ml cell space)$^{-1}$)	k_m (ml s^{-1} (ml cell space)$^{-1}$)
0.37 ± 0.03 (n=8)	0	3.8	75.7 ± 2.9*	1.96 ± 0.15*	0.807 ± 0.130**	0.302 ± 0.128	0.245 ± 0.054
0.38 ± 0.02 (n=8)	100	3.8	32.8 ± 3.5*	0.17 ± 0.03*	0.366 ± 0.059**	0.326 ± 0.034	0.179 ± 0.034
0.37 ± 0.04 (n=8)	0	150	26.6 ± 2.9***	0.027 ± 0.015	0.0775 ± 0.004***	–	–
0.36 ± 0.04 (n=8)	100	150	15.4 ± 3.9***	0.026 ± 0.009	0.0475 ± 0.0124***	–	–
3.12 ± 0.09 (n=4)	0	3.8	36.5 ± 4.3	1.05 ± 0.26****	0.319 ± 0.046	0.191 ± 0.065	0.073 ± 0.003
3.29 ± 0.11 (n=4)	100	3.8	32.0 ± 2.2	0.10 ± 0.01****	0.249 ± 0.018	0.099 ± 0.005	0.087 ± 0.017
3.29 ± 0.04 (n=5)	0	150	4.19 ± 0.92	0.22 ± 0.01*	0.014 ± 0.04	–	–
3.36 ± 0.04 (n=5)	100	150	1.94 ± 1.94	0.03 ± 0.03*	0.006 ± 0.006	–	–

The data were obtained form experiments similar to those shown in Figs. 1 and 2 under the conditions given in the first three columns. Analysis was done by means of Eqs. (1) and (2) as described in the text.

* Statistical analysis: $P<0.001$.

** Statistical analysis: $P<0.01$.

*** Statistical analysis: $P<0.05$.

**** Statistical analysis: $P<0.02$.

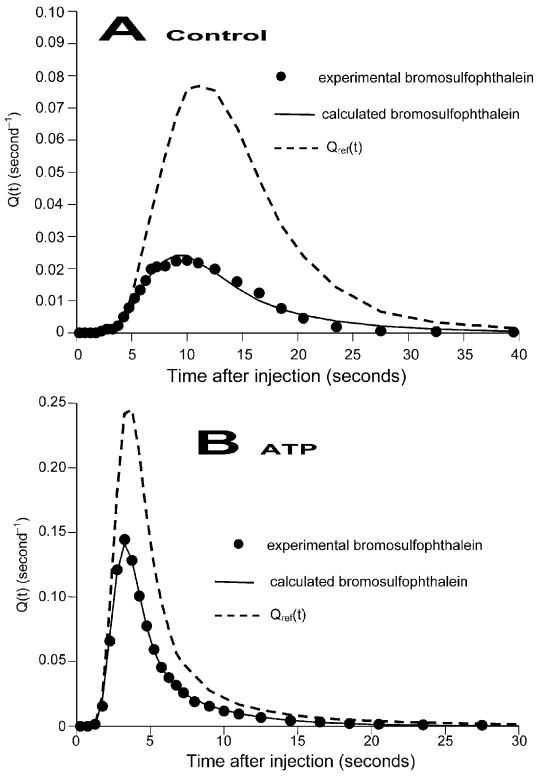

Fig. 4. Experimental and computed bromosulfophthalein curves before and during ATP infusion. Data from the experiments illustrated by Fig. 1 were used. Eqs. (1) and (2) were fitted to the experimental data by means of a least-squares procedure, as described in Materials and methods. The optimized parameters were used for calculating the theoretical bromosulfophthalein outflow profiles using Eq. (1) and the reference curves using Eq. (2). The optimized parameters for the bromosulfophthalein outflow profiles before and during ATP infusion were, respectively: k_{in}, 0.167 and 0.287 ml s^{-1} (ml extracellular bromosulfophthalein distribution space)$^{-1}$; k_{ef}, 0.0558 and 0.153 ml s^{-1} (ml cellular bromosulfophthalein space)$^{-1}$; k_m, 0.126 and 0.198 ml s^{-1} (ml cellular bromosulfophthalein space)$^{-1}$; β, 1.639 and 0.219.

at high albumin concentrations did not contain enough information as to allow the determination of k_{ef} and k_m. These parameters appear only in the second term of Eq. (1), which describes the material that has entered the cell at least once during a single passage through the liver (exchanged fraction; Goresky et al., 1970). Thus, at high albumin concentrations, the exchanged component was negligible, contrary to what happened at low albumin concentrations.

Fig. 4 reveals a great difference between the reference curve for bromosulfophthalein ($Q_{ref}(t)$) in the absence and presence of ATP. The infusion of ATP reduced the peak times and increased the peak values. These changes reflect the values of β which can be appreciated in Fig. 5 and in Table 1. In Fig. 5, the mean β values that were obtained at a low albumin concentration were represented against the bromosulfophthalein-injected dose. In the absence of ATP, the real or apparent space into which bromosulfophthalein undergoes flow-limited distribution exceeded the sucrose space by factors approaching 2 at the lowest doses. The difference decreased when the injected dose was increased. In the presence of ATP, however, the β values were very small (see also Table 1) so that the difference between the sucrose ($Q_{suc}(t)$) and the computed reference ($Q_{ref}(t)$) curves became minimal. The reduction of the space into which bromosulfophthalein undergoes flow-limited distribution is thus the main reason for the pronounced changes that were observed in the outflow profiles of bromosulfophthalein when ATP was infused (Fig. 1).

Raising the albumin concentration in the perfusate from 3.8 to 150 μM changed the form of the bromosulfophthalein outflow profiles in a way that seems to be similar to the changes caused by ATP (compare Figs. 1A and 3A). Quantitatively, this phenomenon can be evaluated by comparing the β values in Table 1. At the high albumin concentration, the β values were considerably lower. When the injected bromosulfophthalein dose was increased from 0.37 to 3.29 μmol, however, the value of β increased by a factor of 8.1 ($P < 0.001$). Apparently, the action of ATP on β was not abolished by raising the albumin concentration. When the agonist was introduced in the perfusate containing 150 μM albumin, injection of a high bromosulfophthalein dose revealed a clear reduction in β.

3.3. Transfer and sequestration coefficients and the influence of ATP

It is clear from Fig. 2 that ATP decreases net uptake of bromosulfophthalein. This could be due to inhibition of influx, inhibition of intracellular sequestration (conjugation + excretion into bile) or both. Examination of the coefficients for transfer and sequestration should provide an answer to this question. The transfer coefficient for influx k_{in} obtained from Eq. (1) is referred to the real or apparent extracellular space into which bromosulfophthalein under-

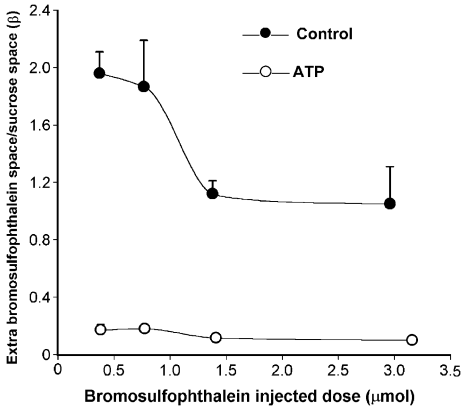

Fig. 5. The parameter β as a function of the bromosulfophthalein-injected dose before and after ATP infusion. Values for β were optimized by fitting Eqs. (1) and (2) to the bromosulfophthalein experimental outflow profiles as described in Materials and methods and in the legend to Fig. 4. The bovine serum albumin concentration in the perfusate was 3.8 μM. Each data point corresponds to a minimum of five and a maximum of eight multiple-indicator dilution experiments. Bars are standard errors of the mean. They were omitted when smaller than the symbol sizes.

goes flow-limited distribution, i.e., ml time^{-1} (ml bromosulfophthalein extracellular space)$^{-1}$. Since ATP markedly decreased this space, comparison of the transfer coefficients for influx rates needs a common denominator. This common denominator could be the extracellular sucrose space which, as demonstrated previously, is not significantly reduced by ATP (Fernandes et al., 2002). One way of accomplishing this is to multiply all k_{in} values by the factor $(1+\beta)$ which, as shown by Eq. (3), equals the ratio (flow-limited bromosulfophthalein space)/(sucrose space). The product $k_{in}(1+\beta)$ has the dimensions ml time^{-1} (ml sucrose space)$^{-1}$. In Fig. 6, the various values of $k_{in}(1+\beta)$ were represented against the bromosulfophthalein-injected dose for the experiments done at low albumin concentrations. Values for the experiments done at low and high albumin concentrations can be seen in Table 1. Fig. 6 reveals that ATP had a marked negative influence on $k_{in}(1+\beta)$, but the difference decreased as the dose was increased. This phenomenon reflects the fact that saturation was more pronounced in the control condition. In this sense, the dose dependencies of single pass extraction (Fig. 2) and $k_{in}(1+\beta)$ (Fig. 6) were similar. The k_m values in the absence and presence of ATP did not differ statistically from each other, although a tendency toward smaller values in the latter condition were apparent at low bromosulfophthalein doses. The mean standard errors of k_m tended to be higher (in relative terms) than those of $k_{in}(1+\beta)$. The same is true for k_{ef}, for which no systematic and significant influence of ATP could be demonstrated (Table 1). As already mentioned, at high albumin concentrations, only k_{in} could be determined. As revealed by Table 1, ATP also decreased $k_{in}(1+\beta)$ in the presence of 150 μM albumin at low bromosulfophthalein doses.

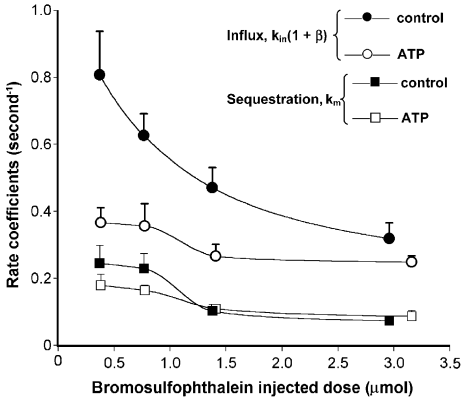

Fig. 6. Rate coefficients for unidirectional influx $[k_{in}(1+\beta)]$ and irreversible sequestration (k_m) as a function of the bromosulfophthalein-injected dose before and after ATP infusion. Values for k_{in}, k_m and β were obtained by fitting Eqs. (1) and (2) to the bromosulfophthalein experimental outflow profiles as described in Materials and methods and in the legend to Fig. 4. Multiplication of k_{in} by $(1+\beta)$ refers all values to the extracellular sucrose space. The bovine serum albumin concentration in the perfusate was 3.8 μM. Each data point corresponds to a minimum of five and a maximum of eight multiple-indicator dilution experiments. Bars are standard errors of the mean. They were omitted when smaller than the symbol sizes.

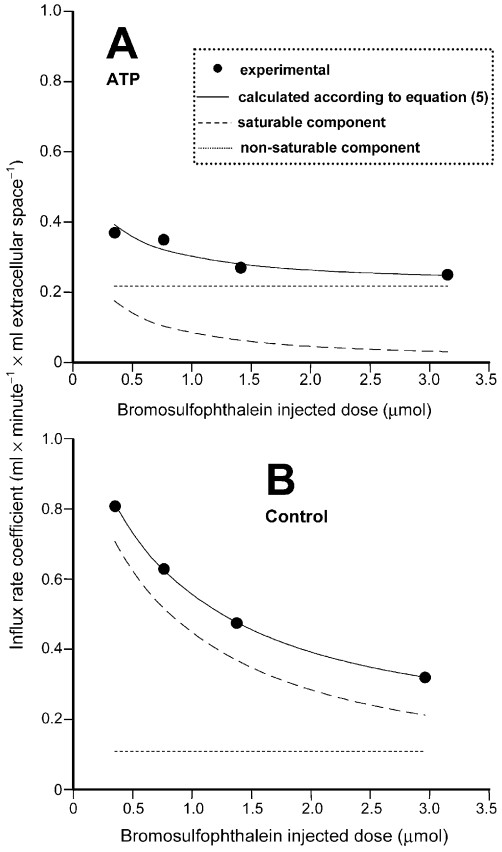

Fig. 7. Analysis of the influx rate coefficients $k_{in}(1+\beta)$ according to Eq. (4). Eq. (4) was fitted by means of a nonlinear least-squares procedure to the experimental $k_{in}(1+\beta)$ versus D relationships shown in Fig. 6. The saturable component corresponds to the term $R/(D+K_D)$ in Eq. (4), whereas the non-saturable component corresponds to α. Optimized values of R, K_D and α are given and discussed in the text.

3.4. Analysis of bromosulfophthalein influx saturation

Comparison of the forms of the control $k_{in}(1+\beta)$ versus injected dose curves suggest that inhibition of bromosulfophthalein transport is characterized by apparent or real changes in the affinity of the transport system for bromosulfophthalein. A simple quantitative way of testing this hypothesis is to analyze the $k_{in}(1+\beta)$ versus injected dose (D) curves as it has been done by Goresky (1964) in terms of the Michaelis–Menten equation, normalized by the injected dose. In agreement with more recent studies, however, it seems adequate to introduce a non-saturable component (α; Satlin et al., 1997), whereby the following equation can be written:

$$k_{in}(1+\beta) = \frac{R}{K_D + D} + \alpha \quad (4)$$

K_D corresponds to the dose for 50% saturation and D the injected dose. The parameter R is proportional to the maximal influx rate of the saturable component, but it is not a true maximal velocity because its dimensions are

ml × min^{-1} × [μmol/ml extracellular space]. Nonlinear fits of Eq. (4) to the experimental $k_{in}(1+\beta)$ data revealed saturable and non-saturable components for both conditions, absence and presence of ATP. Fig. 7 allows to compare experimental data and theoretical curves. It also shows a resolution of the saturable and non-saturable components. No good fits were obtained when the non-saturable component (α) was omitted from Eq. (4) (not shown). In the presence of ATP (Fig. 7A), the contribution of the non-saturable component was more prominent. ATP treatment had a pronounced effect on R (0.791 ± 0.032, control versus 0.105 ± 0.062 ml × min^{-1} × [μmol/ml extracellular space], ATP infusion, $P<0.001$). In contrast, K_D, the dose for 50% saturation, was not significantly reduced in statistical terms due to the high standard errors (0.770 ± 0.043, control versus 0.250 ± 0.388 μmol, ATP infusion, $P<0.23$). The constant for non-saturable influx α, was increased by ATP infusion (0.108 ± 0.006, control versus 0.218 ± 0.017 ml × min^{-1} × (ml extracellular space)$^{-1}$, ATP infusion, $P<0.001$). Only the saturable component was thus inhibited by ATP. The degree of this inhibition varied between 75% for the lowest bromosulfophthalein dose and 85% for the highest bromosulfophthalein dose.

4. Discussion

The results of this work demonstrate that ATP inhibits bromosulfophthalein transport, and probably organic anion transport, at a concentration that is within the same concentration range for which the purinergic action of the agonist has been observed (Buxton et al., 1986; Häussinger et al., 1987; Minguetti-Câmara et al., 1998). Inhibition of net uptake of bromosulfophthalein seems to be mainly the consequence of influx inhibition because only small and poorly significant changes in the sequestration rate coefficients were detected. Previous studies with isolated hepatocytes, also using bromosulfophthalein as the model substance, have found inhibition at much higher concentrations, more precisely in the range between 1 and 5 mM (Campbell et al., 1993; Glavy et al., 2000). Our results thus reinforce the proposition that inhibition of organic anion transport could be representing one of the physiologic effects of ATP, even though the precise physiological significance of this inhibition remains obscure. The present study also reinforces the importance of measuring transport in the intact liver, because the difference in sensitivity to ATP between isolated hepatocytes and the perfused liver exceeds one order of magnitude. Isolation of hepatocytes eliminates at least interactions between hepatocytes and other liver cells, but changes in the cell surfaces are equally a likely phenomenon.

Glavy et al. (2000) have proposed that inhibition of bromosulfophthalein transport by ATP in isolated hepatocytes is caused by phosphorylation of the serine residue of the oatp1 protein, which is responsible for this transport route. A question that can be formulated is if the inhibition phenomenon detected in the present work represents the same phenomenon occurring at much higher ATP concentrations in isolated hepatocytes. Concerning this question, two alternatives must be considered: (a) both inhibition of organic anion transport found in the present work and that one detected by Glavy et al. (2000) are basically the same event, but the absence of some factors in isolated cells reduces sensitivity, thus requiring much higher ATP concentrations; (b) inhibition found in the present work and that one found by Glavy et al. (2000) are different events with different mechanisms. A definitive answer to this question depends on additional evidence. However, the kinetics of inhibition seems to favour the second hypothesis. Glavy et al. (2000) reported that inhibition of the saturable component was mainly due to a decrease in the maximal transport rate (V_{max}; −75.6%) plus a similar proportional decrease in the rate constant for non-saturable incorporation of bromosulfophthalein (−71.4%). No significant change of the concentration for half-maximal transport rates (K_M) was found. This means that saturation of the overall bromosulfophthalein incorporation was practically not affected by ATP in isolated hepatocytes. The opposite occurred in the perfused liver. Both the fractional net uptake and the influx rate coefficient dependencies on the injected bromosulfophthalein doses were changed by ATP in a way that clearly suggests a reduction in the overall degree of saturation. Quantitative analysis confirmed this deduction: besides an 86.7% reduction in the maximal rate of saturable influx, the non-saturable influx rate coefficient was more than doubled. This explains and justifies quite well the fact that the rate coefficient for influx and the fractional single pass extraction of bromosulfophthalein in the presence of ATP in the perfused liver were almost independent of the injected dose. Apparently, the non-saturable or low-affinity component predominates in the presence of ATP. A non-saturable or low-affinity component was also observed by Schwenk et al. (1976) is their study with isolated hepatocytes. These authors have interpreted the failure for achieving saturation at high bromosulfophthalein concentrations as a consequence of the existence of two translocating sites, one with high and the other one with low affinity for bromosulfophthalein.

ATP did not only inhibit bromosulfophthalein influx but it also affected the flow-limited distribution of the compound. The existence of this space can be predicted from the early experiments of Schwenk et al. (1976). In isolated hepatocytes, these authors have detected a saturable rapid adsorption of bromosulfophthalein to the surface of hepatocytes. Saturation was also apparent in our experiments: the β values diminished as the bromosulfophthalein-injected dose was increased at low albumin concentrations. The action of ATP on the flow-limited bromosulfophthalein distribution was quite pronounced. Possibly, it reflects the same phenomenon which also affects the flow-limited distribution of labeled water, as described and analyzed in

detail by previous work (Fernandes et al., 2002). The flow-limited space probably comprises the glycocalix and the external regions of the plasma membrane. Its reduction by ATP could be caused by a contraction of the stellate cells, for example, or by the mechanical constraints caused by the very pronounced vasodilation that occurs in the arterial bed (Fernandes et al., 2002). It seems also that this effect is not exerted by ATP itself, but by an effector that is released in presinusoidal regions, because it was absent when ATP was infused into the hepatic vein in retrograde perfusion (Fernandes, 2002). Apparently, however, virtual elimination of the flow-limited distribution did not affect bromosulfophthalein influx in a significant way, at least not at high bromosulfophthalein doses. In fact, at the highest bromosulfophthalein dose, around 3 μmol, influx and intracellular sequestration of the substance were minimally affected by ATP, whereas the flow-limited distribution was reduced to a minimum. Strong binding to albumin is expected to diminish adsorption of bromosulfophthalein to the cell surface, a prediction that was actually confirmed by our experiments. Binding to albumin also diminishes bromosulfophthalein influx, as originally found by Schwenk et al. (1976) in isolated hepatocytes. The behaviour of bromosulfophthalein in our experiments with high albumin concentrations in the perfusate was similar to that found by Goresky (1964) in his pioneer multiple-indicator dilution experiments. These were done in anesthetized dogs, with the normal high in vivo albumin concentration (450–600 μM). Strong binding of bromosulfophthalein to albumin generated outflow profiles that were entirely contained within the envelope of the labeled albumin curve and exchange with the cellular space was minimal so that only the coefficient for influx could be determined (Goresky, 1964). It is worth to mention that we have found the same phenomena when the albumin concentration was raised from 3.8 to 150 μM. Possible alterations of bromosulfophthalein binding to albumin are a further possibility which deserves some comments. If ATP diminishes bromosulfophthalein binding to albumin, it should increase β. However, no increases in β caused by ATP in the presence of high albumin concentrations were found. An increase of bromosulfophthalein binding to albumin, on the other hand, would reflect in a decrease in the β values. However, ATP itself exerts this effect and it would be very difficult to distinguish such an additional factor. It should be recalled that at low albumin concentrations, binding of bromosulfophthalein to the protein is negligible. Consequently, the action of ATP on the β values at low albumin concentrations cannot be attributed to changes in the binding degree.

The behavior of bromosulfophthalein in the liver is similar to the that of octanoate (Ferraresi-Filho et al., 1997). Octanoate also has an apparently great flow-limited distribution space in addition to another one into which distribution is barrier limited. Moreover, the size of the flow-limited space is reduced when the albumin concentration is raised. Octanoate and bromosulfophthalein have in common lipophilic structures associated with negatively charged groups. The behaviour of bromosulfophthalein and octanoate, however, differs from another group of substances which also possess lipophilic structures associated with negatively charged groups, as for example, niflumic acid (Kelmer-Bracht et al., 1993) and salicylamide (Pang et al., 1994). The distribution of these substances is entirely flow limited into an apparent space which can exceed the water space by factors of 20 or more depending on the concentration. Solely the combination of lipophilic nature with electrical negative charge is not enough for predicting the exact behavior of a given substance in the liver. The latter probably results from a complex combination of lipophilicity degree, specific interactions with carrier or ligand proteins and other factors.

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