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EFFECTS OF BILIRUBIN ON POTASSIUM ($^{86}Rb^+$) INFLUX AND IONIC CONTENT IN EHRLICH ASCITES CELLS

JUAN LELIO CORCHS, RAQUEL E. SERRANI and MAURICIO PALCHICK

Depto. de Ciencias Fisiológicas, Fisiología, Fac. Cs. Médicas, Universidad Nacional de Rosario y Centro de Medicina Nuclear, Hosp. Provincial de Rosario, Santa Fe 3100 y Alem 1450, Rosario 2000 (Argentina)

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Summary

Potassium influx, intracellular potassium and sodium content and cellular volume were determined in vitro in Ehrlich ascites cells in the presence of up to 0.8 mM bilirubin in the incubation medium. Bilirubin uptake into cells as a function of bilirubin concentration in the incubation medium increased linearly with a molar bilirubin/albumin ratio of 20 : 1. Potassium influx and intracellular content decreased while cellular volume increased after 180 min of incubation of cells in bilirubin at a molar bilirubin/albumin ratio of 20 : 1. At a bilirubin/albumin ratio 2 : 1, potassium influx decreased, cellular volume remained unchanged, and bilirubin uptake into cells became saturated at bilirubin concentrations greater than 0.3 mM. It is suggested that bilirubin-induced alterations in potassium gradients across cell membranes may play a role in toxic effects of bilirubin on cells.

Introduction

Bilirubin can cause morphological and functional alterations in several types of cells [1—6]. Although bilirubin has a high affinity for albumin [7], bilirubin is also bound by cells [8—11]. Bratlid [11] suggested that toxic effects of bilirubin on cellular processes may be due mainly to the fraction of bilirubin bound to cells. Since cation transport processes play a key role in cellular metabolism, effects of bilirubin on cellular ionic transport processes may be involved in bilirubin toxicity. The present study was carried out to determine whether bilirubin affects potassium influx and ionic composition of cells. Potassium influx was estimated using $^{86}Rb^+$ which was shown previously to replace potassium as external activator of the sodium pump [12].

Materials and Methods

Ehrlich ascites cells were maintained by weekly transplantation in adult mice of either sex. Cells were obtained by aspiration from the peritoneal cavities of several mice carrying populations of 7—10-day-old cells. The mixed cell suspension was freed of red cells by differential centrifugation. The Ehrlich ascites cells were resuspended in a saline medium [13] to give a cytocrit of 3%. Unconjugated bilirubin was dissolved in 0.1—0.2 ml of 0.1 M NaOH [14] and added to cellular suspensions, which also contained serum albumin, to give a molar ratio of bilirubin to serum albumin of 2 : 1 or 20 : 1. The final concentration of bilirubin in incubation medium ranged from 0.02 to 0.8 mM. Cellular suspensions were incubated in a shaker bath (Dubnoff Model) in erlenmeyer flasks at 21°C. Cells were separated from aliquots of cell suspension by centrifugation. Cellular potassium was determined using specially designed tubes with a capillary end where cells were located [13]. Cellular sodium was determined in cells washed three times by resuspension in isoosmotic $MgCl_2$ [15]. The packed cells were lysed in distilled water [13]. Sodium and potassium were measured by flame photometry. Cytocrit was measured in Van Allen tubes [16] (30 min, 630 g). Cellular sodium and potassium contents were expressed as mmol $\cdot$ l^{-1} (original number of cells — that number of cells which initially occupied 1 l) [17]. Intracellular water was calculated as previously described [18]. 10—30 μl of a solution $^{86}RbCl$ of high specific activity (0.5—10 Ci/g) was added to known concentrations of cells at t_0. 1 ml samples of incubation medium were taken at intervals and centrifuged for 30 s at 2500 $\times g$ to obtain a cell pellet. The cells were washed twice by resuspension in 0.3 osM $MgCl_2$ and finally centrifuged for 30 s at 2500 $\times g$. Cellular isotope loss was assessed [19]. The radioactivity of packed cells and aliquots of incubation medium was determined in a well-shaped scintillation counter. Cellular uptake of $^{86}Rb^+$ was shown to be linear with time up to 3 min of incubation (unpublished observations). Potassium ($^{86}Rb^+$) influx was calculated from the radioactivity incorporated by cells within this time interval and the specific activity of the incubation medium. The recovery of isotope averaged 95% (mean of three determinations). Fluxes were expressed in pmol/s per 10^5 cells [20].

The binding of bilirubin by cells was determined in suspensions of Ehrlich ascites cells diluted with bilirubin to the desired bilirubin concentration. The volume of cells in an aliquot of each sample was measured. At room temperature (21°C) the equilibrium of bilirubin was established at 45 min (after this period bilirubin concentration in the medium was independent of time). The cells were then separated by centrifugation for 30 s at 2500 $\times g$. Control experiments were carried out in the absence of cells in the bilirubin solution in order to determine the spontaneous loss of bilirubin. All experiments were carried out without glucose in the medium so as to minimize possible effects of glycolysis on pH [21,22]. Bilirubin in the supernatant was measured by its absorbance [23—25] in a DU Beckman spectrophotometer. The amount of bilirubin bound by cells was estimated from the difference between the total (previous to) and free (after separation ofcells) bilirubin concentration in the medium [26,27]. Recuperation of bilirubin added to the system (cells plus medium [26] averaged 95% (mean of three determinations). The amount of bilirubin

bound by cells was expressed as mmol · l^{-1} cells. The viability of cells incubated with and without bilirubin was determined using nigrosin [28] and was found to be at least 90% in all experiments, with no significant differences between the experimental conditions. Cell numbers were determined in a cell-counting chamber under phase optics. Bilirubin and bovine serum albumin were purchased from Sigma (St. Louis, MO, U.S.A.). Radioactive rubidium was obtained through the Comision Nacional de Energía Atómica of Argentine as $^{86}Rb^{+}$ of high specific activity (0.5—10 Ci/g) in sterile solution. All the other reagents were also of analytical grade.

Results

Cellular water, sodium and potassium content and cell volume

Intracellular water averaged 0.736 ml (±0.005 ml)/ml of packed cells. In the absence of bilirubin, a steady state for intracellular sodium and potassium content was obtained in cell suspension during 180 min of incubation. In the presence of bilirubin (0.3 mM) at a molar bilirubin/albumin ratio of 20 : 1, intracellular potassium content decreased significantly ($P < 0.001$) while intracellular sodium content increased significantly ($P < 0.001$) (Fig. 1). The sum of intracellular potassium plus sodium contents was not decreased after 180 min of incubation compared to t_0 values at a bilirubin/albumin ratio of 20 : 1. In the presence of bilirubin (0.3 mM) at a molar bilirubin/albumin ratio of only 2 : 1, intracellular potassium content decreased significantly to 70.53 ± 4.04

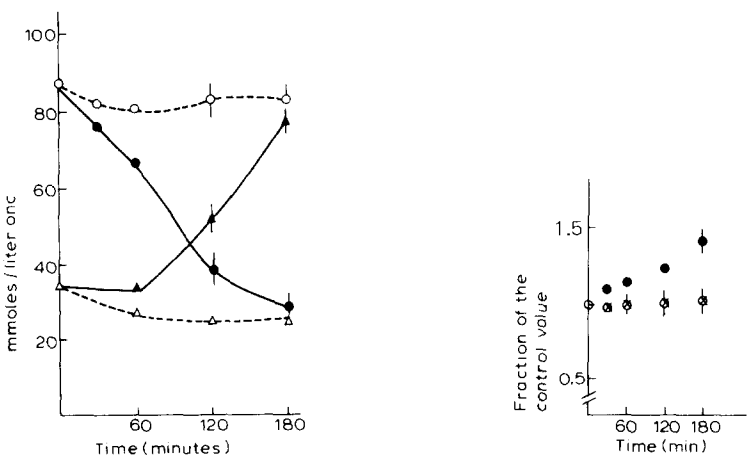

Fig. 1. Effect of bilirubin on intracellular sodium (△,▲) and potassium (○,●) content in Ehrlich ascites cells in vitro. △,○, the absence of bilirubin in the medium; ▲,●, the presence of bilirubin at an initial concentrations of 0.3 mM and a bilirubin/albumin molar ratio of 20 : 1. The values shown are means ± S.E. for nine experiments. ONC, original number of cells.

Fig. 2. Effect of bilirubin at a molar bilirubin/albumin ratio 20 : 1 (●) and 2 : 1 (X) on the packed cell volume of 1 ml of suspension. Data are expressed as a fraction of the control value (packed cell volume at t_0 in the absence of bilirubin (○). Initial bilirubin concentration in the incubation medium: 0.3 mM. The values shown are mean ± S.E. for 5—7 experiments.

(n = 6) mmol · l^{-1} original number of cells ($P < 0.01$), but no significant change in intracellular sodium content occurred. The decrease in potassium content (180 min incubation) at a molar bilirubin/albumin ratio of 20 : 1 was significantly greater than the decrease seen at a ratio 2 : 1 ($P < 0.001$). Cell volume increased significantly during 180 min of incubation in a medium with a bilirubin/albumin ratio of 20 : 1 ($P < 0.001$), while no significant change in cell volume occurred in cells incubated in a bilirubin/albumin ratio of 2 : 1 (Fig. 2).

Potassium influx (Table I)

Potassium influx measured using $^{86}Rb^{+}$ was determined after 180 min of incubation under control conditions as well as in the presence of bilirubin at molar bilirubin/albumin ratios of 2 : 1 and 20 : 1. Control influx averaged 15.8 pmol/s per 10^5 cells, which was similar to previous reports [20]. Influx was significantly reduced in the presence of bilirubin in the medium and was also dependent of the bilirubin/albumin molar ratio; it was 58.2% and 27.8% of the control value at bilirubin/albumin molar ratio 2 : 1 and 20 : 1, respectively.

Cellular binding of bilirubin

Bilirubin bound by cells was measured after 45 min of incubation. The

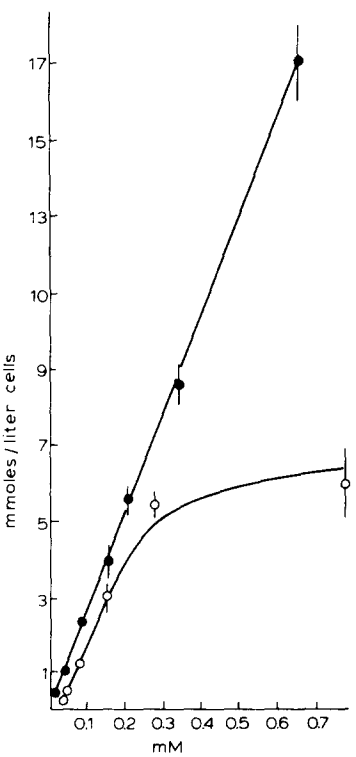

Fig. 3. The amount of bilirubin bound by cells as a function of the bilirubin concentration in the incubation medium at molar bilirubin/albumin ratios of 20 : 1 (•) and 2 : 1 (○). The values show means ± S.E. for seven experiments.

TABLE I

POTASSIUM INFLUX MEASURED USING $^{86}Rb^+$ IN VITRO IN EHRLICH ASCITES CELLS

Incubated for 180 min in a bilirubin solution with an initial bilirubin concentration of 0.3 mM and at molar bilirubin/albumin ratios of 2 or 20. The molar bilirubin/albumin ratio of 0 indicates control conditions, i.e. without bilirubin in the incubation medium. The data are means ± S.E. for the number of experiments shown in parenthesis.

Molar bilirubin/albumin ratio	Potassium influx ($^{86}Rb^+$) (pmol/s per 10^5 cells)
0	15.8 ± 1.4 (7)
2	9.2 ± 1.7 * (5)
20	4.4 ± 0.5 ** (5)

*,** Significantly less than control values at P values less than 0.05 and 0.001, respectively. Mean corresponding to molar ratio 2 differs significantly ($P < 0.05$) from that corresponding to molar ratio 20.

bilirubin concentration in the medium decreased only 2.3 (±0.05) % (n = 10) in the absence of cells, while in the presence of cells, the bilirubin concentration in the medium decreased by 75%. Bilirubin binding by cells increased significantly as the bilirubin concentration in the incubation medium increased (Fig. 3). The binding of bilirubin by cells depended on the molar bilirubin/albumin ratio; no increase in binding of bilirubin occurred at bilirubin concentrations higher than 300 μM at a ratio of 2 : 1, while binding increased linearly up to bilirubin concentrations of 800 μM at a ratio of 20 : 1.

Discussion

The present findings show that bilirubin was bound by Ehrlich ascites cells *. The cell surface and/or the intracellular phase could contain the pigment. The main evidence for cellular binding of bilirubin was that the concentration of bilirubin in the medium decreased by 75% (except at the higher concentration tested at molar ratio 2 : 1) during incubation of cells for 45 min and the concentration of bilirubin in the cells exceeded the initial bilirubin concentration in the incubation medium. This suggests that, according to the evidence of others [26] intracellular binding is more likely. Cell volume was unchanged by bilirubin except after 180 min of incubation in the medium with a bilirubin/albumin ratio of 20 : 1. We consider the present findings suggest that although bilirubin could be bound intracellularly [25], at the molar ratio and incubation period above referred, the pigment is incorporated at least partially in an osmotically active form.

The dependence of intracellular binding of bilirubin on the bilirubin/albumin ratio is related to the fact that at the high molar ratio (20 ; 1) albumin served to disperse bilirubin through nonspecific absorption [24], while at the low molar ratio (2 : 1) 2 mol of bilirubin were bound to albumin in a tight form [7]. At this ratio non-albumin-bound bilirubin in the medium was less than 8 μM (same reference). The saturation of bilirubin binding by cells observed at

* Bilirubin was firmly bound by cells. After three elution procedures with a 3% albumin solution [11], the cells remained strongly stained by the pigment.

bilirubin concentrations greater than 300 μM at the low molar ratio (2 : 1) suggests that the bilirubin-albumin complex occupied cellular bindings sites and thereby reduced further bilirubin transport into cells.

The reduction of $^{86}Rb^{+}$ influx into cells in the presence of bilirubin suggests that bilirubin impaired the pump component of potassium influx [12]. The opposite effects of bilirubin on intracellular potassium and sodium contents also suggests that the effects of bilirubin on cell electrolytes were mediated by alterations in pump mechanisms.

Furthermore, an alteration of the sodium pump by bilirubin as a cause of the swelling of cells incubated for 180 min in the high molar bilirubin/albumin ratio (20 : 1) cannot be excluded, because active cation translocation across the cell membrane regulates cell volume [29]. However, under the present circumstances such an alteration is unlikely. It is to be noted that the cell volume did not increase at a 2 : 1 molar bilirubin/albumin ratio. Although less probable, a rather general effect of the pigment on metabolism [30] as an alternative explanation of cellular alterations, is not excluded. The effects of bilirubin on potassium influx and intracellular potassium content (this last effect similar to that referred in erythrocytes [4]) may be related to other effects of bilirubin on cellular processes. For example, the decrease in glycine incorporation to protein in cells treated with bilirubin [5] may be due to alteration in nitrogen fixation caused by effects of bilirubin on potassium gradients [31,32]. Thus, alterations induced by bilirubin in cellular ionic transport processes may play a role in bilirubin toxicity.

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