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Na^+,K^+-ATPase and Acetylcholinesterase Activities: Changes in Postnatally Developing Rat Brain Induced by Bilirubin

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TSAKIRIS, S. *Na^+,K^+-ATPase and acetylcholinesterase activities: Changes in postnatally developing rat brain induced by bilirubin.* PHARMACOL BIOCHEM BEHAV 45(2) 363–368, 1993.—Na^+,K^+-ATPase, Mg^{++}-ATPase, and acetylcholinesterase activities were determined in brain homogenates of rats in different ages, which were decapitated 30 min after administration of various bilirubin doses. Bilirubin serum and brain tissue levels should be dependent upon the dose administered. At these concentrations, a progressive enzyme inactivation was observed, which reached 25–30% for acetylcholinesterase and 70–80% for Na^+,K^+-ATPase in neonate rats and 15–20% for acetylcholinesterase and only 30–40% for Na^+,K^+-ATPase in the brain of aged rats (20 mo). However, Mg^{++}-ATPase activity was not affected by bilirubin deposition in the developing brain. Moreover, brain albumin content increased 53% in suckling, 40% in adult, and 33% in aged rats at high drug administration. These results may indicate an opening of the blood–brain barrier and a bilirubin entry into the rat brain. The bilirubin immediate toxic effects on brain acetylcholinesterase and Na^+,K^+-ATPase, and probably on brain electrical activity, may be modulated by the developmental state of membrane-bound enzymes.

Na^+,K^+-ATPase Mg^{++}-ATPase Acetylcholinesterase Bilirubin Postnatally developing rat brain

FORTY-TWO years after the indictment of bilirubin as the cause of kernicterus (2), our understanding of the pathogenesis of bilirubin encephalopathy remains incomplete. Total bilirubin concentration (23), free bilirubin concentration (9,12), and reduced serum bilirubin binding (24,38,47) have been implicated as critical elements in the pathogenesis of this condition but fail to explain fully the variable response of infants to hyperbilirubinemia. More recently, an opening of the blood–brain barrier from hyperosmolality, prematurity, asphyxia, hypoxia, hypercapnia, brain blood flow, respiratory distress syndrome, acidosis, shock, and the use of certain drugs has been proposed to be the cause of bilirubin toxicity (3,4,10, 11,21,28,34,48).

Bilirubin is slightly water soluble in the physiological pH range. It possesses a high affinity for lipids in nature (8,36) and can bind efficiently to cellular and intracellular membrane lipids as phosphatidylcholine, phosphatidylserine, gangliosides, and sphingomyelin (22,35,37), modulating the activity of several membrane-bound enzymes (25) as Na^+,K^+-ATPase (26,27). Bilirubin can enter through the endothelial cells of the brain capillaries (42) and diffuse through lipid membranes (22), passing in this way possibly the blood–brain barrier.

It is known that unconjugated hyperbilirubinemia occurs in newborns in association with kernicterus and in some adults with hemolytic jaundice, hepatitis, prolonged cholestasis, Gilbert's syndrome, etc. The purpose of this study was to investigate whether unconjugated hyperbilirubinemia could cause in newborns and adults immediate toxic effects on cholinergic and adrenergic mechanisms (39,43) and brain electrical activity (1,48). Changes in acetylcholinesterase (AChE) and Na^+,K^+-ATPase activities in postnatally developing rat brain induced by administration of various bilirubin doses were studied in the present work.

METHOD

Animals

Male Albino Wistar rats of both sexes were used in all experiments. Their body weight was 6–10 (SD ± 0.6) g in suckling (2 and 8 days), 125 (SD ± 10) g in adult (4 mo), and 326 (SD ± 34) g in aged adult (20 mo). Rats were housed four to a cage, at constant room temperature (22 ± 1°C), under a 12 L : 12 D (light 0800–2000) cycle and acclimated 1 week before use. Food and water were provided ad lib. Animals were cared for in accordance with the principles of the *Guide to the Care and Use of Experimental Animals.*

Experimental Hyperbilirubinemia

Animals were anesthetized with ethyl ether about 5 min, when bilirubin administration solvent of 0.2 ml was injected

IV. In other experiments with suckling rats (2 and 8 days), bilirubin at the indicating doses was injected IP too and similar results were observed with both drug administrations. The solvent for the control was isotonic saline solution adjusted to pH 8.0 with Tris-HCl buffer of 50 mM. The desired amount of crystalline bilirubin was dissolved in 0.1 N NaOH in the dark adjusted to pH 8.0 with HCl and Tris-HCl buffer of 50 mM. Drug administration was: bilirubin 0.02–0.27 mg/rat in neonate and 0.20–0.84 mg/rat in adult and senescent animals.

A few minutes after receiving the injection, all animals treated with bilirubin developed neurological signs of sluggishness and lethargy (in suckling) and showed differences in locomotor activity, ataxia, hypotonia, feeding difficulty, and sluggishness (in adult and senescent rats). These characteristics were proportional to the dose administered. Behavior observations were performed on awake animals (about 25 min), permitting full recovery from anesthesia effects.

Immediately after each treatment, animals were sacrificed by decapitation 30 min after drug administration. Indirect and direct bilirubin was determined in the serum (32) and the unconjugated levels were dependent upon the dose administered. The serum direct bilirubin values for all animals, controls and experimentals, remained constant. All preparations and biochemical determinations were done in dim light to protect bilirubin photooxidation.

Tissue Preparation and Biochemical Determinations

The whole brain was removed, weighed, and thoroughly perfused with isotonic saline. In the case of bilirubin determination (5), the half brain was homogenized with 8 ml chloroform per g wet weight. In the case of albumin determination (14) and enzyme activities measurements, the other half brain was homogenized in 10 vol ice-cold (0–4°C) medium containing 50 mM Tris-HCl, pH 7.4, and 300 mM sucrose using an ice-chilled glass homogenizing vessel. Then, the homogenate was centrifuged at $1,000 \times g$ for 10 min to remove nuclei and debris. In the resulting supernatant, the protein content was determined according to Lowry et al. (30) and then the enzyme activities were measured. The enzyme incubation temperature mixture was kept at 37°C.

Na^+,K^+-ATPase was calculated from the difference between total ATPase activity (Na^+,K^+, Mg^{++}-dependent) minus Mg^{++}-dependent ATPase activity incubated in a mixture without NaCl and KCl (16,46). The values of Mg^{++}-dependent ATPase activity were similar in the presence or absence in the reaction mixture 1 mM ouabain and 0.1 mM ethylene glycol bis(2-amino-ethylether)-*N,N,N',N'*-tetraacetic acid (EGTA). Enzyme activity was not found without $MgCl_2$, NaCl, and KCl in the incubation mixture. Total ATPase activity was assayed in an incubation medium consisting of 50 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 7 mM KCl, 120 mM NaCl, 240 mM sucrose, 3 mM disodium ATP, and 80–100 μg protein of the homogenate, in a final volume of 1 ml. Incubation were performed in the reaction medium for 20 min under continuous magnetic stirring. The reaction was started by the addition of ATP and stopped with 0.2 ml 50% trichloroacetic acid. The liberated Pi was measured by the method of Fiske and Subbarow (17).

AChE activity was determined according to Ellmann's method (15). The reaction mixture (1 ml) contained 50 mM Tris-HCl, pH 8.0, and 240 mM sucrose in the presence of 120 mM NaCl. Protein concentration was 80–100 μg/1 ml incubation mixture. Then, 0.030 ml 5-5'-dithionitrobenzoic acid (DTNB) and 0.050 ml acetylthiocholine iodide, used as substrate, were added and the reaction was started. The final concentration of DTNB and substrate were 0.125 and 0.5 mM, respectively. The reaction was followed spectrophotometrically by the increase in absorbance [difference in optical density ($\Delta\overline{OD}$)] at 412 nm by using a Beckman Acta MVI spectrophotometer.

Statistical Analysis

The data were analyzed by using two-tailed Student's *t*-test.

RESULTS

Figure 1 presents dependence of bilirubin levels in the brain of 2-day-old rats upon dose administered. The curve is biphasic, with a higher slope from 0.3–1 mg bilirubin per 100 g body wt (0.020–0.064 mg/rat). Serum total bilirubin levels ranged from 20–78 mg/dl and brain levels from 1.1–6.5 mg per 100 g brain tissue. Direct bilirubin values for all animals, controls and experimentals, remained constant. Therefore, unconjugated bilirubin levels in the serum and brain were dependent upon the dose administered.

Effects of brain bilirubin deposition on AChE and Na^+, K^+-dependent ATPase activities in homogenated brain of 2-day-old rats are illustrated in Fig. 2. As can be seen, both enzymatic activities were affected from 2–6.5 mg bilirubin per 100 g brain; serum total bilirubin levels ranged from 30–78 mg/dl. A progressive enzyme inactivation was observed, which reached 25–30% for AChE and 70–80% for Na^+,K^+-ATPase.

Effects of bilirubin on AChE and Na^+,K^+-ATPase activities in homogenated brain of different age are presented in Table 1. At high drug administration, total bilirubin values ranged from 30–52 mg/dl in serum and 2–4.3 mg per 100 g brain. High brain bilirubin deposition caused an enzyme inactivation 25% for AChE and 68% for Na^+,K^+-ATPase in 8-day-old rats and 20% for AChE and 52% for Na^+,K^+-ATPase in adult rats of 4 mo.

Figure 3 presents the effects of brain bilirubin deposition on AChE and Na^+,K^+-dependent ATPase activities in ho-

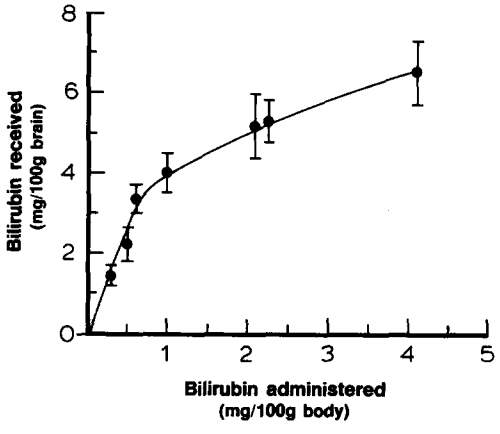

FIG. 1. Dependence of bilirubin levels in the brain of 2-day-old rats upon dose administered. Values represent means ± SD for five independent experiments (five rats). The average value of each experiment came from three determinations in the homogenated brain of each animal.

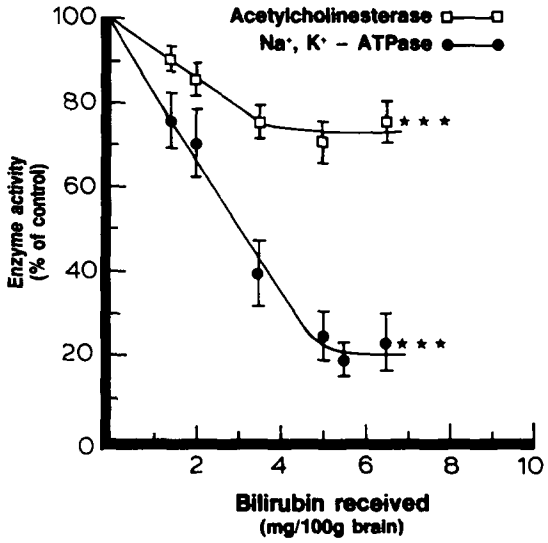

FIG. 2. In vivo effect of bilirubin on the activities of acetylcholinesterase (□ – □) and Na^+,K^+-ATPase (● – ●) determined in homogenated brain of 2-day-old rats. Values represent means ± SD for five independent experiments (five rats). The average value of each experiment came from seven determinations in the homogenated brain of each animal. The values in high concentrations of bilirubin are statistically significant compared with those of control (***$p < 0.001$).

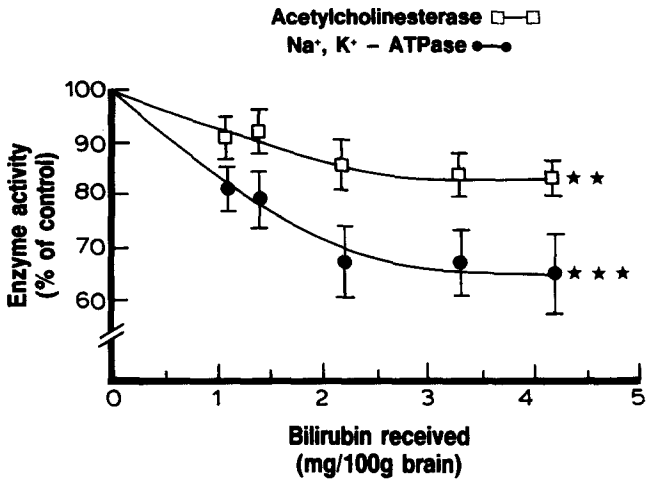

FIG. 3. In vivo effect of bilirubin on the activities of acetylcholinesterase (□ – □) and Na^+,K^+-ATPase (● – ●) determined in homogenated brain of 20-mo-old rats. Values represent means ± SD for five independent experiments (five rats). The average value of each experiment came from seven determinations in the homogenated brain of each animal. The values in high concentrations of bilirubin are statistically significant compared with those of control (**$0.001 < p < 0.01$, ***$p < 0.001$).

mogenated brain of 20-mo-old rats. It was found that both enzymatic activities were affected from 2.2–4.2 mg bilirubin per 100 g brain; serum total bilirubin levels ranged from 33–63 mg/dl. The observed enzyme inactivation should be dependent upon the dose administered (0.20–0.84 mg/rat) and reached 15–20% for AChE and only 30–40% for Na^+,K^+-ATPase.

Mg^{++}-ATPase activity was found to be 5.74 ± 0.38 μmol Pi/h/mg protein in 2-day-old rats and remained constant in adult and senescent animals. Various bilirubin doses produced in the brain an increased bilirubin uptake, which was not able to affect the enzyme activity in the developing brain ($p > 0.05$).

Brain albumin content in relation to bilirubin administra-

TABLE 1

IN VIVO EFFECT OF BILIRUBIN ON THE ACTIVITIES OF ACETYLCHOLINESTERASE AND Na^+,K^+-ATPASE DETERMINED IN HOMOGENATED RAT BRAINS OF DIFFERENT AGES

Time of Development	Bilirubin Received (mg/100 g brain)	Activity: Acetylcholinesterase (ΔOD/min/mg protein)	Activity: Na^+,K^+-ATPase (μmol Pi/h/mg protein)
2 days control	0	0.458 ± 0.030	1.45 ± 0.06
	2.0 ± 0.3	0.380 ± 0.025*	0.99 ± 0.08†
	4.3 ± 0.5	0.326 ± 0.011†	0.44 ± 0.02†
8 days control	0	1.00 ± 0.04	1.56 ± 0.12
	2.0 ± 0.2	0.85 ± 0.03*	1.01 ± 0.10*
	4.0 ± 0.5	0.75 ± 0.04†	0.50 ± 0.06†
4 mo control (adult)	0	0.70 ± 0.04	2.07 ± 0.15
	2.2 ± 0.4	0.63 ± 0.04‡	1.15 ± 0.12†
	4.2 ± 0.6	0.56 ± 0.03†	0.99 ± 0.07†
20 mo control (aged adult)	0	0.573 ± 0.043	2.76 ± 0.23
	2.2 ± 0.3	0.490 ± 0.020*	1.91 ± 0.13†
	4.2 ± 0.5	0.479 ± 0.013*	1.81 ± 0.13†

Values represent means ± SD for three independent experiments (three rats) using 8-day-old rats and five experiments (five rats) using 2-days-, 4-, and 20-month-old rats. The average value of each experiment came from seven determinations in the homogenated brain of each animal.

The values in high concentrations of bilirubin are statistically significantly compared with those of controls: *$0.001 < p < 0.01$, †$p < 0.001$, ‡$0.01 < p < 0.05$.

TABLE 2

BRAIN BILIRUBIN AND ALBUMIN CONTENT IN RELATION TO BILIRUBIN ADMINISTRATION IN RATS OF DIFFERENT AGES

Time of Development	Body Weight (g)	Brain Weight (g)	Bilirubin Administered (mg/rat)	Bilirubin Received (mg/100 g brain)	Albumin Content (g/100 g brain)
2 days control	6.4 ± 0.4	0.36 ± 0.03	0	0	2.55 ± 0.10
	6.2 ± 0.6	0.38 ± 0.04	0.064	4.3 ± 0.5	3.91 ± 0.19*
4 mo control (adult)	125.0 ± 10.2	0.94 ± 0.04	0	0	3.58 ± 0.22
	134.5 ± 10.9	0.98 ± 0.05	0.840	4.2 ± 0.6	5.02 ± 0.30*
20 mo control (aged adult)	326.5 ± 34.0	1.53 ± 0.08	0	0	3.94 ± 0.26
	300.0 ± 30.2	1.48 ± 0.07	0.840	4.2 ± 0.5	5.24 ± 0.28*

Administration solvent of 0.2 ml was isotonic saline solution for the control and bilirubin was dissolved in 0.1 N NaOH in the dark adjusted to pH 8.0 with HCl and Tris-HCl buffer of 50 mM. Values represent means ± SD for five independent experiments (five rats). The average value of each experiment came from five determinations in the homogenated brain of each animal.

In the case of bilirubin administration, the values of brain albumin content are statistically significantly compared with those of controls (*$p < 0.001$).

tion in rats of different ages is presented in Table 2. High drug administration caused in the brain a bilirubin uptake and an increase in albumin content 53% in suckling, 40% in adult, and 33% in aged rats.

DISCUSSION

AChE is a biologically significant component of the membrane, contributing to its integrity and to the permeability changes occurring during synaptic transmission and conduction (20); it is a membrane-bound enzyme with its active side exposed at the external leaflet of the bilayer (ectoenzyme) (13). Moreover, phosphatidylcholine and sphingomyelin are at the outside of the membrane surface and phosphatidylserine is confined to the inner surface of the plasma membrane (7, 19,49). Bilirubin can bind efficiently to these lipids (22,35, 37), which are components of rat brain synaptosomal plasma membranes (6). Therefore, bilirubin binding to phosphatidylcholine and sphingomyelin of plasma membrane may cause the observed inhibition of AChE (25–30%) in newborns. Further, it has been reported that the fatty acid composition of phosphatidylcholine and sphingomyelin in rat brain is significantly influenced by brain development (31). Subsequently, the observed low inhibition of brain AChE from bilirubin (15–20%) in senescent rats may be caused by developmental changes in the composition of the membrane lipids, which could interact with the enzyme protein and thus modulate its activity (18,40).

Na^+,K^+-ATPase, the enzymatic basis of univalent cation transport (44) and of activity-dependent energy utilization in nerve (33), appears to be stimulated by catecholamines in vitro and in vivo (39,43). Therefore, bilirubin toxic effects on adrenergic mechanisms could affect brain Na^+,K^+-ATPase activity, as found in this work. The activity of NaI-treated microsomal Na^+,K^+-ATPase from young rat cerebrum was inhibited in vitro by bilirubin (27). In this study, in vivo bilirubin incorporation in the brain can cause the maximum enzyme inhibition (70–80%) during the neonatal period. Moreover, during development the increase in the enzyme activity correlates with the onset of electrical activity in the brain (1). Therefore, the observed enzyme inhibition from bilirubin could influence brain electrical activity (48). Subsequently, the low inactivation of Na^+,K^+-ATPase (30–40%) at high bilirubin uptake in the aged brain could be due to developmental-related modifications of cellular and intracellular plasma membranes. Indeed, bilirubin acts differently on the Arrhenius plots for Na^+,K^+-ATPase activities of young and adult rat cerebra because of different lipid environments surrounding the enzyme during membrane development (26), modulating its activity (40,45).

Mg^{++}-ATPase and Na^+,K^+-ATPase are different enzymes in brain nerve endings (29,41). Brain intracellular Mg^{++} concentration (about 1 mM) is maintained at a high level because of Mg^{++}-pump activity. It was found that Mg^{++}-ATPase activity was not affected by bilirubin deposition in the developing brain. Therefore, bilirubin uptake may not be able to influence brain intracellular Mg^{++} concentration.

The increase in brain bilirubin uptake and albumin content and yellow staining of the tissue in newborn, adult, and senescent animals may be caused by an opening of the blood–brain barrier and a bilirubin entry [free and albumin-bound (21)] into the tissue. The anesthesia used during drug administration may cause hypoxia, asphyxia, and hypercapnia in the rat, conditions of which can open the blood–brain barrier in adult animals (11,34). Moreover, it is yet not known which factors can open the blood–brain barrier in senescent rats.

In conclusion, the results of this study suggest that: a) Bilirubin immediate toxic effects on brain neurotransmission may be caused by inhibition of AChE and mainly of Na^+,K^+-ATPase. Toxic effects on brain AChE and Na^+,K^+-ATPase and probably on brain electrical activity (1,48) may be modulated by the developmental state of membrane-bound enzymes. b) During the neonatal period, where the activities of AChE and Na^+,K^+-ATPase were found to have low values, bilirubin incorporation in the brain can cause the maximum enzyme inhibition. c) The observed low inactivation of Na^+,K^+-ATPase at high bilirubin levels in the aged brain could be due to developmental-related modifications of cellular and intracellular plasma membranes (26,31,40,45). d) The increase in brain bilirubin uptake and albumin content and yellow staining of the tissue in newborn, adult, and senescent animals may be caused by an opening of the blood–brain barrier and a bilirubin entry into the tissue.

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