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Short-Term Exposure to Bilirubin Reduces Synaptic Activation in Rat Transverse Hippocampal Slices

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ABSTRACT. We evaluated the feasibility of using the *in vitro* transverse rat hippocampal slice as a model to study the effect of bilirubin on neuronal activity. Bilirubin in concentrations from 100 μmol/liter to 1 mmol/liter with bovine serum albumin as a stabilizer caused a significant decrease in the slope of the field excitatory postsynaptic potentials, concomitant with a significant increase in the peak latency of the population spike. These changes were partially reversible when bilirubin was removed from the incubation fluid. A partially reversible shift to the right of the presynaptic fiber volley/field excitatory postsynaptic potential relationship was interpreted as an expression of a reduction in synaptic activation. A partially reversible shift to the left of the field exitatory postsynaptic potential population spike relationship was interpreted as an expression of increased postsynaptic excitability. In conclusion the *in vitro* rat hippocampal slice was used successfully to study the effect of bilirubin on neuronal activity. A depressive effect of bilirubin was observed. (*Pediatr Res* 23: 453–456, 1988)

Abbreviations

BSA, bovine serum albumin
ACSF, artificial cerebrospinal fluid
PV/prevolley, presynaptic fiber volley
fEPSP, field excitatory postsynaptic potential
PS, population spike

The neurotoxicity of bilirubin is well recognized (1–5). There is considerable evidence that bilirubin depresses neuronal function. Both auditory and visual evoked responses have been used to follow the course of hyperbilirubinemia (6–8). Studies of the auditory brainstem response have shown reductions of amplitude and increases in peak latencies in jaundiced neonates. These changes are reversed when the serum bilirubin level is lowered (7, 9, 10). Similar results have been obtained in monkeys and rats during experimentally induced hyperbilirubinemia (11, 12). In addition, high bilirubin levels have been shown to suppress electrical activity in rat brain after opening of the blood brain barrier (13). However, the basic neurophysiological mechanism involved in the depressive effect of bilirubin still is unknown.

The *in vitro* rat hippocampal slice preparation offers a technique to study the mechanisms involved in the activation of a specific neuronal population (14). The aim herein was to evaluate whether this model might be suitable for analyzing neurophysiological effects of bilirubin. Preliminary observations from some of the experiments have been published (15, 16).

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METHODS

Bilirubin and BSA were obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals used were analytical grade.

Young adult male Sprague-Dawley rats were purchased from Dyrlæge Møllegaards Avlslaboratorium, Skensved, Denmark. Their weights at the time of use ranged from 80–350 g (mean 139 g).

Transverse hippocampal slices were prepared as described by Skrede and Westgaard (14). The slices were placed on a nylon net in an incubation chamber at 30 to 32° C and superfused with ACSF of the following composition (mM): Na^+ 151; K^+ 5; Mg^{2+} 2; Ca^{2+} 2; Cl^- 129; HCO_3^- 26; SO_4^{2-} 2; phosphate 2; and glucose 10, preequilibrated with 95% O_2/5% CO_2 to a pH of 7.4. In some experiments the calcium concentration in the ACSF was reduced to 1–1.25 mmol/liter. The flow rate was 1 ml/min. A humidified stream of 95% O_2/5% CO_2 was directed over the upper surface of the slices at a rate of approximately 500 ml/min.

After a recovery period of not less than 1 h, platinum-coated tungsten electrodes were placed visually. The stimulating electrode was inserted among the Schaffer collaterals in the stratum radiatum; the recording electrodes were placed in the dendritic field of the stratum radiatum and in the corresponding cell body layer in the stratum pyramidale of the CA1 region (Fig. 1).

The pathways were stimulated using 0.8 Hz single shock pulses of 0.1 ms duration. These were delivered by constant voltage stimulators in the initial experiments, later with constant current stimulators. Intensities were adjusted to produce submaximal responses. We measured the amplitude of the PV/prevolley, the slope of the fEPSP, measured as the difference in voltage between two points on the curve, each point having a fixed time relationship to the stimulus, and the amplitude of the PS, as shown in Figure 1. We analyzed and recorded the data on-line with an Apple II+ microcomputer. Analog signals were displayed on an oscilloscope, and photographs of these were taken at intervals for subsequent off-line evaluation.

Bilirubin dissolved in 0.1N NaOH was added to the ACSF to achieve final bilirubin concentrations of 50–1000 μmol/liter. To retard flocculation of bilirubin in these supersaturated solutions, BSA was added in a molar ratio of 1:8 (BSA:bilirubin). Control solutions did not contain bilirubin, but were otherwise identical. All experiments were performed in subdued light, and bilirubin-containing vessels and infusion tubes were wrapped in tin foil.

Alterations in the PV/fEPSP and the fEPSP/PS relationships were studied by recording the responses to different stimulus

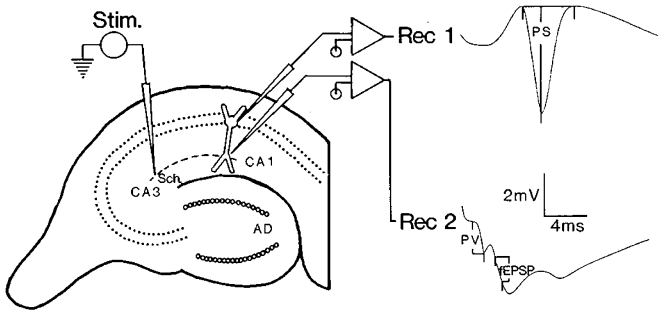

Fig. 1. Schematic illustration of the transverse hippocampal slice. The stimulatory electrode (*Stim.*) was placed among the Schaffer collaterals (*Sch.*) in the CA3 region. The two recording electrodes were placed in the cell body layer (*Rec 1*) and in the dendritic layer (*Rec 2*) of the CA1 region. The following parameters were recorded: PV, fEPSP. and PS. *AD*, area dentata.

intensities. Changes in the fEPSP slope and the PS peak latency were calculated as a percentage of the starting values after approximately 60 min exposure to bilirubin, and again after subsequent exposure to bilirubin-free, BSA-containing ACSF. The statistical significance of these changes was evaluated by calculating the confidence intervals.

RESULTS

Bilirubin caused a significant reduction in the slope of the fEPSP (mean −30%, 99.9% confidence interval −18.2 to −42.6; $n = 31$). Concomitantly the peak latency of the population spike increased significantly (mean +12%, 99.9% confidence interval 3.2 to 21.4; $n = 23$). A typical experiment is illustrated in Figure 2. The changes appeared gradually after intervals of from 10 to 30 min, and increased with time and with the concentration of bilirubin. The lowest concentration of bilirubin associated with such changes was 100 μmol/liter. The effects of 100 μmol/liter bilirubin were seen under low calcium conditions. In 40% of the experiments exposure to bilirubin was associated with the appearance of multiple spikes.

The decrease in the fEPSP slope elicited by bilirubin could be partly reversed when the slices were exposed to bilirubin-free ACSF with the same content of BSA (mean +14%; 95% confidence interval 1.2 to 27.4; $n = 16$). Similarly, the increase in the PS peak latency caused by bilirubin could be reversed by BSA (mean −11%; 99% confidence interval −19.9 to −2.7; $n = 10$). Renewed exposure to bilirubin once again reduced the fEPSP slope and increased the PS peak latency (Fig. 3 *a* and *b*).

To further investigate the mechanisms underlying the observed depressive effect of bilirubin, the responses to stepwise changes in the stimulus intensity were plotted. The PV/fEPSP curve was shifted to the right and the fEPSP/PS curve was shifted to the left by exposure to bilirubin. These shifts were observed in seven of nine experiments, and could be partly reversed by exposing the slices to bilirubin-free, BSA-containing ACSF. Data from a representative experiment using 1 mmol/liter bilirubin, 125 μmol/liter BSA, and 1 mmol/liter extracellular calcium are shown in Figure 4 *a* and *b*.

DISCUSSION

The results herein show that a depressive effect of bilirubin is observed and may be studied in the *in vitro* rat transverse hippocampal slice model. Bilirubin produced a significant reduction in the slope of the fEPSP in rat transverse hippocampal slices, concomitant with a significant increase in the peak latency of the PS. These effects were seen at bilirubin concentrations of as little as 100 μmol/liter in the ACSF after a time span of 10–30 min. These changes could be partly reversed by BSA. The

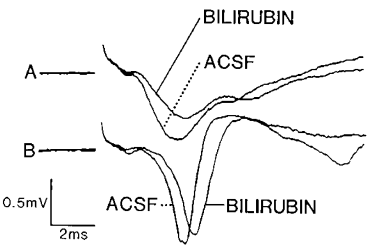

Fig. 2. Effect of bilirubin on extracellular potentials of the CA1 region. Potentials during initial exposure to ACSF and after 55 min exposure to bilirubin 1000 μmol/liter with bovine serum albumin 125 μmol/liter as stabilizer (*BILIRUBIN*). The bilirubin-induced decrease in the slope of the fEPSP is illustrated in *A*, whereas the concomitant increase in the latency of the PS is shown in *B*.

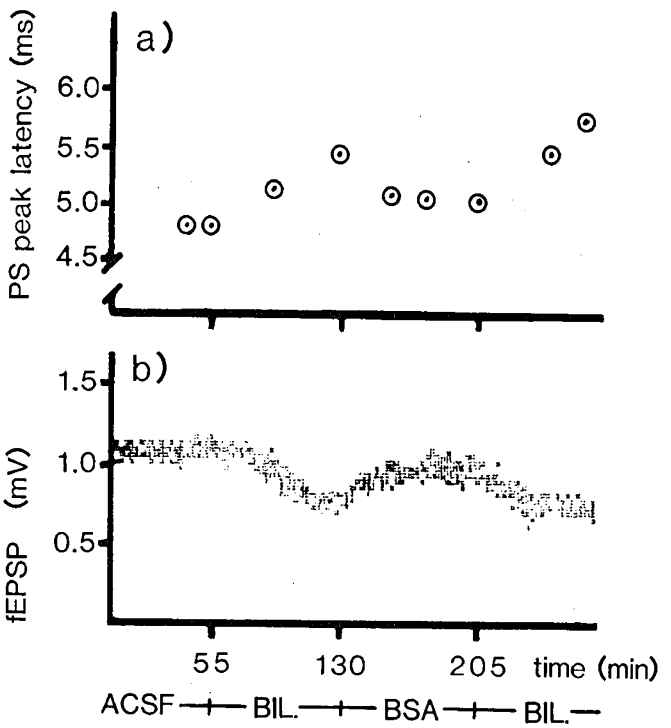

Fig. 3. Reversible effect of bilirubin on population spike peak latency and field EPSP. *a*, changes in the PS peak latency as measured off-line from photographs of the oscilloscope screen. *b*, continuous computer tracing of the field EPSP. *ACSF*, artificial cerebrospinal fluid with 1.25 mmol/liter Ca^{++}. *BIL.* = 500 μmol/liter bilirubin with 67.5 μmol/liter BSA in ACSF. *BSA*, 67.5 μmol/liter BSA in ACSF.

reduction in the slope of the fEPSP together with a shift to the right of the PV/fEPSP curve indicates that bilirubin inhibits synaptic activation. Our data do not permit a definite conclusion on whether this inhibition is due to impaired neurotransmitter release or to an effect on the neuronal membrane.

To the best of our knowledge direct effects of bilirubin on neurotransmitter release have not yet been demonstrated. It has, however, recently been shown that bilirubin inhibits phosphorylation of synapsin I (17, 18), which is thought to play a role in neurotransmitter release (19). Also, jaundiced Gunn rats have been found to have a higher concentration of norepinephrine in their hippocampi than their nonicteric (heterozygous) littermates (20). Brann *et al.* (21) showed that bilirubin inhibits the cAMP-stimulated synthesis of dopamine in rat striatal synaptosomes.

The left shift of the fEPSP/PS curve seen during bilirubin exposure indicates an increase in the postsynaptic excitability. The tendency to multiple spikes under these conditions points in the same direction. One possible explanation for this observation might be a reduction in the resting potential. However, it

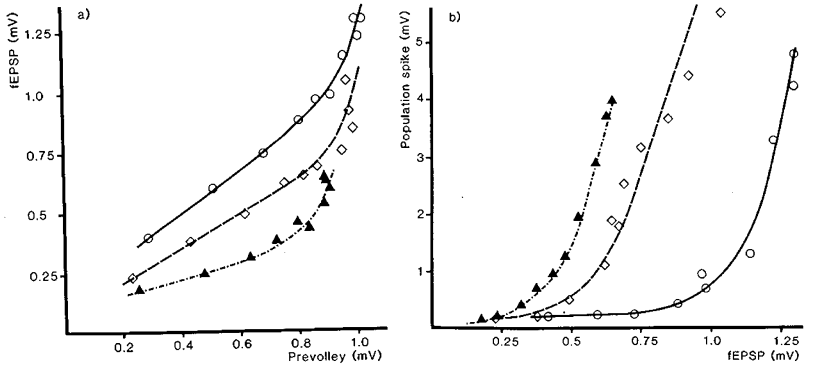

Fig. 4. Effect of bilirubin on PV/prevolley, field EPSP, and population spike at different stimulus strengths. *a*, relationship between the amplitude of the prevolley and the slope of the fEPSP. *b*, relationship between the slope of the fEPSP and the amplitude of the PS. O, during initial exposure to ACSF containing 1 mmol/liter Ca^{++}; ▲, 40 min after start of exposure to bilirubin 1000 μmol/liter with BSA 125 μmol/liter in ACSF. ◇, 55 min after removal of bilirubin solution that was replaced with a solution containing 125 μmol/liter BSA in ACSF. The *lines* have been drawn by visual approximation.

is known that inhibitory synapses are conserved in transverse hippocampal slices of the thickness used in our experiments (22, 23). The increase in postsynaptic excitability observed in our experiments could therefore also be explained on the basis of inhibition of transmission in these inhibitory synapses.

There is a considerable body of evidence indicating that bilirubin may exert an effect on neuronal membranes. Mayor *et al.* (24) have shown that bilirubin (final concentration 40 μmol/liter) induces a rapid depolarization of rat forebrain synaptosomes as reflected by an efflux of previously accumulated ^{3}H-tetraphenylphosphonium. BSA in a molar ratio of 1:1 to bilirubin could prevent and reverse this effect of bilirubin. Cowger (25) has shown that for periods of up to 4 h the membranes of bilirubin-exposed cells become increasingly permeable even to such large molecules as enzymes.

In the experiments reported herein we observed effects of bilirubin down to concentrations of 100 μmol/liter, but most of the experiments were run at higher concentrations. Although it is unlikely that brain bilirubin concentrations as high as these are attained in the clinical situation, high levels can be found in experimental situations (26–30). Regional variations in brain bilirubin concentrations have been observed, and the hippocampus is among the structures with high bilirubin levels (31). In the basal ganglia of infants dying with hemolytic disease, values of approximately 30–40 μmol/liter have been found (32).

Limited information is available concerning regional differences in brain sensitivity to bilirubin toxicity. In the Gunn rat the cerebellum and the Purkinje cells are primarily affected (33–36). The behavior of homozygous Gunn rats bears a close resemblance to that of rats with experimental lesions in the hippocampus, also pointing to the hippocampus as an area of higher sensitivity (37).

Some limitations are inherent in the rat hippocampal slice model as used herein. Caution is needed in extrapolating from *in vitro* findings to the clinical situation. The period of observation is limited to a few hours, and the effects observed may differ from those occuring after several days' exposure *in vivo*. The presence of albumin as a stabilizer may interfere with the effect of bilirubin. Because of the time element dose-response relationships are difficult to study. Finally, as only one slice can be studied at a time, and as bilirubin is impossible to remove completely once it has been introduced into this system, only one control period is possible per experiment.

In conclusion, we found that bilirubin causes changes in the electrophysiology of rat transverse hippocampal slices, interpreted as evidence of reduced synaptic activation and increased postsynaptic excitability. Our data provide additional evidence that elements of bilirubin neurotoxicity are reversible.

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