0031-3998/88/2302-0219$02.00/0
PEDIATRIC RESEARCH

Vol. 23, No. 2, 1988
Printed in U.S.A.

Bilirubin Decreases Phosphorylation of Synapsin I, a Synaptic Vesicle-Associated Neuronal Phosphoprotein, in Intact Synaptosomes from Rat Cerebral Cortex

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ABSTRACT. The mechanisms by which bilirubin causes neurotoxicity in newborns have not been well defined, but an involvement in synaptic transmission appears possible. Herein we present evidence for an inhibitory effect of bilirubin on both basal and depolarization-induced (50 mM KCl) phosphorylation of synapsin I, a synaptic vesicle-associated protein that may play a role in neurotransmitter release. Synaptosomes from rat cerebral cortices, prelabeled with ^{32}P *in vitro* to label the intraterminal ATP pool, were incubated with or without bilirubin and bovine serum albumin (added as a stabilizer) at varying doses and for different time intervals. Some preparations were also depolarized by high KCl concentrations to induce Ca^{++} influx. The phosphorylation of synapsin I was monitored. Our results show that addition of bilirubin to the medium significantly decreases ^{32}P incorporation into synapsin I, both under basal and depolarizing conditions, in a time- and dose-dependent manner, significant effects being observed already at 10 μM bilirubin after 120-min incubation of the synaptosomes. Separate analysis of the multiple phosphorylation sites in synapsin I showed that the phosphorylation of both the "head" and "tail" regions of the protein was decreased by bilirubin. Removal of the bilirubin-containing incubation medium retarded the decrease in synapsin I ^{32}P content, indicating that the effect observed may be reversible. The nontoxic pyrrole biliverdin had no effect on synapsin I phosphorylation under the experimental conditions used, indicating that the effect was specific to bilirubin. Our results thus suggest that bilirubin may achieve some of its reversible effects on the brain through inhibition of the phosphorylation of the synapsic vesicle-associated protein synapsin I. (*Pediatr Res* 23: 219–223, 1988)

Abbreviations

BSA, bovine serum albumin
SDS, sodium dodecyl sulphate
PAGE, polyacrylamide gel electrophoresis

Bilirubin is a well known cause of brain damage (1). The classical term "kernicterus," denoting yellow staining of the basal ganglia (2), has been used to describe the sometimes severe neurodevelopmental sequelae of neonatal jaundice (3, 4). In addition to such irreversible effects, clinical experience has indicated that bilirubin also may have reversible effects on brain function, as shown by the sluggishness and feeding problems often observed in a jaundiced infant, who reverts to normal when the serum bilirubin concentration is lowered. Modern neurophysiological diagnostic techniques, such as auditory brain stem response and cry analysis, have given objective evidence of a reversible depressive effect of bilirubin on neuronal function (5, 6), suggesting interference with synaptic transmission.

Recent studies have implicated protein phosphorylation as an important regulatory mechanism in the function of nerve cells (7). Stimulation-dependent release of neurotransmitters appears to be one phenomenon that is regulated by phosphorylation of specific intraterminal proteins (cf. Ref. 8 for review). Synapsin I, a neuron-protein known to be specifically associated with synaptic vesicles (9), may be involved in this regulation. Thus, intracellular injection studies have indicated that the dephosphorylated form of synapsin I may inhibit the release process, while phosphorylation of synapsin I, catalyzed by Ca/calmodulin-dependent kinase II, appears to promote stimulation-dependent neurotransmitter release (10).

We considered the possibility that part of the depressive effect of bilirubin on central nervous system function might be due to effects on nerve terminal function. We therefore found it of interest to investigate whether bilirubin would interfere with the protein phosphorylation systems involved in synapsin I phosphorylation in intact nerve terminals, where the protein is known to be associated with the cytosolic surface of synaptic vesicles (9). For this purpose we decided to use an intact synaptosomal preparation, enriched in viable nerve terminals, in which prelabeling with inorganic ^{32}P leads to labeling of the nerve terminal ATP pool (11). In this preparation it is possible to analyze whether phosphate incorporation into synapsin I under basal and depolarized conditions may be influenced by bilirubin. A summary of some of the results has been presented previously (12).

METHODS

Materials. Crystalline bilirubin and BSA were obtained from Sigma Chemical Co, St. Louis, MO; carrier-free $^{32}P_i$ was from New England Nuclear, Boston, MA; and *Staphylococcus aureus* V8 protease was from Miles Laboratories, Inc., Elkhart, IN.

Received July 27, 1987; accepted October 14, 1987.
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Supported by United States Government Grant under public law 87-256; The Fulbright-Hays Act ("Fulbright Fellowship"); Høyesterettsadvokat Per Rygh's Legat; Nathalia og Knut Juul Christiansens Stiftelse; Chr. Christensens Stipendiefond; The Norwegian Research Council for Science and the Humanities Travel Grant.

Other chemicals, of reagent grade, were obtained from standard commercial suppliers.

Animals. Male Sprague-Dawley rats (200–300 g) were used in all experiments. They were stunned and decapitated, and the cortices were dissected out on a cold surface before being placed in ice-cold 0.32 M sucrose.

Preparation of synaptosomes. Each cortex was homogenized in glass-Teflon homogenizers in 25 ml of ice-cold 0.32 M sucrose, and a crude synaptosomal fraction was prepared by differential centrifugation (13). The synaptosome preparation was then resuspended in 6–8 ml of an oxygenated Krebs Ringer buffer (pH 7.4) containing (mM): NaCl 132, $MgSO_4$ 2.4, KCl 4.8, Hepes 20, $CaCl_2$ 1, glucose 10.

Prelabeling of endogenous ATP-pool. A total of 37 MBq of $^{32}P_i$ was added to 3 ml of the synaptosome suspension, which was then incubated at 37° C for 30 min (11). After this the synaptosomes were centrifuged at 2000 rpm for 5 min, the highly radioactive supernatant decanted, and the synaptosomes resuspended in 3.2 ml of Krebs Ringer buffer.

Incubation with bilirubin and BSA. Bilirubin, dissolved in 0.1 N NaOH and stabilized by the addition of BSA in a molar ratio of 1:10 (BSA:bilirubin), was diluted with Krebs Ringer buffer such that addition of 10 μl of the bilirubin/BSA solution to 100 μl of the synaptosome suspension resulted in bilirubin concentrations of 1–160 μM in the final incubate. A BSA concentrate treated similarly was used for the control incubations. During preparation and incubation the bilirubin/BSA-containing solutions were covered by aluminum foil and shielded from light as much as possible.

After the addition of bilirubin and/or BSA, the synaptosome suspensions were incubated for periods of 15, 60, and 120 min, after which the reaction was stopped by adding 50 μl of a SDS-containing "stop solution" (14), followed by boiling for 2 min.

Some incubates were depolarized for 30 s by the addition of a concentrated solution of KCl (final concentration 50 mM) before adding SDS-solution and boiling as described above.

Analysis of synapsin I phosphorylation. The phosphorylated proteins were separated by one-dimensional SDS-PAGE using gels containing 8% acrylamide (15). After removal of the dye-front and brief washing in 10% isopropanol/10% acetic acid in water, the gels were dried and subjected to autoradiography. Synapsin I was identified on the gels as a doublet of 80/86 kD molecular mass, cut out from the gel, and the radioactivity incorporated was measured by liquid scintillation counting of the gel pieces (16).

Analysis of multiple site phosphorylation by peptide mapping. In order to analyze the site-specific phosphorylation of the different domains of synapsin I, the gel pieces containing synapsin I were subjected to incomplete proteolytic digestion using *S. aureus* V8 protease (17). The radioactive peptide fragments were separated by SDS-PAGE on 15% acrylamide gels, visualized by autoradiography of the dried gels and quantitated by liquid scintillation counting as described above.

RESULTS

An initial series of experiments suggested that bilirubin could induce a decrease in the ^{32}P content of synapsin I in the rat cerebral cortex synaptosome preparation, when analyzed by one-dimensional SDS-PAGE (Fig. 1). This phenomenon was therefore studied further.

After 30 min of prelabeling, which has been found to be suitable for nerve terminal labeling in this type of experiment (11), and removal of extrasynaptosomal $^{32}P_i$, exposure to bilirubin for 120 min led to a distinct decrease in the ^{32}P content of phosphoproteins in the molecular mass range of 80/86 kD, which mostly represent synapsin I. When compared to control incubates, this decrease in phosphate incorporation was significant at bilirubin concentrations as low as 10 μM (Fig. 2). The decrease

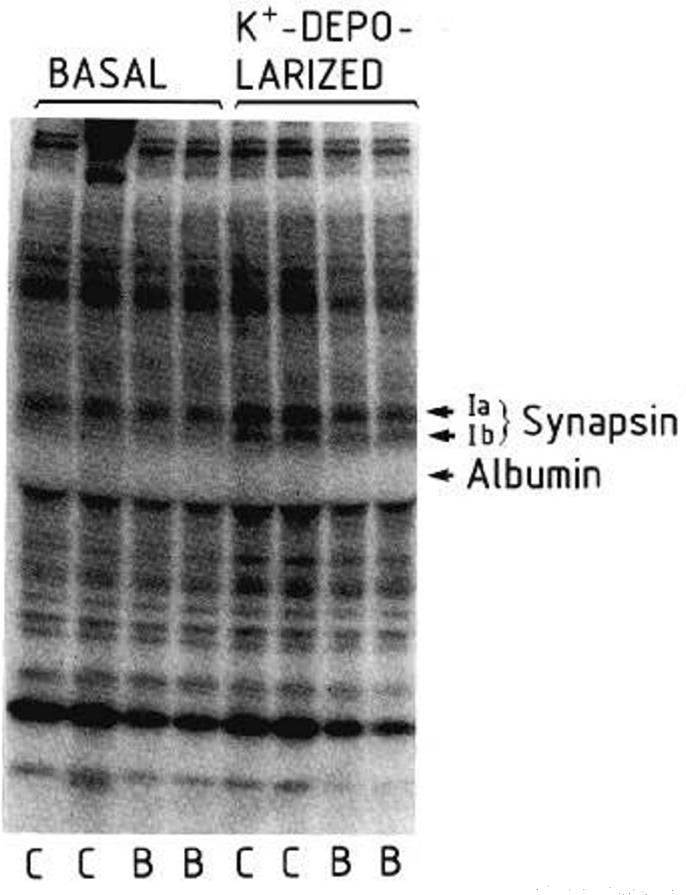

Fig. 1. Autoradiogram showing inhibitory effect of bilirubin on ^{32}P incorporation into proteins in intact synaptosomes from rat cerebral cortex. Synaptosomes were prelabeled with $^{32}P_i$ and incubated with bilirubin (160 μM) plus BSA (16 μM) (*B*) or with BSA only (*C*) for 120 min. Some samples were then depolarized for 30 s with 50 mM KCl to induce Ca^{++} influx into the nerve terminals. Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. Synapsin Ia and Ib are indicated.

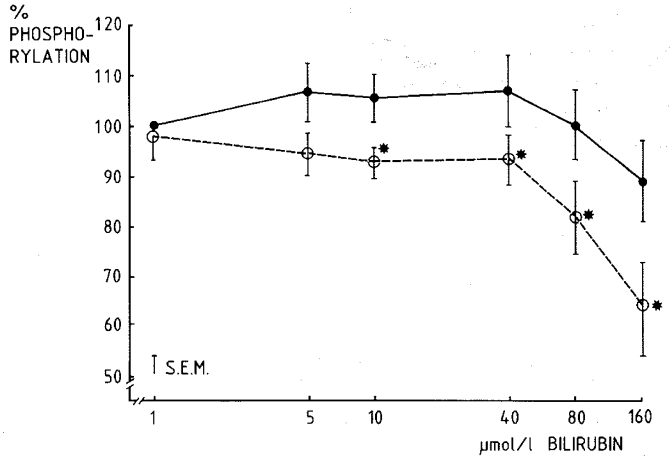

Fig. 2. Effect of varying doses of bilirubin on unstimulated phosphorylation of synapsin I in rat cerebral cortex synaptosomes after 120 min. Synapsin I was phosphorylated and isolated and ^{32}P incorporation was quantitated as described in the text. ●, controls; ○, bilirubin treated. $*p < 0.05$. Degree of phosphorylation given as percentage of controls with 0.1 μM BSA.

in phosphorylation caused by bilirubin was also time dependent, with a significant loss of ^{32}P content observed after 60 min of incubation with 160 μM bilirubin in unstimulated synaptosomes (Fig. 3*A*), and after 120 min in synaptosomes which had been depolarized with KCl 50 mM (final concentration) for 30 s before stopping the reaction (Fig. 3*B*). BSA alone had no significant effect on synapsin I phosphorylation.

Identical experiments with biliverdin, a chemically related pyrrole compound differing from bilirubin in the absence of a hydrogen atom and the corresponding presence of a double binding at the γ position of the molecule, did not lead to changes in the phosphorylation of synapsin I (data not shown).

Inasmuch as the 80/86 kD region of 1-dimensional SDS-PAGE gels derived from synaptosomes also contains proteins other than synapsin I (18), and because synapsin I can be phosphorylated on more than one site (17, 19), we decided to specifically analyze the effect of bilirubin on the distinct sites of phosphorylation of the synapsin I molecule. To this end we used the incomplete proteolytic digestion technique developed by Cleveland *et al.* (20). This technique, when using the *S. aureus* V8 protease, separates site II, which is phosphorylated by Ca/calmodulin-dependent kinase II and is located in the "tail region" of the molecule, from site I, which is phosphorylated by cAMP-dependent kinase and Ca/calmodulin-dependent kinase I, and is located in the "head region" of the molecule (17, 21).

Separate analysis of these phosphorylation sites showed that bilirubin significantly decreased phosphorylation of both sites in a dose- and time-dependent manner. For example, when synaptosomes were exposed to bilirubin 160 μM/BSA 16 μM, both site I and site II phosphorylation were significantly lower than in the controls ($p < 0.05$), at 60 min (not shown) and 120 min, in the unstimulated and in the KCl-depolarized preparations (Fig. 4). Significant reductions in unstimulated phosphorylation were observed from bilirubin concentrations of 10 and 5 μmol/liter for site I and II, respectively, (data not shown).

A final set of experiments was designed to study whether the inhibitory effect of bilirubin on protein phosphorylation was reversible. Synaptosomes were treated with bilirubin/BSA for 60 min, collected by centrifugation, and incubation was then continued for another 60 min, either in bilirubin/BSA or BSA solution. A reference level of phosphorylation was established by incubating synaptosomes for 60 min with BSA alone, without preceding exposure to bilirubin. In these experiments we observed that the rate of decrease of ^{32}P incorporation in bilirubin-treated synaptosomes was reduced on removal of extracellular bilirubin (Fig. 5). Thus, when bilirubin begins to diffuse out of the nerve terminals, phosphorylation of synapsin I appears to recover. This indicates that the effect of bilirubin is at least partly reversible.

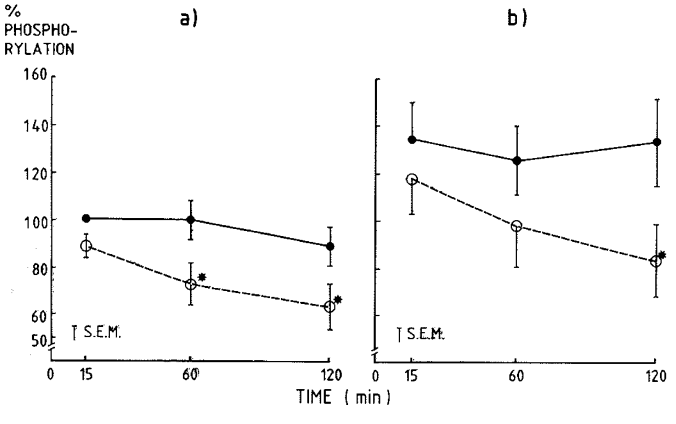

Fig. 3. Effect of increasing incubation time with bilirubin on phosphorylation of synapsin I in rat cerebral cortex synaptosomes. Synapsin I was phosphorylated and isolated and ^{32}P incorporation was quantitated as described in the text. ○, bilirubin (160 μM)/BSA (16 μM); ●, controls (BSA, 16 μM). *$p < 0.05$. Degree of phosphorylation given as percent of unstimulated controls at 15 min. *a*, unstimulated; *b*, depolarized with KCl 50 mM (final concentration) for 30 s before stopping the reaction.

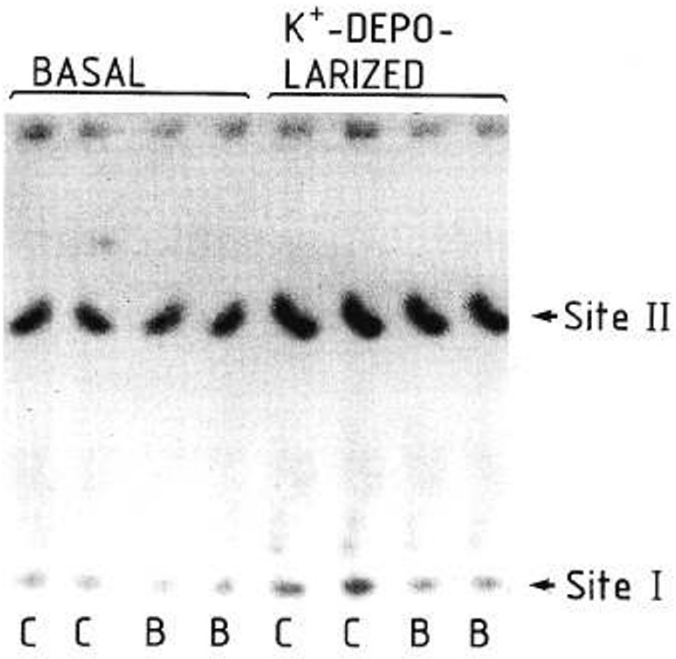

Fig. 4. Autoradiogram showing effect of bilirubin 160 μM for 120 min on phosphorylation of site I and site II of synapsin I in rat cerebral cortex synaptosomes. Synapsin I was phosphorylated and isolated by SDS-PAGE, cut out from the gels after autoradiographic visualization, and subjected to partial proteolysis by *S. aureus* V8 protease (as described in the text). The fragments containing phosphorylation sites I or II were then separated by SDS-PAGE and visualized by autoradiography. *B*, bilirubin incubated; *C*, controls (exposed to 16 μM BSA); *K⁺-depolarization*, KCl 50 mM final concentration added 30 s before termination.

DISCUSSION

Herein we show that bilirubin exerts an inhibitory effect on the incorporation of radioactive phosphate into the neuronal phosphoprotein synapsin I in rat cortex synaptosomes *in vitro*. These results point to several possible targets for bilirubin toxicity in mammalian brain.

First, our data show that the presynaptic terminals are affected by bilirubin. It is well established that synaptosomes, *in vitro* preparations of "pinched-off" nerve terminals, represent good models for the study of physiological processes in nerve terminals (22, 23), and as such might be used for bilirubin studies. Adding to this specificity is the highly restricted distribution of synapsin I, which appears highly enriched only in nerve terminals in association with small, clear synaptic vesicles (24). Recent results, which implicate the phosphorylation of this protein in the regulation of transmitter release (10), further suggest that bilirubin, *inter alia*, may interfere with this particular nerve terminal function.

Use of an *in vitro* preparation, which avoids several problems of *in vivo* studies on bilirubin toxicity, has also allowed us to partly characterize the bilirubin effect regarding dose response, time dependency, and specificity. The significant effects of micromolar bilirubin (but not biliverdin, its immediate precursor) observed after 1–2 h incubation are in general agreement with the range of brain bilirubin achieved in animal experiments. Thus, moderate hyperbilirubinemia in the young healthy rat gives brain bilirubin concentrations of 2–3 μM (25, 26), the use

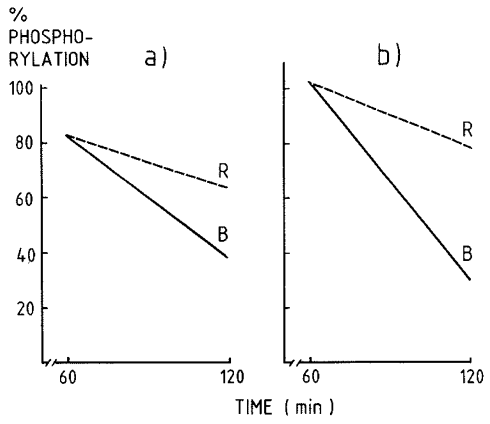

Fig. 5. Effect of removal of extracellular bilirubin on synapsin I phosphorylation in rat cerebral cortex synaptosomes. Synaptosomes, prelabeled with $^{32}P_i$ as described in the text, and incubated with 160 μM bilirubin/16 μM BSA for 60 min, were collected by centrifugation and resuspended in fresh buffer containing 16 μM BSA (*R*) or 160 μM bilirubin/16 μM BSA (*B*). Incubation was continued for another 60 min, after which synapsin I was isolated and ^{32}P incorporation analyzed as described in the text. *a*, standard buffer composition. *b*, 50 mM KCl (final concentration) added 30 s before termination of incubation, to induce Ca^{++} influx into the nerve terminals. The reference level of phosphorylation (100%) was established by incubating synaptosomes with BSA alone for 60 min without preceding exposure to bilirubin.

of a binding competitor can increase brain bilirubin to 10 μM (25), while opening of the blood-brain barrier can give brain bilirubin concentrations in the 50–75 μM range (27). These values refer to extractions of bilirubin from whole brain. Moreover, regional differences in brain bilirubin concentration have also been demonstrated (28, 29), and it is possible that differences in bilirubin concentrations between groups of cells may contribute to the observed difference in sensitivity to bilirubin toxicity (30). Bilirubin enters the brain quite rapidly, and the bilirubin concentration in the brain has been shown to peak 1 h after administration of a bolus dose of bilirubin in animals with opened blood-brain barriers (31). Studies using neuroblastoma (N115) cell cultures have shown that uptake of bilirubin in that system, which probably represents a passive process, is concentration dependent, peaks by 3 h, and is relatively insensitive to temperature (32). The time course observed in our experiments suggests that nerve terminals may be permeated by bilirubin by the same mechanism.

The mechanism by which bilirubin influences synapsin I phosphorylation is not clear. Our studies indicate that several distinct phosphorylation sites on the protein molecule, which are regulated by distinct Ca^{++}-dependent or cyclic AMP-dependent protein kinases, respectively (17, 19), were affected by bilirubin. This suggests that bilirubin might have a pervasive effect on protein phosphorylation in synaptosomes. Other workers have also suggested that protein phosphorylation systems in the brain might be influenced by bilirubin. Analysis of histone kinase activities in extracts from jaundiced rabbit brains showed the presence of a cyclic AMP-dependent activity that appeared to be unchanged, while a cyclic AMP-independent activity was reported to be bilirubin sensitive (33). However, the physiological significance of these observations remains uncertain.

Bilirubin inhibits oxidative phosphorylation *in vitro* (34, 35), but the results of *in vivo* experiments are contradictory (36, 37). Effects on membrane permeability have also been reported (38–40). Several enzymes are inhibited by bilirubin (cf. Ref. 41 for review). Therefore, the effect induced by bilirubin may have multiple causes. However, preliminary *in vitro* studies using purified preparations of synapsin I and the catalytic subunit of cAMP-dependent kinase (not shown), indicate that bilirubin may have a direct influence on the phosphorylation of synapsin I, independently of the presence of intact cells or respiratory activity. Such effects, together with possible effects on oxidative phosphorylation or membrane transport, may contribute to the results found herein.

Regardless of the mechanism involved, the significant and reproducible decrease of synapsin I phosphorylation shows a novel effect of bilirubin in the nervous system, an effect that may be involved in the regulation of neurotransmitter release. Future studies in this area may be of interest in relation to the clinical syndrome of lethargy and neurophysiological changes in jaundiced newborns.

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