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Bilirubin Induces a Calcium-Dependent Inhibition of Multifunctional Ca^{2+}/Calmodulin-Dependent Kinase II Activity *in Vitro*

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ABSTRACT

Excessive bilirubin levels in newborn infants result in long-term neurologic deficits that remain after bilirubin levels return to normal. Much of the observed neurologic deficits can be attributed to bilirubin-induced, delayed neuronal cell death. Inhibition of calcium/calmodulin-dependent kinase II (CaM kinase II) activity that precedes cell death is observed in conditions such as seizure activity, stroke, and glutamate excitotoxicity. Because neonatal bilirubin exposure results in neuronal loss in developing brain systems, we tested whether bilirubin exposure would induce an immediate inhibition of CaM kinase II activity, *in vitro*. P-81 filtration assay of basal and calcium-stimulated kinase activity was performed under standard kinase assay conditions. Bilirubin and/or albumin was added to the reaction vessels to determine the effect of these agents on kinase activity. Bilirubin exposure resulted in a concentration-dependent inhibition of CaM kinase II activity (IC_{50} = 16.78 μM). At concentrations above 50 μM, bilirubin exposure resulted in a 71 ± 8% (mean ± SD) inhibition of kinase activity ($p < 0.001$, t test, $n = 10$). Bilirubin exposure did not result in kinase inhibition if excessive bilirubin was removed by albumin binding before stimulation of kinase activity (106.9 ± 9.6% control activity, $n = 5$). However, removal of bilirubin by binding with albumin after calcium addition did not restore kinase activity. (36.1 ± 3.8% control activity, $n = 5$). Thus, once inhibition was observed, the activity could not be restored by addition of albumin. The data suggest that bilirubin exposure resulted in a calcium-dependent inhibition of CaM kinase II activity that, once induced, was not reversible by removing bilirubin by the addition of albumin. Because inhibition of CaM kinase II activity has been correlated with delayed neuronal cell death in many neuropathologic conditions, bilirubin-induced inhibition of this enzyme may be a cellular mechanism by which bilirubin exposure results in delayed neuronal cell death in developing brain. (***Pediatr Res* 38: 949–954, 1995**)

Abbreviation

CaM kinase II, calcium/calmodulin-dependent kinase II

The neurotoxicity of bilirubin, especially in neonates, is well established (1, 2), and severe neonatal jaundice has been associated with significant neurologic sequelae. Bilirubin has been shown to depress mitochondrial function by interfering with oxidative phosphorylation (1, 2) and disrupting membrane function (3). However, mitochondrial effects alone cannot account for all of the neurotoxic effects of bilirubin (1). Bilirubin has been shown to modulate neuronal second messenger systems that control neurotransmission (4, 5) and synaptic activity (3). Bilirubin exposure has been shown to alter synapsin phosphorylation at both the calcium-dependent and cAMP-dependent phosphorylation site (5). Therefore, characterization of bilirubin effects on neuronal phosphorylation systems may help to elucidate some of the neurotoxic effects of bilirubin.

Multifunctional CaM kinase II is an important second messenger system that regulates many neuronally important functions. CaM kinase II modulates neurotransmitter synthesis and release (6–9), receptor-gated ion channels (10, 11) calcium-dependent ion currents (12, 13), and neuroskeletal elements (14, 15). Therefore, inhibition of this enzyme would have significant effects on neuronal function. Inhibition of CaM kinase II activity has been studied in multiple models of delayed neuronal cell death (16–18), and altered neuronal function such as kindling and epilepsy (19–22). Thus, characterizing the acute and chronic effect of bilirubin exposure on CaM kinase II activity would provide insight into the cellular mechanisms of bilirubin neurotoxicity.

In this report, *in vitro* characterization of bilirubin exposure on CaM kinase II activity was performed. CaM kinase II enriched from rat forebrain was exposed to bilirubin and tested

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for enzymatic activity. In some experiments, human serum albumin was also added to determine whether or not bilirubin toxicity was reversible. The results demonstrate that bilirubin exposure results in an irreversible, calcium-dependent inhibition of CaM kinase II activity. Because inhibition of CaM kinase II activity has been correlated with delayed neuronal cell death and/or altered neuronal function in many neuropathologic conditions, the observations are consistent with the hypothesis that bilirubin-induced inhibition of CaM kinase II may be a cellular mechanism by which bilirubin exposure results in neurotoxicity.

METHODS

Kinase activity quantitation. CaM kinase II was purified from rat forebrain by the method of Goldenring *et al.* as described in detail by Churn *et al.* (23). Briefly, forebrain homogenates were subjected to 100,000 × *g* centrifugation for 1 h. The supernatant was then passed through a series of ion exchange, calmodulin affinity, and Sephacryl 300 molecular weight sieving columns. The resultant kinase-enriched fraction was used for bilirubin toxicity assays.

To determine the effect of bilirubin on CaM kinase II activity, standard autophosphorylation reactions (23) were performed in the presence or absence of bilirubin and albumin. Standard autophosphorylation reaction solutions contained 0.8 μg of protein, 10 mM $MgCl_2$, 7 μM [γ-^{32}P]ATP, 10 mM 1,4-piperazinediethanesulfonic acid (PIPES), pH 7.4, ±5 mM $CaCl_2$, and ±1 μg of calmodulin. Standard reactions were performed in a shaking water bath at 30°C. Reactions were initiated by the addition of calcium, continued for 1 min, and terminated by the addition of 5% SDS stop solution (23). Proteins were resolved by SDS-PAGE, and protein bands were visualized as described previously (23). Stained gels were dried and exposed to x-ray film (XRP-1, Kodak) for autoradiography. The autoradiograph was then used as a template for excising radioactive phosphoproteins for quantitation in a liquid scintillation spectrometer (model LS 2800, Beckman, Fullerton, CA), with a counting efficiency of 80% (17, 23). In some experiments, computer-assisted densitometry was used (Mocha; Jandel Corp., San Rafael, CA), as previously described (23). Inhibition of CaM kinase II activity was analyzed by *t* test. For multiple comparisons, inhibition of kinase activity was analyzed by Bonferroni-corrected, one way analysis of variance (Graphpad, San Diego, CA).

In addition to standard autophosphorylation reactions, CaM kinase-dependent substrate phosphorylation was examined. Substrate phosphorylation was performed by a modification of the method of Hashimoto and Soderling (24) as previously described (25). For kinase activity, standard reactions were performed as described above, except that 60 μM Syntide II (Sigma Chemical Co., St. Louis, MO) was included in the mixture. This concentration of Syntide II was found to provide maximal phosphate incorporation under standard conditions (25). The phosphorylation reaction was initiated by the addition of Ca^{2+}, allowed to continue for 1 min, and stopped by the addition of 20 μM EGTA. An aliquot of 10 μL of the assay solution was immediately blotted onto phosphocellulose filter paper, P-81 (Whatman, Maidstone, England) as described elsewhere (24, 25). Each reaction was quantitated in triplicate. P-81 filter paper was then washed three times in 50 mM phosphoric acid, rinsed with acetone, and allowed to air dry. Radioactive phosphate was quantitated by scintillation counting as described above.

Albumin reversal methods. To determine the Ca^{2+} dependence of bilirubin exposure-induced inhibition of CaM kinase II activity, human serum albumin (Sigma) was added to the kinase reaction mixture either before or after the addition of Ca^{2+}. For Ca^{2+}-independent inhibition studies, bilirubin was added to the kinase reaction mixture and allowed to react for 20 min on ice. The bilirubin-kinase mixture was equilibrated at 0°C to reduce any loss of kinase activity due to incubation at higher temperatures (23). For Ca^{2+} independence of bilirubin toxicity studies, human serum albumin (60 μM) was added to the reaction vessel, and the vessel was transferred to the reaction bath (30°C). The mixture was allowed to equilibrate at 30°C for 60 s at which time Ca^{2+} was added, and the mixture was allowed to equilibrate for another 60 s. To initiate the kinase reaction, ATP was added to the mixture, and standard kinase reactions were performed as described above. For Ca^{2+} dependence of bilirubin-induced inhibition of kinase activity, kinase reactions were performed as described above, except that the order of addition was altered. The bilirubin-kinase mixture was equilibrated for 20 min on ice as described above. Ca^{2+} was added to the reaction vessel, and the vessel was transferred to the reaction water bath (30°C) and allowed to react for 60 s. Human albumin was then added, and the kinase reaction was initiated by the addition of ATP (7 μM). The reaction was continued for 60 s and terminated by solubilizing the proteins as described above. Kinase activity from both reaction groups was compared with sham reactions (no bilirubin added).

RESULTS

Standard kinase autophosphorylation reactions demonstrated a significant calcium-dependent increase in phosphate incorporation into the α (50 kD) and β (60 kD) subunits of CaM kinase II (Fig. 1). The calcium-dependent activity was significantly reduced when kinase was reacted in the presence of 50 μM bilirubin. Maximal kinase activity was 1981.76 ± 157.05 fmol/min in the absence of bilirubin and 558.86 ± 18.73 fmol/min when reacted in the presence of bilirubin ($p < 0.001$, *t* test, $n = 6$). Thus, bilirubin exposure resulted in a significant inhibition of kinase activity.

To further evaluate the effect of bilirubin on kinase activity, substrate phosphorylation reactions, using P-81 quantitation of phosphate incorporation into Syntide II, were performed. Syntide II is a synthetic peptide that corresponds to the consensus phosphorylation sequence of CaM kinase II (24). Previous characterization of Syntide II phosphorylation demonstrated that maximal substrate phosphorylation was obtained with 60 μM Syntide II (data not shown) under standard kinase reactions (see "Methods"). Coincubation of kinase with 50 μM bilirubin resulted in a 71.8 ± 8% inhibition of kinase activity ($p < 0.001$, *t* test, $n = 10$). The observed inhibition of kinase

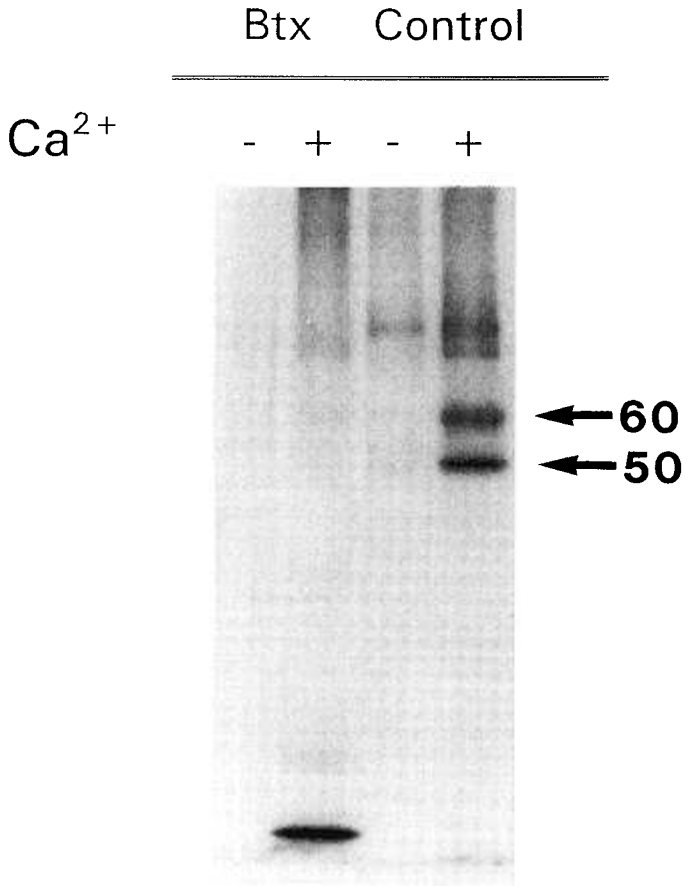

Figure 1. Bilirubin toxicity (*Btx*) resulted in decreased CaM kinase II activity when measured by autophosphorylation. Autoradiograph from SDS-PAGE of CaM kinase II exposed to either bilirubin (*lanes 1* and *2*) or control buffer (*lanes 3* and *4*). Control kinase displayed a Ca^{2+}-dependent increase in phosphate incorporation into the 50- and 60-kD protein bands. Bilirubin exposure resulted in significant inhibition of the Ca^{2+}-dependent activity (1981.76 ± 157.05, control *vs* 558.85 ± 18.73, experimental; $p < 0.001$, *t* test, $n = 6$).

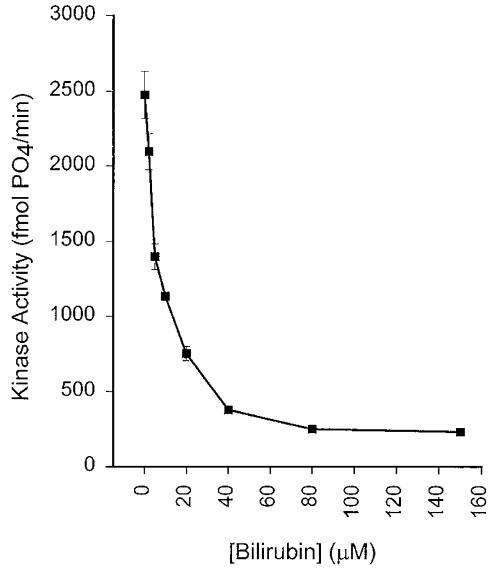

Figure 2. Bilirubin exposure resulted in a concentration-dependent inhibition of CaM kinase II activity. Maximum CaM kinase II-dependent substrate (Syntide II) phosphorylation was inhibited by co-incubation with bilirubin in a concentration-dependent manner. Bilirubin displayed an apparent IC_{50} of 16 μM for kinase inhibition. At 50 μM, bilirubin exposure resulted in a 71.8 + 8% inhibition of Syntide II phosphorylation ($p < 0.001$ *t* test, $n = 10$).

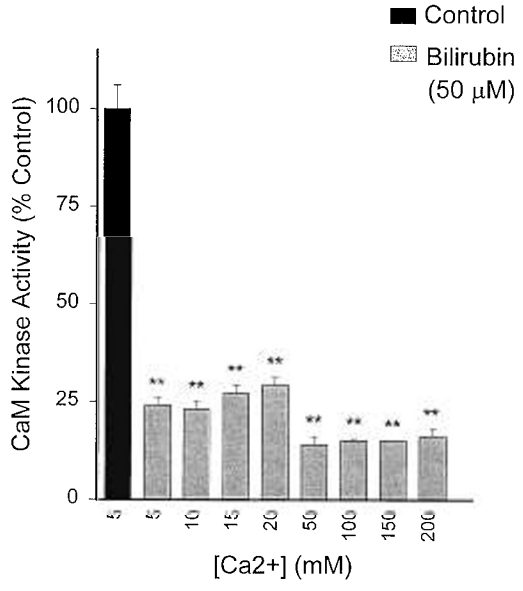

Figure 3. Bilirubin-induced inhibition of CaM kinase II activity could not be overcome by increasing Ca^{2+} concentration. CaM kinase II dependent phosphorylation of Syntide II was determined in the presence of 50 μM bilirubin and increasing concentrations of Ca^{2+}. The bilirubin-induced inhibition could not be overcome by increasing Ca^{2+} to 40 times the maximal control levels of 5 mM. Thus, the bilirubin-induced inhibition of CaM kinase II activity was not due to bilirubin binding to Ca^{2+} and removing this essential component from stimulating kinase activity. $^{**}p < 0.001$.

was dependent on the concentration of bilirubin in the reaction vessel (Fig. 2) with an IC_{50} for bilirubin of 16.78 μM (Fig. 2). Thus, coincubation of CaM kinase II with bilirubin resulted in a concentration-dependent inhibition of kinase activity when measured by autophosphorylation of kinase subunits and by phosphorylation of a exogenously added synthetic kinase substrate.

To determine the mechanism for decreased kinase activity in the presence of bilirubin, standard phosphorylation reactions were performed, and specific parameters of reaction constituents were varied (Fig. 3). Bilirubin-dependent inhibition of kinase activity could not be overcome by increasing the concentration of ATP, Ca^{2+}, substrate, or calmodulin. For instance, increasing the Ca^{2+} concentration to 40 times the optimal concentration for control CaM kinase II activity (23, 26), did not restore Ca^{2+}-dependent activity in the presence of bilirubin (Fig. 3). In addition, coincubation of the kinase-bilirubin mixture did not alter the apparent K_M values for Ca^{2+}, or the time course of the reaction (data not shown). However, bilirubin exposure altered the apparent affinity of CaM kinase II for ATP (Fig. 4). Control kinase displayed an apparent K_M for ATP of 8.4 μM which was similar to that reported for rat kinase when measured by autophosphorylation (26). Coincubation of kinase with bilirubin decreased the apparent affinity to 19.9 μM. Thus, coincubation of the reaction mixture with bilirubin did not remove any of the above constituents and did not prevent them from being used in the phosphorylation reaction. In addition, bilirubin exposure induced a selective alteration in kinase affinity for ATP, without significantly affecting other parameters of kinase activity.

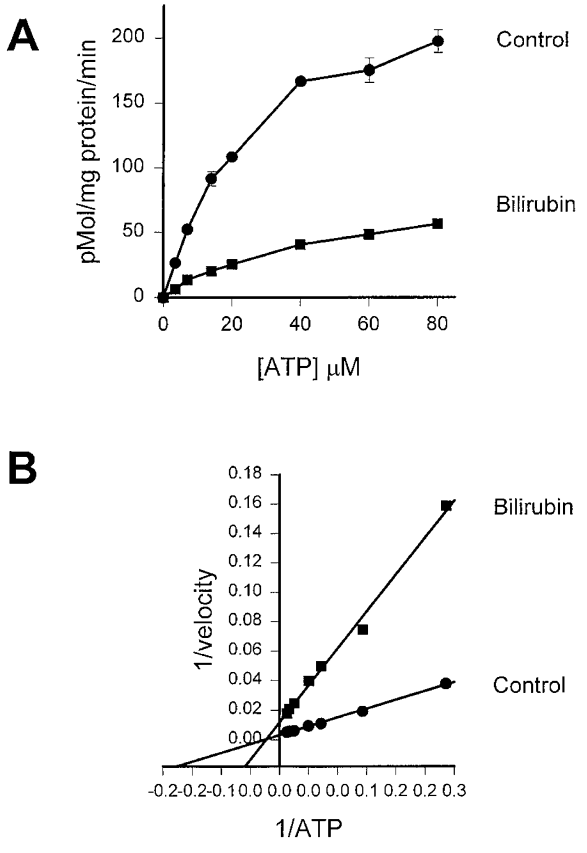

Figure 4. Bilirubin exposure resulted in altered apparent ATP binding kinetics of CaM kinase II. (*A*) Bilirubin-induced inhibition could not be overcome by increasing ATP concentration. CaM kinase II displayed a V_{max} of 321.54 pmol/μg protein/min. Coincubation with bilirubin significantly reduced maximal kinase activity to 85.47 pmol/μg protein/min. (*B*) Double reciprocal plots showing bilirubin exposure resulted in an alteration of apparent ATP binding kinetics from 8.4 μM (control) to 19.9 μM in bilirubin-treated kinase.

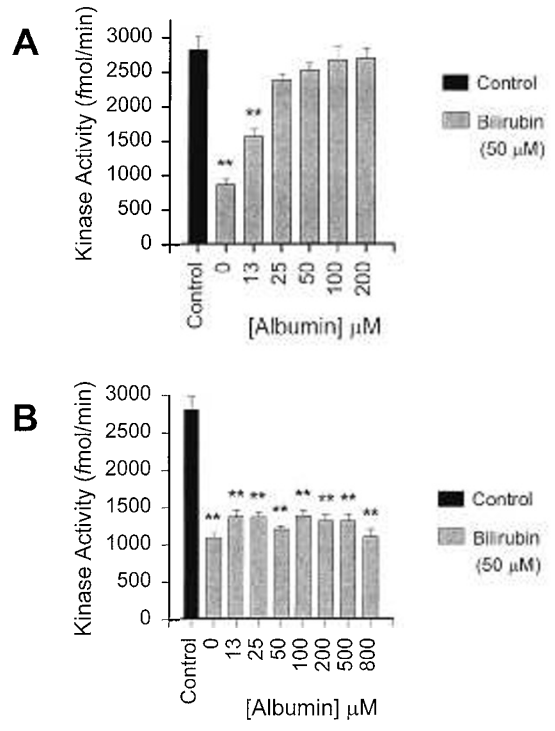

Figure 5. Bilirubin-induced inhibition of CaM kinase II activity was dependent upon Ca^{2+}. (*A*) Removal of bilirubin before Ca^{2+}-dependent activation of CaM kinase II resulted in recovery of enzyme activity. (*B*) Removal of bilirubin after Ca^{2+}-dependent activation of CaM kinase II did not result in recovery of enzyme activity. $^{**}p < 0.001$.

To determine whether CaM kinase II inhibition due to bilirubin exposure was reversible, coincubation experiments were performed in the presence or absence of albumin (Fig. 5). Coincubation of the kinase-bilirubin mixture with albumin before the addition of calcium resulted in a preservation of kinase activity (106.0 ± 9.6% of control, $n = 5$). However, if the albumin was added after activation of the kinase with calcium, removal of bilirubin did not restore kinase activity (36.1 ± 3.8% of control, $n = 5$). Thus, coincubation of activated kinase with bilirubin resulted in an irreversible inhibition of CaM kinase II activity, whereas binding of bilirubin to albumin, before activation of CaM kinase II, did not result in inhibition of enzymatic activity.

DISCUSSION

In this report, *in vitro* analysis of the effect of bilirubin exposure on CaM kinase II activity was examined. Acute exposure of CaM kinase II to bilirubin resulted in a significant inhibition of CaM kinase II activity. Once induced, the inhibition of kinase activity was not reversible by removing the bilirubin with excess albumin. However, the bilirubin exposure-induced inhibition of CaM kinase II activity could be reversed if the bilirubin was removed by the addition of excess albumin before calcium-dependent activation of CaM kinase II. The observation that bilirubin exposure results in an irreversible, Ca^{2+}-dependent inhibition of kinase activity provides a mechanism whereby bilirubin may exert its neurotoxic effects *in vivo*.

This report demonstrates that bilirubin has a direct inhibitory effect on CaM kinase II. The study agrees with earlier studies showing decreased synapsin I phosphorylation after bilirubin exposure (5). In the present study, the observed inhibition was a direct effect of bilirubin exposure on the kinase and was not due to sequestering any of the constituents of the reaction mixture. In addition bilirubin exposure selectively altered the kinase's apparent affinity for ATP without significantly altering the enzyme's affinity for other parameters necessary for maximal kinase activity. A similar change in apparent affinity for ATP has been reported in whole animal ischemia (23, 27). The ischemia-induced posttranslational modification of CaM kinase II that results in alteration of ATP binding may be due to the addition of an inhibitor moiety that can be removed by SDS-PAGE and guanidine-HCl denaturation (28). Therefore, bilirubin exposure may result in inhibition of kinase activity by directly binding to either the ATP binding site of the kinase or to some regulatory site for ATP binding. Because bilirubin exposure results in an inhibition similar to that observed in ischemia, it is tempting to speculate that bilirubin neurotoxicity may occur through inhibition of CaM kinase II. The bilirubin exposure-induced altered ATP recognition would significantly reduce neuronal ability to regulate important neuronal functions, especially at times of high metabolic demand, when

cellular ATP levels may be reduced in specific subcellular regions. This may be one mechanism whereby chronic bilirubin exposure induces delayed neuronal cell death in neonates.

If CaM kinase II is exposed to bilirubin in the unstimulated state (basal activity), no inhibition is observed. The enzyme activity was recoverable if the bilirubin was bound by albumin, and thus functionally removed from the reaction, before Ca^{2+}-dependent activation of the enzyme. Therefore, the data support the hypothesis that activation of CaM kinase II is necessary for the bilirubin-induced inhibition of the enzyme. In addition, the data show that adding excess albumin does not bind up Ca^{2+} necessary for stimulation of kinase activity. Furthermore, once established, the bilirubin-induced inhibition of CaM kinase II activity was not reversible by simple removal of bilirubin from the reaction mixture by the addition of albumin. Thus, the data support the hypothesis that bilirubin exposure results in a Ca^{2+}-dependent inhibition of CaM kinase II activity that is observed when measured by autophosphorylation or exogenously added substrate phosphorylation.

It is interesting to speculate about the mechanisms involved in bilirubin-induced inhibition of kinase activity. The simplest explanation of bilirubin-induced inhibition of kinase activity would be that bilirubin blocks Ca^{2+} stimulation of the enzyme. However, the present study provides data to argue for a more complex mechanism. First, bilirubin-induced inhibition could not be overcome by adding excessive Ca^{2+} to the reaction. Second, if bilirubin is removed from the reaction before addition of Ca^{2+}, then no inhibition is observed. However, if kinase is exposed to both bilirubin and Ca^{2+}, then irreversible inhibition of enzyme activity is observed. Third, the only kinetic parameter that is significantly affected by bilirubin exposure is ATP binding kinetics. Therefore, the data from the present study suggests that bilirubin exposure affects the ability of kinase to recognize ATP. The mechanism by which bilirubin exposure produces this inhibition cannot be determined in the present study. However, the data suggest that Ca^{2+}/calmodulin-dependent stimulation of CaM kinase II may open up the enzyme and expose a bilirubin binding site at or near the ATP binding site. Bilirubin binding to this site may be very tight, because adding excessive amounts of albumin do not remove bilirubin from this site. Thus, the data support the hypothesis that bilirubin exposure results in inhibition of CaM kinase II activity in a Ca^{2+}-dependent manner and that once induced, the inhibition is irreversible.

CaM kinase II has been shown to be involved in the regulation of neurotransmission (7–9), receptor-gated ion channels (10, 11), and Ca^{2+}-dependent ion currents (12, 13), and alterations of CaM kinase II activity have been associated with modulation of membrane excitability (19–22). In addition, inhibition of CaM kinase II activity correlates with delayed neuronal cell death in glutamate-induced neuroexcitotoxicity (18, 25), global forebrain ischemia (17, 29, 30), and focal cerebral ischemia (31). Thus, bilirubin exposure-induced inhibition of CaM kinase II activity would be expected to modulate many neuronal functions. Exposure of neurons to bilirubin results in decreased synaptic activity (3, 4), decreased synapsin I phosphorylation (5) and in, severe cases, delayed neuronal cell death (1, 2). Therefore, it is interesting to speculate that some of the *in vivo* and *in vitro* neurotoxic effects of bilirubin may be due to inhibition of CaM kinase II activity.

To establish any causal effects of bilirubin-induced kinase inhibition and bilirubin-induced delayed neuronal cell death, *in vivo* models of bilirubin toxicity must be used. The Gunn rat model (32) is an excellent whole animal model to study bilirubin toxicity (33–35), bilirubin-induced alterations in neuronal physiology (3, 36–38), and biochemical alterations (39–41). Future studies will be directed toward correlating bilirubin-induced alterations in all three attributes mentioned above.

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