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Bilirubin facilitates depolarizing GABA/glycinergic synaptic transmission in the ventral cochlear nucleus of rats

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ABSTRACT

Excitotoxicity contributes to bilirubin-induced central nervous system injury; however, the mechanisms involved remain controversial. Previous studies from our lab have demonstrated that in juvenile rats bilirubin faciliates γ-aminobutyric acid (GABA)/glycinergic synaptic transmission through activation of presynaptic protein kinase A (PKA) in isolated neurons of the ventral cochlear nucleus (VCN). However, the descending mechanism and physiological effects of bilirubin-induced potentiation remain unclear. Here, whole-cell recordings show that 3×10^{-6} M bilirubin increased the frequency of both spontaneous (sPSCs) and miniature (mPSCs) GABA/glycinergic postsynaptic currents in VCN neurons of postnatal day 12–14 (P12–14) rats. This action was dependent on the concentration and duration of exposure to bilirubin and was only partially suppressed by 10^{-5} M bicuculline. The potentiation effect on mPSCs persisted in a Ca^{2+}-free solution, but was fully occluded by pretreatment with 1,2 bis-(2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), an intracellular Ca^{2+} chelator. Following pretreatment of the neurons with BAPTA-AM, forskolin, a PKA activator, had no effect on the frequency or amplitude of mPSCs. This suggests that Ca^{2+} release from presynaptic stores is part of the descending pathway of PKA activation and is responsible for biliurbin-induced potentiation of cell activity. Using gramicidin-perforated patch recordings, the reversal potential of GABA-evoked currents (E_{GABA}) was also investigated. The GABA response resulted in depolarization of 12 of 20 recorded VCN neurons from P12–14 rats. Therefore, potentiation of depolarizing GABA/glycinergic transmission by bilirubin may underlie bilirubin excitotoxicity, which may play a role in the hearing impairment observed among hyperbilirubinemic neonates.

1. Introduction

Bilirubin encephalopathy is a neurotoxic syndrome attributable to the deposition of unconjugated bilirubin in selective brain areas during neonatal hyperbilirubinemia (Hansen, 2001; Ostrow et al., 2003). Bilirubin-induced damage to sites known to be susceptible to this influence, such as the inferior colliculus, globus pallidus, oculomotor nuclei, subthalamic nucleus, hippocampus, substantia nigra reticulata, lateral superior olivary nuclei, especially the ventral cochlear nuclei (VCN), results in temporary or permanent impairment of auditory, motor, and other disorders of brain functions (Gourley, 1997; Shapiro, 2010). Bilirubin toxicity remains a significant problem in clinical populations (Shapiro, 2003).

Excitotoxicity is a primary component of bilirubin-induced central neural system injury (Ostrow et al., 2004; Watchko, 2006), but the underlying mechanism of action has yet to be clarified. Previous studies have yielded conflicting evidence regarding whether glutamatergic transmission is a direct cause of bilirubin-induced excitotoxicity (Grojean et al., 2000, 2001; Shapiro et al., 2007; Warr et al., 2000). As a result, further investigation is necessary to determine alternative mechanisms by which bilirubin-induced excitotoxicity might be expressed.

Our previous studies have demonstrated that bilirubin facilitates GABA/glycinergic synaptic transmission in the VCN neurons of juvenile rats through activation of the presynaptic protein kinase A (PKA) signal transduction pathway (Li et al., 2010). However, the mechanism by which bilirubin influences presynaptic activity and the subsequent physiological effects remains unclear. An increase in presynaptic intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is the principal signal in regulated exocytosis, and an elevation of $[Ca^{2+}]_i$ in nerve endings causes an increase in transmitter release probability (Ivanov and Calabrese, 2003). PKA signal transduction has been shown to play an important role in $[Ca^{2+}]_i$ regulation (Bugrim, 1999; Zirpel et al., 1998) as well as the Ca^{2+}-dependent regulation of exocytosis in several types of neurons (Evans and Morgan, 2003; Sedej et al., 2005; Takahashi et al., 1999). Thus, it is possible that Ca^{2+} is a critical factor in

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bilirubin-induced potentiation of cell activity and may be responsible for the PKA-activating mechanism underlying bilirubin action.

The GABA/glycinergic receptor, traditionally thought to be inhibitory, can evoke membrane depolarization and play an excitatory role in a wide range of immature neurons due to an inverted chloride (Cl^-) gradient (Chen et al., 1996; Kakazu et al., 1999; Rohrbough and Spitzer, 1996; Ye, 2008). In such neurons, GABA-evoked currents (E_{GABA}) are relatively more depolarized than the resting membrane potential and the action potential threshold (Kandler et al., 2002). Considering that the role of the GABA/glycinergic receptor in neuronal excitation has not been investigated in VCN neurons of juvenile rats before, a clarification of GABA/glycine action is important for the understanding of the physiological effects of bilirubin action.

In the present study, the specific molecular mechanism supporting the bilirubin-potentiation effect on GABA/glycinergic synaptic transmission was investigated in the VCN neurons of juvenile rats aged between postnatal day 12 (P12) and postnatal day 14 (P14). This is a period in juvenile rats in which physiological hearing has begun (Grecova et al., 2009; Sun et al., 2008). The GABA/glycine action was also identified to clarify the possible correlation between billirubin acivity and neuronal hyperexcitation.

2. Materials and methods

Male and female Sprague–Dawley rats aged between P12 and P14 were included in this expriment. All experimental protocols complied with the guiding principles for the care and use of animals approved by the Ethics Review Committee for Animal Experimentation of Shanghai Jiaotong University. Every effort was made to minimize the suffering of the animals.

2.1. Neuron preparation

Each subject was decapitated under pentobarbital anesthesia (55 mg/kg, i.p.), and the brain was removed quickly and transversely dissected at a thickness of 300 μm using a microslicer. Slices containing the VCN were kept in an incubation solution (see Solution) at room temperature (21–26 °C) for at least 20–30 min. Slices were then transferred to Petri dishes (Primaria 3801; Becton Dickinson, Rutherford, NJ) filled with standard solution or with a solution free of extracellular Ca^{2+} ($[Ca^{2+}]_o$; see Solutions). The region of the VCN to be dissected was identified using a dissecting scope (XTL-2400; SOIEC, Shanghai, China). Mechanical dissociation of the VCN was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at 50 Hz (0.1–0.2 mm). The tip of the fire-polished glass pipette was placed close to the surface of the VCN region identified for removal and vibrated horizontally for 2 min. The mechanically dissociated neurons were left for 10 min to allow adhesion to the bottom of the Petri dishes (Li et al., 2010; Shi et al., 2006). Neurons with remaining dendritic processes were used in the experiments presented herein.

2.2. Solutions

The incubation solution for the slices was saturated with 95% O_2 and 5% CO_2 and contained (in mM) 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 24 $NaHCO_3$, and 10 glucose. The standard external solution contained (in mM) 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 MgCl2, 10 glucose, and 10 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES). The $[Ca^{2+}]_o$- free external solution contained (in mM) 140 NaCl, 5 KCl, 6 $MgCl_2$, 2 ethylene glycol-bis (β-aminoethyl ethel)-N,N,N′,N′-tetraacetic acid (EGTA), 10 glucose, and 10 HEPES. The pH of these external solutions was adjusted to 7.4 with NaOH. Except when recording sPSCs, 3×10^{-7} M tetrodotoxin (TTX) was added to all external solutions. The ionic composition of the internal (patch pipette) solution contained (in mM) 92 CsCl, 50 Cs methanesulfonate, 5 TEA-Cl, 2 EGTA, 4 ATP-Mg, and 10 HEPES. The patch pipette solution for the gramicidin-perforated patch recording contained (in mM) 150 KCl and 10 HEPES. All pipette solutions were buffered to a pH of 7.2 using Tris–OH. Gramicidin was first dissolved in methanol to prepare a stock solution of 100 mg/ml and then diluted to a final concentration of 10 mg/ml in the pipette solutions (Kakazu et al., 1999). The gramicidin-containing solution was prepared just prior to use.

2.3. Electrical measurements

Membrane currents were recorded using a patch-clamp amplifier (EPC-10; HEKA, Germany). Patch pipettes were pulled from borosilicate capillary glass to have resistances of 6–8 M for whole-cell recordings and 5–7 M for gramicidin-perforated recordings through the use of two stages on a vertical pipette puller (P-9; Narishige, Tokyo, Japan). A whole-cell voltage clamp was used to record all postsynaptic currents (PSCs). Neurons were voltage clamped at a holding potential (V_H) of 60 mV, and electrode capacitance and liquid junction potential were compensated for. Neurons were visualized under phase contrast on an inverted microscope (TE-2000U; Nikon, Japan). All data were sampled at 3–10 kHz and filtered at 1–3 kHz using a Dell computer equipped with Pulse 6.0 (HEKA, Lambrecht, Germany). Gramicidin-perforated patch recording was used to determine E_{GABA}.

After recording the resting membrane potential in current clamp mode with zero current, the amplitude of the ionic current was activated by puffing 30 μM of GABA (10 s) onto isolated VCN neurons. Currents were measured at different holding potentials (−90–30 mV) in voltage-clamp mode, with a step of 30 mV. Application intervals lasted 60 s. For all experiments, series resistance compensation was used (75–80%). The gramicidin-perforated patch-clamp recording was confirmed by rupturing the neuronal membrane at the end of the experiment. All experiments were performed at room temperature (21–26 °C).

2.4. Drugs

The drugs used in the experiments included free bilirubin, bicuculline, strychnine, 1,2 bis-(2-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), GABA, and TTX (all from Sigma, St. Louis, MO). Bilirubin was dissolved in 0.1 M NaOH at 10^{-3} M as a stock solution, stored in single-use aliquots in the dark at −20 °C, and diluted into the final solution prior to application. Because bilirubin is light sensitive, all samples were protected from the light. To make stock solutions, BAPTA-AM and bicuculline were dissolved in dimethyl sulfoxide (DMSO) with a final DMSO concentration of ≤0.1%. TTX and strychnine were dissolved in distilled water. All drug-containing solutions were applied to the isolated neurons using a Y-tube method (Nabekura et al., 2004). The external solution could be completely exchanged within 20 msec using this method.

2.5. Data analysis

The MiniAnalysis Program (Synaptosoft, NJ, USA) was utilized for counting and analyzing GABA/glycinergic mPSCs and sPSCs. The currents were visually identified and accepted for further analysis if they exceeded a certain amplitude (≥5 pA) and area (>100 pA^2) in addition to exhibiting specific decay times (≥6.0 ms). The average values of the GABA/glycinergic frequencies and amplitudes during the control period were calculated and scaled to 1.0. All subsequent amplitudes and frequencies recorded during different experimental conditions and washes were normalized to these control values. To investigate the time course of changes in the GABA/glycinergic PSC parameters, the GABA/glycinergic PSCs were analyzed in 2-min bins. Differences in these PSC parameters were examined using Wilcoxon signed-ranks tests for comparison between groups. Values of $P<0.05$ were considered significant. Numerical values are provided as mean standard deviation (S.D.). To determine E_{GABA}, fitting of I–V relations