In vitro assessment of the effect of methylene blue on voltage-gated sodium channels and action potentials in rat hippocampal CA1 pyramidal neurons

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ABSTRACT

Methylene blue (MB) is a vital dye to allow better visualization and marker of parathyroid glands. The compound causes a toxic encephalopathy in clinical observations and some neuronal adverse effects in experimental studies. Of neurotoxic effects, reduced field excitatory postsynaptic potentials (fEPSPs) in hippocampal slice cultures and apoptosis induced in neurons by MB, suggest that MB may affect electrophysiological properties in neurons. Consequently, studies were undertaken to characterize the effects of MB on voltage-gated sodium currents ($I_{Na}$) in hippocampal CA1 neurons. MB was tested at a clinically-relevant concentration (10 $\mu$M), of which as a surgical marker of the parathyroid glands, and other concentrations (0.25 $\mu$M, 1 $\mu$M, and 100 $\mu$M). The results showed that MB reduced the amplitude of $I_{Na}$ at the concentrations of 10 $\mu$M and 100 $\mu$M. No significant changes were found with the other two concentrations of MB, 10 $\mu$M of MB did not produce a shift in the activation–voltage curve of $I_{Na}$ but produced a hyperpolarizing shift in the inactivation–voltage curve of $I_{Na}$ and delayed the recovery of $I_{Na}$ from inactivation. Action potential (AP) properties and the pattern of repetitive firing were examined using whole-cell current-clamp recordings. Peak amplitude, overshoot and maximum velocity of depolarization ($V_{max}$) of the evoked single AP decreased in the presence of the 10 $\mu$M MB solution. The rate of repetitive firing also decreased. The results suggest MB as a surgical marker of the parathyroid glands, may cause sodium channel inhibition on neurons in the nervous system.

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1. Introduction

Methylene blue (MB) is a phenothiazine compound; it represents a vital dye to allow better visualization of tissue structures. MB has been used to facilitate parathyroid surgery for over 30 years. When injected intravenously (preoperatively), MB (5–10 mg/kg) selectively accumulates in parathyroid glands (Derom et al., 1993; Meekin, 1998). Indeed, preoperative intravenous administration of MB is now a universally used practice in parathyroid gland surgery. The previous investigators have suggested the safety of this technique when MB was used as a marker of the parathyroid glands (5–10 mg/kg). An emerging number of clinical observations, however, have suggested that MB intravenous infusion (5–10 mg/kg) induced a toxic encephalopathy during parathyroidectomy. In these cases, neurological signs includedaphasia, prolonged disorientation and confusion (Karthap et al., 2006; Khan et al., 2007; Majithia and Stearns, 2006; Mathew et al., 2006). Even in healthy individuals, intravenous injection of 500 mg MB induced mental confusion for several hours in the majority of the subjects (Nadler et al., 1934). Consistent with the clinical observations, neuronal adverse effects of MB have also been identified in vitro experimental data (Vutskits et al., 2008). In vivo, MB intravenous infusion (5 mg/kg and 50 mg/kg) induced cell death indicated by Fluoro-Jade B staining and activated caspase-3 immunohistochemistry in neurons of the cerebral cortex. In vitro, MB (10 $\mu$M) abolished evoked field excitatory postsynaptic potentials (fEPSPs) recorded in hippocampal CA1 region (Schaffer collateral-CA1 synapses) and induced cell death in hippocampal slice preparations of rats. MB (1 $\mu$M and 10 $\mu$M) could also induce significant retraction of dendritic arbor of cultured gamma-aminobutyric acid-mediated neurons (Vutskits et al., 2008).

Although it is now evident that MB causes some neurotoxic effects, little information is available about its mechanisms on neurons in the central nervous system (CNS). Of neuronal adverse effects, reduced fEPSPs in hippocampal slice cultures suggests that MB may affect electrophysiological properties of neurons. Of electrophysiological properties of neurons, voltage-gated sodium current ($I_{Na}$) is responsible for the generation and conduction of

Abbreviations: MB, methylene blue; $I_{Na}$, voltage-gated sodium current; AP, action potential; CNS, central nervous system; $V_{max}$, maximum velocity of depolarization.

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APs, which are fundamental properties of excitable cells. \( I_{Na} \) also modifies neuronal cellular and network excitability. In fact, phenothiazine derivatives, such as trifluoperazine, fluphenazine and chlorpromazine, were reported to block neuronal voltage-gated sodium channels (VGSCs) (Ogata et al., 1989, 1990; Zhou et al., 2006). Furthermore, one previous study observed that MB induced a shift in the inactivation–voltage curve of \( I_{Na} \) in invertebrate (crayfish) preparations (Starkus et al., 1984). It might be possible that the neurotoxic effects of MB are partly attributed to the characteristic changes of AP and VGCS in neurons. Therefore, we suggested that MB reduced the voltage-gated sodium currents and modified some properties of APs on hippocampal CA1 neurons.

2. Materials and methods

2.1. Slice preparation

Male Wistar rats, bred in the Experimental Animal Center of Chinese Academy of Medical Sciences, were used on postnatal days 10–14 (Zhao et al., 2009). The experiments were conducted in accordance with the guidelines made by the Medical Experimental Animal Administrative Committee of Nation. Horizontal slices that included the entire hippocampus and subiculum (400 \( \mu \)m in thickness) were prepared with a vibratome (VT1000M/EL, Leica, Germany) and incubated with artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 25 NaHCO\(_3\), 1.25 KCl, 1.25 KH\(_2\)PO\(_4\), 1.5 MgCl\(_2\), 2.0 CaCl\(_2\), and 16 glucose. The standard pipette solution for recording sodium current contained (in mM): CsCl 140, MgCl\(_2\) 2, 4-(1-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) 10, EGTA 10, tetraethyl ammonium chloride (TEA-Cl) 20, Mg-ATP 2, buffered to pH 7.2 with CsOH. For recording the amplitude of \( I_{Na} \), cells were held at −70 mV, current traces were obtained by applying depolarizing pulses from −70 to +30 mV for 20 ms (Liu et al., 2009). The activated inward current was completely and reversibly blocked by bath application of 1 \( \mu \)M TTX (data not shown), indicating that the sodium channels expressed in hippocampal CA1 neurons were brain-type TTX-sensitive.

2.2. Electrophysiological recordings and drug application

For whole-cell recording, slices were transferred to a recording chamber (200 \( \mu \)l volume) placed on the stage of a modified upright infrared DIC microscope equipped with Nomarski optics. Hippocampal CA1 neurons were visualized on a television monitor connected to a low light sensitive CCD camera (Stuart et al., 1993). Electrophysiological data were low-pass filtered at 2.9 kHz, digitized at 10 kHz (four pole Bessel filter) and stored in an Intel-based computer using Pulse 8.74 software (HEKA, Germany). The series resistance was compensated at least 80%. Leakage and capacitive currents were subtracted on-line using a P/4 subtraction procedure.

Data acquisition was performed on computer using EPC10 patch-clamp amplifier (HEKA, Germany). After seal formation and membrane rupture, the cells were allowed to stabilize for 10 min before starting pulse protocols. Afterwards, slices were superfused with a solution of MB in ACSF for 10 min in the dark. MB was dissolved and diluted in ACSF and concentrations of 0.25 \( \mu \)M, 1 \( \mu \)M, 10 \( \mu \)M and 100 \( \mu \)M were obtained, which were based on the reference data (Vutskits et al., 2008).

Tetrodotoxin (TTX)-sensitive sodium channels carry the largest inward current. For recording the amplitude of \( I_{Na} \), cells were held at −70 mV, current traces were obtained by applying depolarizing pulses from −70 to +30 mV for 20 ms (Liu et al., 2009). The activated inward current was completely and reversibly blocked by bath application of 1 \( \mu \)M TTX (data not shown), indicating that the sodium channels expressed in hippocampal CA1 neurons were brain-type TTX-sensitive.

2.3. Data analysis

All data were analyzed by Clampfit 9.0 and Origin 7.5. For activation, currents were converted to conductance (G) at each test potential using the following formula G = I/(V – Vt), where Vt is reversal potential. The peak conductance value was normalized to Gmax and plotted against the test potential for each test potential to produce a voltage–conductance relationship curves, which was fitted using Boltzmann functions G/Gmax = 1/[1 + exp((V – V0)/k)], where V0 is the voltage at which conductance being half-maximal, and k is slope factor describing the slope of the steady-state inactivation curves.

The current–voltage (I–V) curves and the steady-state activation of \( I_{Na} \) were obtained by applying depolarizing pulses from −80 mV to +50 mV at 10 mV steps for 20 ms. Cells were held at −70 mV.

The steady-state inactivation of \( I_{Na} \) was obtained by a double-pulse protocol; applying 50 ms conditioning prepulse was applied to potentials between −100 mV and −15 mV in 5 mV increments, followed by a 20 ms pulse to −10 mV, and holding potential at −70 mV. The peak amplitudes for \( I_{Na} \) currents were normalized and plotted vs. prepulse potentials.

In order to determine the kinetics of recovery from the inactivated channel state, cells were held at −90 mV. We used a double-pulse protocol; a 50 ms conditioning pulse to −40 mV was applied to inactivate the sodium channels fully, and then a 50 ms test pulse to −40 mV was applied after a series of −90 mV pulses in intervals varying from 2 ms to 36 ms (in 2 ms increments). All experiments were performed at room temperature (22–24 °C).

In the current-clamp mode, neurons were held at −70 mV. The single action potential was elicited using a depolarizing current pulse (5 ms, 100 pA) (Zhao et al., 2009). To observe the effect of MB on action potential firing rate, a long-term depolarizing current (500 ms, 20 pA) was given to the neuron.

TTX was purchased from the Research Institute of the Aquatic Products of Hebe (China). MB, 1-H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), 4-AP, TEA-Cl, CsOH, CdCl\(_2\), EGTA, Heps, Mg-ATP and 3-(5-hydroxymethyl-2-furyl)-1-benzylindazole (YC-1) were purchased from Sigma (USA), and other reagents were analytical research (AR) grade.
normalized current, $V_h$ is the potential for half-maximal inactivation, and $k$ is the slope factor describing the slope of the steady-state inactivation curves.

The time course of recovery of the $I_{Na}$ currents from inactivation was fitted with a monoexponential function: $I/I_{max} = A \times \left(1 - \exp\left[-t/\tau\right]\right)$, where $I_{max}$ is the maximal current amplitude, $I$ is the current after a recovery period of $\Delta t$, $\tau$ is the time constant and $A$ is the amplitude coefficient.

Data are presented as mean ± SEM. Statistical significance was assessed using a Student’s paired t-test when there were only two groups involved. In the remaining cases the results were statistically analyzed using one-way ANOVA followed Tukey’s multiple comparison and $P < 0.05$ was considered significant. All data analyses were performed using the software SPSS 16.0.

3. Results

3.1. Effects of MB on the peak amplitude of $I_{Na}$

The sodium currents were recorded from a holding potential of $-70 \text{ mV}$ by 20 ms test steps to $+30 \text{ mV}$. MB was tested at increasing concentrations (0.25 μM, 1 μM, 10 μM and 100 μM), the concentration–response results showed MB decreased the amplitude of $I_{Na}$ by approximately 30% at 10 μM and by approximately 45% at 100 μM (Fig. 1). These currents were reversibly blocked by bath application of 1 μM TTX (not shown). The concentration of 10 μM was used in further experiments. Recordings were always started at 10 min after obtaining the whole-cell configuration to allow sodium current to stabilize.

3.2. Effects of MB on I–V relationships of $I_{Na}$

After the sodium current was stable, MB was applied in the extracellular solution at a final concentration of 10 μM. The peak amplitudes of $I_{Na}$ were decreased significantly from $-40 \text{ mV}$ to $+30 \text{ mV}$ ($n=7$, $P < 0.05$ vs. control, Fig. 2A and B).

3.3. Effects of MB on the steady-state activation curves for $I_{Na}$

The steady-state activation curves for $I_{Na}$ under control and after exposure to MB (10 μM) were shown in Fig. 3. The values of $V_h$ were $-52.05 \pm 2.18 \text{ mV}$ and $-52.31 \pm 1.84 \text{ mV}$ for activation of $I_{Na}$ in control and MB (10 μM) ($n=7$, $P > 0.05$), with a slope factor $k$ of $5.50 \pm 2.02$ and $4.07 \pm 1.66$ ($n=7$, $P < 0.05$), respectively. MB (10 μM) did not produce a significant shift in the activation–voltage curve but produced a change in slope factor on $I_{Na}$ currents.
3.4. Effects of MB on the inactivation kinetics of $I_{Na}$

The effects of MB (10 μM) were examined on the inactivation kinetics of $I_{Na}$ (Fig. 4A). The inactivation curves were obtained through normalizing the test current amplitudes by taking the maximum value under each condition as unity (Fig. 4B). $V_h$ for inactivation of $I_{Na}$ were $/C0 58.75/C6 1.21$ mV and $/C0 62.73/C6 1.51$ mV in the control and 10 μM MB group ($n = 8$, $P < 0.05$), and $k$ values were $8.48/C6 1.13$ and $11.60/C6 1.58$ ($n = 8$, $P < 0.01$), respectively. These results indicated that MB accelerated the inactivation of $I_{Na}$.

3.5. Effects of MB on the recovery of $I_{Na}$

The effects of MB on the recovery time course of $I_{Na}$ from inactivation were examined (Fig. 5A). To analyze the recovery time course of $I_{Na}$, normalized current amplitudes were plotted against the inter-pulse interval durations, and were fitted with mono-exponential functions. The time constants ($\tau$) describing the recovery time course were presented. The $\tau$ values were $2.10 \pm 0.35$ ms and $2.98 \pm 0.28$ ms ($n = 8$, $P < 0.05$) before and after application of MB (10 μM, Fig. 5B). Therefore, in addition to enhancing steady-state sodium channel inactivation, MB slowed the transition from the inactivated to recovered state.

3.6. Effects of MB on AP and AP firing rate

Action potential properties and the pattern of repetitive firing were examined using whole-cell current-clamp recordings. Peak amplitude, overshoot (after hyperpolarization), peak rise time and maximum velocity of depolarization ($V_{max}$) of APs as well as the number of repetitive firings were measured before and after MB (10 μM) application. Peak amplitude, overshoot and $V_{max}$ of evoked single AP were decreased ($n = 7$, $P < 0.05$, Figs. 6 and 7, Table 1) by MB. The peak rise time did not change in the present of the 10 μM MB solution ($n = 7$, $P > 0.05$). The rate of repetitive firing...
was also decreased (n = 7, P < 0.05) by 10 μM MB. To determine if the effects were transient or persistent, MB treatment was measured for 30 min. The results showed the effects of MB on APs persisted throughout the experiment (Fig. 7). Furthermore, the effect of MB on APs was not reversible, since the number of APs did not return to the control levels following washout of MB for up to 30 min.

Fig. 8. Effect of 10 μM ODQ (A) or 50 μM YC-1 (B) on the amplitude of I_{Na}. Cells were held at −70 mV, current traces were obtained by applying depolarizing pulses from −70 mV to +30 mV for 20 ms.

3.7. Mechanisms of MB inhibition of VGSCs

MB has long been known as an inhibitor of soluble guanylyl cyclase (sGC) and has been demonstrated to inhibit the enzyme entirely at 10 μM concentration (Pickard et al., 1991). For this reason it has been used to deplete cells of internal cGMP (cyclic guanosine monophosphate) in order to investigate the role of the cyclic nucleotide as an intracellular second messenger (Garthwaite and Garthwaite, 1988). Two pharmacological tools are frequently used to manipulate the NO-cGMP signaling pathway at the level of sGC: an inhibitor, ODQ (1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one) (Garthwaite et al., 1999) and an ‘allosteric’ activator, YC-1 [3-(5-hydroxymethyl-2-furyl)-1-benzylindazole] (Ko et al., 1994). In the present studies, blocking of internal cGMP by 10 μM ODQ or increasing internal cGMP by 50 μM YC-1 for 10 min did not affect the amplitude of I_{Na}, even to 30 min after application ODQ or YC-1 (Fig. 8), indicating other mechanisms might be responsible for MB induced sodium channel block effects on neurons in CNS. Concentrations of 10 μM ODQ or 50 μM YC-1 are adequate to change internal cGMP content (Bonthius et al., 2004).

4. Discussion

MB infusion is frequently used to localize the parathyroid glands during parathyroidectomy and is generally considered safe. Like all chemicals, there is an associated risk of developing adverse effects with use of MB. An increasing number of clinical observations reported that single bolus MB injection induced adverse neurologic outcomes, including dizziness, headache, tremors, and mental confusion (Kartha et al., 2006; Khan et al., 2007; Majithia and Stearns, 2006; Mathew et al., 2006). In experimental studies, MB induced widespread neuronal apoptosis in the brains of these animals and a rapid suppression of evoked EPSPs (Vutskits et al., 2008). To date, however, there is only one literature which describes the effect of MB on fEPSPs in the mammalian CNS (Vutskits et al., 2008), much electrophysiological information about ion channels and APs of MB on mammalian neurons receives less research attention. In the present study, we tested the effect of MB on the properties of VGSCs and APs in neurons recorded in hippocampal slices.

According to Vutskits et al. (2008), the concentration of 10 μM MB used in hippocampal slice preparations was a clinically-relevant concentration. In other words, the concentration of 10 μM MB may mimic the actual MB concentration in brain tissues when MB was used as a marker of the parathyroid glands (5–10 mg/kg). The present results showed that both concentrations of 0.25 μM and 1 μM MB did not affect the amplitude of I_{Na}, but 10 μM and 100 μM MB decreased I_{Na}. These findings suggested that MB produced a concentration-dependent inhibition of I_{Na}. The most important finding was that MB at concentration of 10 μM significantly reduced I_{Na} in rat hippocampal CA1 neurons. Because MB used in hippocampal slice preparations was at clinically-relevant concentration, I_{Na} recorded in hippocampal slice preparations at the concentration of 10 μM MB may mimic the effect of intravenous MB as a surgical marker of the parathyroid glands on neurons in the CNS.

The further kinetic studies suggested that the feature of the inhibited I_{Na} by MB (10 μM) observed in hippocampal CA1 neurons was by enhancing the inactivation course and slowing recovery from inactivation of voltage-gated sodium channels, while no discernible change was observed in the activation course of the channel. Combined with the different gating state of ion channel (at least the resting, open and inactivated states) (Ouyang et al., 2009), we can conclude that MB affect inactivation state and recovery from inactivation state.

The AP is a fundamental property of excitable cells in the mammalian CNS. The inhibition of I_{Na} by MB (10 μM) was also
reflected by AP properties and the rate of repetitive firing, at least in part. The peak amplitude and overshoot of the evoked single AP were decreased in the presence of 10 μM MB solution. MB (10 μM) also reduced the maximum velocity of depolarization (V_{max}) of a single AP, an indirect index of the fast inward sodium current. The recovery from inactivation of I_{Na} was slowed by MB (10 μM), which would decrease the rate of repetitive firing by limiting the availability of sodium channels on a time scale.

VGSCs play a critical role in the generation of action potentials in excitable cells throughout the nervous system. Modifications of sodium channel gating properties modify excitability and the generation and conduction of APs on neurons, as a consequence, alter mental state and evoked EPSP. Therefore, it might be possible that the neurotoxic effects of MB are partly attributed to the characteristic profiles of voltage-gated sodium channel in neurons.

The mechanism of MB inhibition of VGSC was independent of sGC, since ODQ or YC-1 did not affect the amplitude of I_{Na}. In invertebrate (crayfish) preparations, Starkus et al. (1984) reported that MB inhibited I_{Na} that was reversible following washout. However, the present study regarding AP inhibiting effect by MB was not reversible, suggesting that there were different mechanisms regarding effects of MB on VGSCs in mammalian neurons compared to invertebrate giant axons. Recently, some studies demonstrated that MB could attenuate the metabolism of serotonin through inhibition of monoamine oxidase (Ng et al., 2008; Ramsay et al., 2007). Moreover, serotonin receptor activation inhibited I_{Na} by reducing maximal current amplitude and shifting the inactivation curve in a hyperpolarizing direction (Carr et al., 2002). Serotonin also reduced the amplitude of AP in CA1 hippocampal pyramidal neurons (Sandler and Ross, 1999). A possible pharmacological mechanism underlying these effects is that MB, as a monoamine oxidase inhibitor, increases the content of serotonin, which leads to inhibitory effects on VGSCs. The inhibitory effect of MB on VGSCs is an important and unresolved question. Further studies are needed to elucidate this question.

Extrapolation the current results to the clinical condition must be done with caution. One should not underestimate the importance of interspecies differences (Berde and Cairns, 2000) and therefore, we cannot exclude an increased sensitivity to MB in rats compared with humans. Nevertheless, in vitro models provide a useful complementary approach to clinical observations in the evaluation of potential drug-induced adverse effects. In addition, studies in characterization of ion channels provide a possible strategy for future preventing or diminishing adverse effects. In the present study, low concentrations of MB (such as 0.25 μM and 1 μM) did not affect the amplitude of I_{Na}, suggesting that the reducing amount of used MB as a surgical marker of the parathyroid glands may diminish or reduce the neurotoxic effects by MB. In practice, a low dose of MB (3 mg/kg) was still adequate to stain the parathyroid, though the intensity of staining was definitely less (Khan et al., 2007).

MB-associated encephalopathy raises concerns about the safety of this compound. The present results showed that MB produced a concentration-dependent inhibition of I_{Na}. Additionally, MB (10 μM) enhanced the inactivation course and slowing recovery from inactivation of VGSCs. MB also inhibited the rate of repetitive firing of APs observed in hippocampal CA1 neurons. The modulation of VGSCs and APs by MB suggests that MB affects the neuronal cellular and network excitability as well as the generation and the propagation of APs.

Conflict of interest

There are none.

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