

The Effect of 4-hydroxybenzaldehyde on the γ -aminobutyric Acid Type A Receptor

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Abstract

The $\alpha_1\beta_2\gamma_2$ subtype of GABA_A receptors is the most commonly found GABA_A receptor subtype in the mammalian cortex and hippocampus. It is expressed heterologously in the *Xenopus laevis* oocyte as a $\alpha_1\beta_2\gamma_2$ S/L subtype for application as an in vitro model for the screening of compounds that modulate receptor activities. In fact, 4-hydroxybenzaldehyde (4-HB) has been identified as one of the major components in *Dendrocalamus asper* bamboo shoots in our previous study, and the current study showed that at 101.7 μ M, 4-HB significantly reduced the GABA-induced chloride current of GABA_A receptors expressed on *Xenopus* oocytes, indicating a possible GABAergic antagonistic effect at high concentrations.

Keywords: $\alpha_1\beta_2\gamma_2$ GABA_A receptor, electrophysiology, *Xenopus* oocytes, bamboo shoot, GABAergic agents

Introduction

GABA_A receptors, major contributors of neuronal inhibitory responses, are activated by an endogenous neurotransmitter GABA that leads to a rapid influx of chloride ions while the binding of GABA onto metabotropic GABA_B receptors leads to slow neurotransmission of potassium ion flux (1). The isoform of GABA_A receptors commonly consists of five subunits — two α_1 , two β_2 and one γ_2 — and it is the most

abundantly expressed combinations in the brain (2, 3). Due to the rapid inhibitory responses of GABA_A receptors, it is widely expressed as $\alpha_1\beta_2\gamma_2$ S/L on the membrane of *Xenopus laevis* oocytes and used as an in vitro model coupled with the two-electrode voltage clamp method to screen for potential modulatory compounds that could enhance or reduce chloride current through GABA_A receptors (4, 5). In traditional Chinese medicine (TCM), bamboo is used as a component to reduce the energy of 'fire' and

used in TCM as treatment for hypertension, cardiovascular disease and arteriosclerosis. Its extract contains phytochemicals, such as flavanoids, saponin and triterpenoids, that have been shown to possess an antiepileptic effect, suggesting its potential for the treatment of epilepsy (6). As the GABAergic function plays an important role in the mechanism and treatment of epilepsy (7), this study investigated the effect of 4-hydroxybenzaldehyde (4-HB) identified in *Dendrocalamus asper* bamboo shoots on GABA_A receptors of the $\alpha_1\beta_2\gamma_2S$ subtype.

Materials and Methods

Expression in Xenopus oocytes

The procedures to prepare DH5 α competent cells were carried out and the cDNA of subunits α_1 , β_2 and γ_2S were cloned in the pCMV vector using the competent DH5 α bacteria. Linearisation and in vitro transcription were performed and the cRNA of each subunit was then isolated and purified. The final 12 μ L ($\alpha_1:\beta_2:\gamma_2S$ by 1:1:10) aliquot (with the final concentration of 20–200 ng/ μ L for each cRNA) were stored at -80 °C (8, 9). Surgery was performed on an anaesthetised *Xenopus laevis* female frog to obtain oocyte lobes. The oocyte lobes were cut into small pieces and placed in a modified OR-2 solution (in mM: 82 NaCl, 5 HEPES, 1 MgCl₂, 2 KCl, 1 Na₂HPO₄, 1 ascorbic acid, pH 7.5), treated with type II collagenase (1 mg/mL) for 1 hour under 16 °C on a rotator. The oocytes were kept in a modified ND96 solution (in mM: 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, 1 Na-Pyruvate, 0.01 mg/mL Gentamycin, pH 7.5) in the incubator at 18 °C overnight. Oocytes of stage V–VI were selected for the cRNA injection. Each oocyte was injected with 15 nL–50 nL of cRNA and stored in the modified ND96 solution in the incubator at 18 °C for 48 hours for up to 5 days (8, 9).

Drugs

A total of 44 kg of *Dendrocalamus asper* bamboo shoots were harvested from Post Brooke village in Gua Musang, Kelantan and identified by Assistant Botanist, Muhammad bin Deraman from the South Kelantan Development Authority (KESEDAR), with the voucher specimen labeled DAPB52014 and kept in the Department of Neurosciences, School of Medical Sciences, Universiti Sains Malaysia. The bamboo shoots were chopped into 1 cm³–2 cm³ pieces and were then lyophilised and ground to yield 6 kg dried

powder of bamboo shoots. One kg of dried bamboo shoot powder was extracted three times using 800 mL of dichloromethane for each extraction. The filtered extracts were dried using a rotary evaporator (Buchi, Germany) under reduced pressure, below 40 °C to yield 31.5 g of dichloromethane extract. Next, 30 g of dichloromethane extract were subjected to purification using column chromatography (4 cm \times 30 cm, dxh) packed with silica gel (Merck 1.09385.1000) and eluted with chloroform, and then gradually increased using ethyl acetate and methanol. The fractions were monitored using thin layer chromatography (Merck 1.05735 silica gel 60 F254). The fractions containing the same spot were combined. Fraction K (2.5 g) containing spot with R_f value 0.4 (chloroform/ethyl acetate, 9:1 v/v) were then subjected to repeated column chromatography (1 cm \times 25 cm, dxh) using silica gel (Merck 1.09385.1000) and eluted with hexane/acetone, 7:3 v/v. 200 mg of 4-hydroxybenzaldehyde (4-HB) were purified after re-crystallisation of fraction K5 from hexane/methanol, with a near 98% purity (Figure 1). 5 mg of 4-HB were dissolved in 0.5 mL of chloroform. 24.84 μ L of the stock solution above were dissolved into 20 mL ND96 with 5 μ M GABA inside for the bath solution in drug groups in two electrode voltage clamp tests.

Animal ethics

This study was approved by the Animal Ethics Committee Universiti Sains Malaysia on the research project titled 'The Anti-Epilepsy Effects of DA1, DA2 and Fraction 57 et al. from Petung Bamboo (*Dendrocalamus asper*) Shoots on GABA (A) Receptor, GluA1 Receptor and Kv1.4 Channel' for 40 female *Xenopus laevis* [No. of Animal Ethics Approval: USM/Animal Ethics Approval/ 2014/ (90) (578)] with a duration of study from November 2014 to November 2017.

Oocyte electrophysiology – two-electrode voltage clamp (TEVC)

The enhancement effect of the compounds was shown through the TEVC GABA_A current recording at a holding potential of -70 mV. TURBO TEC-03X amplifier (NPI Electronic GmbH, Tamm, Germany), Digidata 1440A (Molecular Devices, LLC, Sunnyvale, CA, USA) and pClamp 10.3 software (Molecular Devices, LLC, Sunnyvale, CA, USA) were used for data recording. The data were filtered at 100 Hz and sampled at 500 Hz. Glass pipettes were pulled with vertical puller Narishige PC-10 (Japan) and filled with 3 M KCl, giving a resistance of

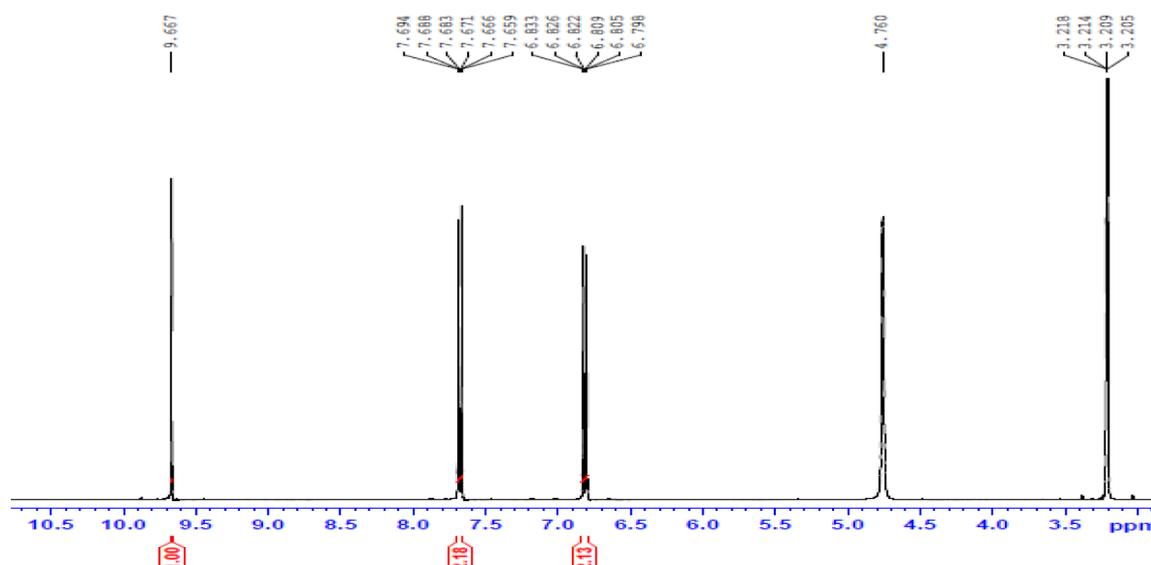


Figure 1. ¹H-NMR spectrum of p-hydroxybenzaldehyde in Deuterated-methanol (MeOD). Based on the NMR data, there is no impurity peak in the spectrum with purity of 4-HB near to 98%.

0.6 MΩ–1.5 MΩ of the electrode. ND96 (in mM: 96 NaCl, 2 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, pH 7.5) was used as a working physiological solution. The GABA_A response of the control group (in ND96: EC₃ [5 μM] GABA + vehicle chloroform) was compared with the drug groups (in ND96: EC₃ GABA + 4-HB). By means of an automated fast perfusion system flowing at the rate of 5 mL/min, the oocytes were exposed to a 4-HB in addition to GABA for 20 seconds with a simultaneous -5 mV voltage step and followed by a wash-out period of 5 minutes–10 minutes to ensure recovery from desensitisation. The GABA concentration of EC₃ (3% of the maximal current amplitudes) was applied to measure the enhancement effects of the chloride currents by 4-HB (10). Dose-response curves were generated and SigmaPlot software was applied to fit the data by nonlinear regression analysis. The data were fitted into the Hill equation:

$$I = 1 + \frac{I_{max}}{1 + \left(\frac{EC_{50}}{[CPD]}\right)^{n_H}}$$

I is the current amplitude at a given concentration of agonist [CPD]. *I*_{max} is the maximum current response, EC₅₀ is the concentration of the compound which elicits a half-maximal activation, and *n*_H is the Hill coefficient. Normalised current amplitude was reported as mean ± SEM for at least 6 oocytes and ≥ 2 batches of oocytes.

Results

Effect of 4-HB on a GABA-induced current

A dose-response of GABA (in μM: 0.01, 0.1, 1, 5, 10, 100, and 1,000 in 20 mL ND96) was tested on empty oocytes (*n* = 12) and GABA_A receptor cRNA-injected oocytes (*n* = 13) with a concurrent -5 mV voltage step (Figure 2). In empty oocytes, the means of normalised current amplitudes for seven concentrations of GABA were 1.0139, 0.9998, 0.9883, 0.9751, 0.9808, 0.9937 and 0.9669, showing no tendency to increase in the current amplitude as we increased the GABA concentration. In cRNA-injected oocytes, the means of normalised current amplitudes were 0.9491, 0.8757, 0.9871, 1.032, 1.0255, 1.0191 and 1.077. A tendency to increase in the current amplitude with increasing GABA concentration was observed, indicating a GABA_{sf} response in GABA_A cRNA-injected oocytes, a response not observed in empty oocytes. This demonstrated that there are few native GABA_A receptors in *Xenopus laevis* oocytes without the injection of GABA_A cRNA and the GABA-induced current was not only due to -5 mV steps but was also a response of GABA_A receptors expressed on the oocyte membrane. Based on the mean values of the cRNA-injected oocytes and empty oocytes, a fit of the equation was made. The peak amplitude was partly voltage command-dependent and partly a GABA-induced response; hence, the dose-response curve was different from that which was automatic ligand-induced.

As the values of the empty oocytes did not suit a reasonable fit, its fit equation was not shown in this paper. For the fit of the GABA_A cRNA-injected oocytes, the EC₅₀ is 1.521 mM, the nH is 0.6081 and the absolute value of the I_{max} is 0.1743 (normalised value). The fit equation for cRNA injected group is: I (normalised value) = $1 + 0.1743 / (1 + (0.001521 \text{ M} / [\text{Comp}])^{0.6081})$. With the input of 5 μM GABA into the equation, it was shown that the current amplitude by 5 μM GABA was approximately EC₃ of the GABA response. The main component of the peak amplitude was

the voltage command response in nature. The quickly increased curve phase of the distribution of the peak amplitude did not appear and therefore the EC₅₀ was 1.521 mM after the Hill equation fit. Based on this protocol, 100 μM of 4-HB was applied to the perfusion system (ND96 with 5 μM GABA) concurrently with a -5 mV voltage step, and this reduced the GABA-induced chloride current to 0.984 ± 0.006 (mean and SEM) as compared with the vehicle control group (Figure 3).

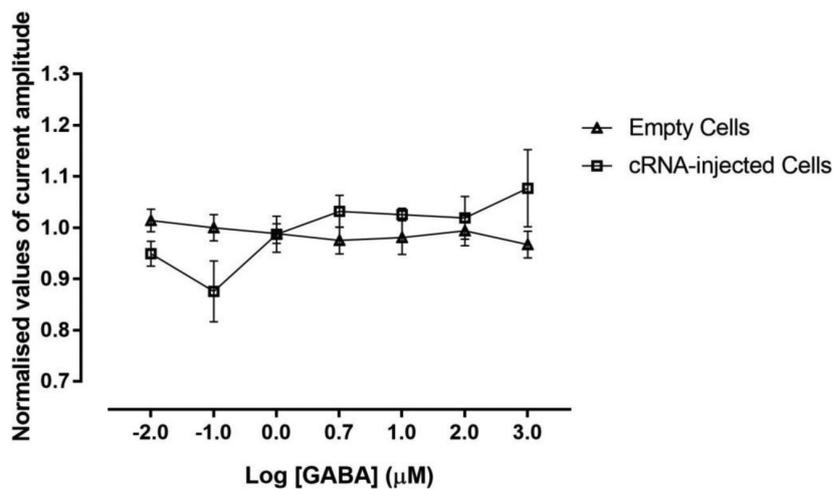


Figure 2. Dose-response of GABA-induced chloride current on empty oocytes and cRNA-injected oocytes. The series of GABA concentrations ranging from 0.01 to 1000 μM was tested with a concurrent -5 mV voltage step for 20 milliseconds during the acute application of GABA in ND96. There was a tendency of an increase in the current amplitude of the GABA-induced current in cRNA-injected cells with increasing GABA concentration, a tendency which was not observed in empty cells.

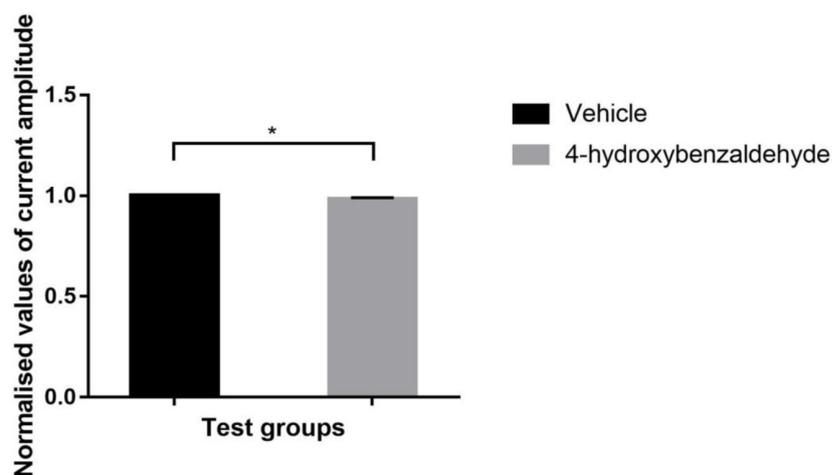


Figure 3. Effect of a 101.7 μM 4-HB on GABA-induced chloride current. 4-HB was applied onto recording oocytes expressed with the GABA_A α₁β₂γ₂S subtype through an automated fast perfusion system and reduced the GABA-induced chloride current (0.984 ± 0.006) as compared with the vehicle group (Student's *t*-test, $P = 0.019$, $n = 8$).

Discussion

Pharmacological evidence suggested interactions between TCM and GABA_A receptors; for example, studies on *Gastrodia elata* demonstrated the potential anticonvulsant activities of this plant with 4-HB, one of the major phytochemicals that displayed a positive modulatory effect on GABAergic responses (11, 12). It was proposed that the carbonyl and hydroxyl groups enable 4-HB to exert its inhibitory effect on GABA-transaminase through competitive binding, with no indication of reversibility (13, 14). At low concentrations, 4-HB was demonstrated to increase chloride influx as well as the expression of α and β subunits (15). 4-HB reduced the onset of sleep and the counts of the sleep-wake cycle; it also increased total sleep time and non-rapid eye movement in rodent models, attributable to an enhanced GABAergic function (15). However, our study on GABA_A receptors expressed on *Xenopus* oocytes demonstrated a reduction of GABAergic function when 101.7 μ M of 4-HB was applied acutely onto the cells, as it lowered the GABA-induced chloride currents. This indicated a potential antagonistic effect of 4-HB at high concentrations and more in-depth studies are warranted to identify the dose-response effect of 4-HB as well as its binding mechanism and reversibility on a GABA_A $\alpha_1\beta_2\gamma_2$ S receptor subtype.

Conclusion

This study demonstrated that acute application of 101.7 μ M of 4-hydroxybenzaldehyde led to a reduction of a GABA-induced chloride current tested on a GABA_A receptor of a $\alpha_1\beta_2\gamma_2$ S subtype expressed on *Xenopus* oocytes, suggesting an inhibitory effect of GABAergic responses at a high 4-HB concentration. The findings provided leads for future studies to verify the dose-response effect of 4-HB on a GABA-induced chloride current.

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research into its biocompounds and its effects in epilepsy and other neurological diseases. Associate Professor Dr. Kamarul Imran Musa from the Department of Community Medicine, School of Medical Sciences, USM, for his helpful advice in statistical analyses. Ms. Sui Mei Kee and Ms. Nuraza Othman, et al., of the Department of Neurosciences, School of Medical Sciences, USM, helped the experiment technologically. Professor Dr. Sharharum Shamsuddin and his lab in PPSK, Universiti Sains Malaysia helped us with cDNA clone and cRNA synthesis. The work was funded by the incentive grant (from Universiti Sains Malaysia), short term grant (304/PPSP/61312047, from Universiti Sains Malaysia), and FRGS grant (203/PPSP/6171155, from the Department of Higher Education, Malaysia) to Dr. Jingli Zhang and Professor Dato' Dr. Jafri Malin Abdullah et al.

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Critical revision of the article for important intellectual content: JZ, HM
Final approval of the article: JZ, HM
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