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Muscarinic M2 receptor inhibition of calcium current in rat nucleus tractus solitarius

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Running title: Ca\(^{2+}\) current modulation by ACh
Abstracts

The cholinergic system in the CNS plays important roles in higher brain functions, through muscarinic receptors. The nucleus tractus solitarius is known to play a major role in the regulation of cardiovascular, respiratory, gustatory, hepatic and swallowing functions. Voltage-dependent Ca\(^{2+}\) channels (VDCCs) serve as crucial mediators of membrane excitability and Ca\(^{2+}\)-dependent functions such as neurotransmitter release, enzyme activity and gene expression. The purpose of this study was to investigate the effects of ACh on VDCCs currents (I\(_{\text{Ca}}\)) in the nucleus tractus solitarius using patch-clamp recording methods. In 68 of 99 neurons, an application of ACh caused inhibition of N- and P/Q-types I\(_{\text{Ba}}\) in a concentration-dependent manner. Pretreatments with AF-DX116 (muscarinic M2 receptor antagonist) attenuated the ACh-induced inhibition of I\(_{\text{Ba}}\). Intracellular dialysis of the G-\(\alpha\)-protein antibody also attenuated the ACh-induced inhibition of I\(_{\text{Ba}}\). These results indicate that ACh inhibits N- and P/Q-types VDCCs via Gi-protein \(\alpha\) subunits mediated by M2 receptors in nucleus tractus solitarius.

(911 words)

Key words: Nucleus tractus solitarius, calcium channel currents, acetylcholine
Introduction

The nucleus tractus solitarius is known to play a major role in the regulation of cardiovascular, respiratory, gustatory, hepatic and swallowing functions [1]. This nucleus receives primary afferent input from a wide variety of peripheral organs and tissues and is essential in the integration of autonomic nervous system functions. The nucleus tractus solitarius contains multiple of putative neurotransmitters and many have been implicated in cardiovascular regulation [2].

Acetylcholine (ACh) is one of only a few neurotransmitters that profoundly decrease arterial pressure and heart rate when injected into the nucleus tractus solitarius [3,4]. Recent investigation demonstrated that muscarinic M2 ACh receptors modulated G-protein-gated inwardly rectifying potassium (GIRK) channels in nucleus tractus solitarius [5].

Voltage-dependent Ca\(^{2+}\) channels (VDCCs) serve as crucial mediators of membrane excitability and Ca\(^{2+}\)-dependent functions such as neurotransmitter release, enzyme activity and gene expression. The modulation of VDCCs is believed to be an important means of regulating Ca\(^{2+}\) influx and thus has a direct influence on many Ca\(^{2+}\)-dependent processes [6]. Modulation of VDCCs current (I\(_{\text{Ca}}\)) by ACh has been described previously in various types of cells [7]. However, the mechanism of ACh
effects on VDCCs in nucleus tractus solitarius has been extensively studied, but remains unclear and even controversial. Consequently, it is the purpose of this study to investigate the effects of ACh on I_Ca in nucleus tractus solitarius.

Materials and methods

Cell preparation

Experiments were conducted according to international guidelines on the use of animals for experimentation. nucleus tractus solitarius neurons were acutely dissociated from neonatal rat as previously [8]. Young Wistar rats (7-18 days old) were decapitated and their brains were quickly removed and submerged in ice cold artificial cerebrospinal fluid (aCSF) saturated with 95% O_2 and 5% CO_2 of the following composition (in mM): NaCl 126, NaHCO_3 26.2, NaH_2PO_4 1, KCl 3, MgSO_4 1.5, CaCl_2 1.5 and glucose 30; pH 7.4. Thin transverse slices from brainstems, 400 μm in thickness, were prepared by a tissue slicer (DTK-1000; Dosaka EM Co., Ltd, Kyoto). After being sectioned, 3-5 slices obtained from a single brain were transferred to a holding chamber and stored in oxygenated aCSF at room temperature for at least 40 min before use. Slices were then transferred to a conical tube containing gently bubbled aCSF at 36 ºC to which 1.8 U/ml dispase (grade II; 0.75 ml/slice) was added. After 60 min incubation, slices were
rinsed with enzyme-free aCSF. Under a dissecting microscope, the nucleus tractus solitarius region was micropunched and placed on a poly-l-lysine-coated coverslip. The cells were then dissociated by trituration using progressively smaller diameter pipettes and allowed to settle on a coverslip for 20 min.

**Whole-cell patch-clamp recordings**

Voltage-clamp recordings were conducted using the whole-cell configuration of the patch-clamp technique [9]. Fabricated recording pipettes (2-3 MΩ) were filled with the internal solution of the following composition (in mM): 100 CsCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 3.6 MgATP, 14 Tris₂-phosphocreatine (CP), 0.1 GTP, and 50 U/ml creatine phosphokinase (CPK). The pH was adjusted to 7.2 with CsOH. After the formation of a giga seal, in order to record I_{Ca} carried by Ba^{2+} (I_{Ba}), the extracellular solution was replaced from Krebs solution to a solution containing the following (in mM): 151 tetraethylammonium (TEA) chloride, 5 BaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose. The pH was adjusted to 7.4 with Tris base. Command voltage protocols were generated with a computer software pCLAMP version 8 (Axon Instruments, Union City, CA, U.S.A.) and transformed to an analogue signal using a DigiData 1200 interface (Axon Instruments, Union City, CA, U.S.A.). The command pulses were applied to cells
through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany). The currents were recorded with the amplifier and a computer software pCLAMP 8 acquisition system. Access resistance (\(< 15 \, \text{M} \Omega\)) was determined by transient responses to voltage commands. Access resistance compensation was not used. To ascertain that no major changes in the access resistance had occurred during the recordings a 5 mV, 10 msec pulses was used before $I_{\text{Ba}}$ was evoked.

**Materials**

ACh and Nifedipine (Nif) were purchased from sigma. Telenzepine and AF-DX116 were purchased from Tocris. Anti-G $\alpha_i$ antibodies, anti-G $\alpha_s$ antibodies and anti-G $\alpha_{q11}$ antibodies were purchased from Upstate biotechnology (Lake Placid, NY, U.S.A.). Each antibodies were from rabbits immunized with a synthetic peptide corresponding to the COOH-terminal sequence of the human G $\alpha_i$, G $\alpha_s$ and G $\alpha_{q11}$, respectively. $\alpha$-conotoxin G $\delta$A ($\alpha$-CgTx G $\delta$A) and $\alpha$-agatoxin $\delta$A ($\alpha$-Aga $\delta$A) were purchased from Peptide Institute.

**Analysis and statistics**

All data analysis were performed using pCLAMP 8.0 acquisition system. Values in text
and figures are expressed as mean ± SEM. Statistical analysis was made by student t-test for comparisons between pairs of groups and by one-way analysis of variance (ANOVA) followed by Dunnett’s test. Probability (p) values of less than 0.05 were considered significant.

Results

ACh-induced inhibition of I_{Ba}

Representative examples of superimposed I_{Ba} traces in the absence and presence of 1 μM ACh are shown in Fig. 1. I_{Ba} was evoked every 20 sec with a 100 msec depolarizing voltage step to 0 mV from a holding potential of -80 mV. As shown in Fig. 1, application of ACh rapidly and reversibly inhibits I_{Ba}. To investigate the voltage dependency of inhibition of I_{Ba} by ACh, I used a double-pulse voltage protocol as shown in Fig. 1a. As shown in Fig. 1a and b, the application of a strong depolarizing voltage prepulse attenuated ACh-induced inhibition of I_{Ba}.

The current-voltage relationships for I_{Ba} in the absence and presence of 1 μM ACh are shown in Fig. 1c. From a holding potential of -80 mV, the I_{Ba} was activated after -30 mV with a peak current amplitude at 0 mV. As shown in Fig. 1c, ACh-induced inhibition resulted in a shift in the voltage dependence of the I_{Ba} to more positive potentials.
The dose-response relations in the ACh-induced inhibition of $I_{Ba}$ is shown in Fig. 1d. For the generation of the concentration-response curve, ACh concentrations were applied randomly, and not all concentrations in a single neuron were tested. Fig. 1d shows that progressive increases in ACh concentration resulted in progressively greater inhibition of $I_{Ba}$.

**Pharmacological Characterization of ACh receptors in ACh-induced inhibition of $I_{Ba}$**

In the next series of experiments, I analyzed the effects of ACh on $I_{Ba}$ in neurons treated with specific antagonists. In this experiment, specific antagonists were applied prior to the ACh. Treatment with muscarinic M1 receptor antagonist Telenzepine ($1 \text{ M for 3 min after assuming the whole-cell configuration}$) did not attenuate the ACh-induced inhibition of $I_{Ba}$. In contrast, treatment with muscarinic M2 receptor antagonist AF-DX116 ($1 \text{ M for 3 min after assuming the whole-cell configuration}$) attenuated the ACh-induced inhibition of $I_{Ba}$. These results indicated that ACh-induced inhibition of $I_{Ba}$ was mediated by muscarinic M2 receptors in nucleus tractus solitarius.

**Characterization of G-protein subtypes in ACh-induced inhibition of $I_{Ba}$**

The G-protein is heterotrimeric molecules with $\alpha$, $\beta$, and $\gamma$ subunits. The $\alpha$
subunit can be classified into families, \( G_{i/o} \), \( G_{q/11} \), or \( G_s \). To characterize the G-protein subtypes in ACh-induced inhibition of \( I_{Ba} \), specific antibody raised against the \( G_{i/o} \), \( G_{q/11} \) and \( G_s \)-protein were used. Experiments were performed using a solution in a pipette containing each G-protein antibody. In these experiments, the G-protein antibody (1:50 dilution; the final concentration was approximately 0.5 mg/ml) was dissolved in the internal solution. The tip of the recording pipette was filled with the standard internal solution, and the pipette was then backfilled with solution which containing the G-protein antibody. In order to obtain the effect of antibody, ACh were applied 7 min after assuming the whole-cell configuration.

As shown in Fig. 2c, intracellular dialysis of the \( G_{i/o} \)-protein antibody attenuated the ACh-induced inhibition \( I_{Ba} \). In contrast, intracellular dialysis of and \( G_s \)- and \( G_{q/11} \)-proteins antibodies did not attenuate the ACh-induced inhibition of \( I_{Ba} \). These results suggest that the \( G_{i/o} \)-proteins are involved in the ACh-induced inhibition of \( I_{Ba} \) in nucleus tractus solitarius but \( G_{q/11} \)- and \( G_s \)-proteins are not.

**Characterization of VDCC subtypes in ACh-induced inhibition of \( I_{Ba} \)**

Several studies have defined pharmacological distinct high voltage-activated (HVA) VDCCs on neuronal cell bodies, such as L-, N-, P-, Q- and R-type VDCCs. In this study,
specific VDCCs blockers were used to isolate each VDCCs current component. Mean percentages of L-type \( I_{Ba-L} \), N-type \( I_{Ba-N} \), P/Q-type \( I_{Ba-P/Q} \) and R-type \( I_{Ba-R} \) components of total \( I_{Ba} \) is 42.2 ± 3.8%, 28.4 ± 3.4%, 19.3 ± 3.2% and 10.1 ± 1.4%, respectively in nucleus tractus solitarius [10]. Therefore, it was investigated about which types of the VDCCs were inhibited by ACh. The effect of ACh on the \( I_{Ba-L} \) was investigated using a neuron treated with \( \delta \)-CgTx G‡Y (1 μM) and \( \delta \)-Aga ⧵A (1 μM). The effect of ACh on the \( I_{Ba-N} \) was investigated using a neuron treated with Nif (10 μM) and \( \delta \)-Aga ⧵A (1 μM). The effect of ACh on the \( I_{Ba-P/Q} \) was investigated using a neuron treated with Nif (10 μM) and \( \delta \)-CgTx G‡Y (1 μM). Each of the \( I_{Ba} \) components and the percentage of the inhibition by ACh are summarized in Fig. 3e. Results shown in Fig.3 demonstrate that ACh inhibited \( I_{Ba-N} \) and \( I_{Ba-P/Q} \) in nucleus tractus solitarius neurons.

**Discussion**

This study has shown that ACh inhibits N- and P/Q-types VDCCs via G protein in nucleus tractus solitarius mediated by muscarinic M2 receptors.

ACh inhibited VDCCs currents through the activation of muscarinic M2 receptors via a voltage-sensitive, G-protein-dependent mechanism. ACh-induced \( I_{Ba} \) inhibition was
dose-dependent (Fig. 1), associated with the slowing of activation kinetics (Fig. 1a) and exhibited voltage dependence and prepulse facilitation (Fig. 1a & b). Moreover, I found that Ba inhibition by ACh via G protein (Fig. 2c). I also found that the effects of ACh were relieved, albeit incompletely, by a depolarizing prepulse. Such an effect is usually interpreted as an indication that VDCCs inhibition is mediated by a rapid membrane delimitied pathway, possibly involving an interaction between the G-protein subunits with the VDCCs 1-subunit [11].

Several electrophysiological recordings from nucleus tractus solitarius described an inhibitory effect of ACh on nucleus tractus solitarius. As mentioned above, it has been reported that ACh activates GIRK channels mediated by muscarinic M2 receptors in rostral portion of nucleus tractus solitarius. If the GIRK channels is activated by ACh, so that neuronal activity may be suppressed. In this study, GIRK channels actions were masked, since the Ba introduced for the measurements can block the GIRK channels. In normal state, both inhibition of VDCCs and activation of GIRK channels deduced to occur with ACh on nucleus tractus solitarius. Both caudal nucleus tractus solitarius and rostral nucleus tractus solitarius of rats have shown ACh responses [4]. In this study, it is not possible to identify distinct nucleus tractus solitarius subgroups. It will be important to clarify the mechanisms of integration of such G-protein signallings in the
neuronal excitation.

What is the physiological relevance of ACh in the nucleus tractus solitarius? ACh injected into the nucleus tractus solitarius of rat elicits a decrease in arterial pressure (AP) and heart rate (HR) similar to that seen with activation of the baroreflex [12]. nucleus tractus solitarius neurons can be divided into three groups, GABAergic, glutamatergic and cholinergic [5,13,14]. In this study, ACh-induced inhibition of N- and P/Q-types VDCCs in nucleus tractus solitarius was observed. N-, P- and Q-types VDCCs are implicated in transmitter release [15]. Presynaptic muscarinic M2 receptor depresses glutamate release and ACh release in CNS [16,17]. Allen proposed that auto-inhibition of ACh release via muscarinic M2 receptors may be the main effect in the control of transmitter release in forebrain [17]. Thus, it can be considered that inhibition of N- and P/Q-types VDCCs may inhibit glutamate or ACh release in nucleus tractus solitarius.

**Conclusion**

ACh inhibits N- and P/Q-types VDCCs via G protein subunits mediated by muscarinic M2 receptors in nucleus tractus solitarius.
References


10. Endoh T. Involvement of Src tyrosine kinase and mitogen-activated protein kinase in the facilitation of calcium channels in rat nucleus tractus solitarius by angiotensin


**Fig. 1** ACh-induced inhibition of I$_{Ba}$. (a) Typical superimposed I$_{Ba}$ traces recorded using a double-pulse voltage protocol at the times indicated in the time course graph (b). Paired I$_{Ba}$ were evoked from a holding potential of -80 mV by a 100 msec voltage step to 0 mV at 20 sec intervals. An intervening strong depolarizing prepulse (100 mV, 30 msec) ended 5 msec prior to the second I$_{Ba}$ activation. (b) Typical time course of ACh-induced I$_{Ba}$ inhibition. Opened circle and triangles in the graph indicate I$_{Ba}$ without prepulse and I$_{Ba}$ with prepulse, respectively. ACh (1 μM) was bath-applied during the time indicated by the filled bar. (c) Current-voltage relations and dose-dependency of ACh-induced inhibition of I$_{Ba}$. Current-voltage relations of I$_{Ba}$ evoked by a series of voltage steps from a holding potential of -80 mV to test pulses between -80 mV and +40 mV in +10 mV increments in the absence (opened points) and presence (filled points) of ACh (1 μM). (d) Concentration-response curves for I$_{Ba}$ inhibition induced by ACh. The inhibition (%) was normalized to that induced by ACh at a maximal concentration. The curve was obtained from fitting to a single-site binding isotherm with least-squares nonlinear regression. Numbers in parentheses indicate the number of neurons tested.
**Fig. 2** ACh-induced inhibition of $I_{Ba}$ under various conditions. (a) Typical superimposed $I_{Ba}$ traces at the times indicated in the time course graph (b). (b) Typical time course of ACh-induced $I_{Ba}$ inhibition in a neuron treated with AF-DX116 (muscarinic M2 receptor antagonist, 1 $\mu$M). AF-DX116 (1 $\mu$M) and ACh (1 $\mu$M) were bath-applied during the time indicated by the open and filled bars, respectively. (c) Summary of ACh-induced inhibition of $I_{Ba}$ under various conditions. $I_{Ba}$ inhibition by 1 $\mu$M ACh in control (untreated neurons), after Telenzepine (muscarinic M1 receptor antagonist), after AF-DX116 (muscarinic M2 receptor antagonist), intracellular dialysis with anti-G$q$ antibody, intracellular dialysis with anti-G$s$ antibody and intracellular dialysis with anti-G$q/11$ antibody,
**Fig. 3** ACh-induced inhibition of distinct $I_{Ba}$. (a) Typical superimposed $I_{Ba}$ traces recorded at the times indicated in the time course graph (b). (b) Typical time course of ACh-induced $I_{Ba}$ inhibition in a neuron treated with VDCCs blockers. $\alpha$-CgTx G $\alpha$A (N-type VDCCs blocker, 1 $\mu$M) + $\delta$-Aga $\delta$A (P/Q-types VDCCs blocker, 1 $\mu$M) and ACh (1 $\mu$M) were bath-applied during the time indicated by the open and filled bars, respectively. (c) Typical superimposed $I_{Ba}$ traces recorded using a double-pulse voltage protocol at the times indicated in the time course graph (d). (d) Typical time course of ACh-induced $I_{Ba}$ inhibition in a neuron treated with VDCCs blockers. Nif (L-type VDCCs blocker, 10 $\mu$M) + $\delta$-Aga $\delta$A (P/Q-types VDCCs blocker, 1 $\mu$M) and ACh (1 $\mu$M) were bath-applied during the time indicated by the open and filled bars, respectively. (e) Fractional components of L-, N-, P/Q- and R-types $I_{Ba}$ and those inhibited by ACh (1 $\mu$M). The total height of the bars (open and hatched) represents the mean $\pm$ SEM contribution of the indicated VDCCs type to the total $I_{Ba}$. The hatched bars represent the mean $\pm$ SEM inhibition by ACh of the corresponding VDCCs type. Numbers in parentheses indicate the number of neurons tested.
Inhibition of $I_{Ba}$ by ACh

(a) Voltage-clamp protocol and currents recorded.

(b) Time course of peak $I_{Ba}$ with and without prepulse, showing inhibition by ACh (1 μM).

(c) Summary graph showing peak $I_{Ba}$ as a function of test potential before and after ACh application.

(d) Concentration-dependent inhibition of $I_{Ba}$ by ACh, indicated by percentage inhibition at different concentrations.
Inhibition of $I_{Ba}$ by ACh

(a) Graph showing current traces.
(b) Graph showing time course of peak $I_{Ba}$ with ACh and AF-DX116 in solution.
(c) Bar graph showing percentage inhibition of $I_{Ba}$ by ACh with different treatments.
(b) 
\(\sigma\text{-CgTx GVIA (1 \mu M) + \sigma\text{-Aga IVA (1 \mu M)}, bath solution}\)

- Peak \(I_{ba}\) (pA)

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(d) 
\(\text{Nif (10 \mu M) + \sigma\text{-Aga IVA (1 \mu M), bath solution}}\)

- Peak \(I_{ba}\) (pA)

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