Short communication

Inhibition of voltage-gated K^+ channels and Ca^{2+} channels by diphenidol

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Abstract:

Background: Although diphenidol has long been deployed as an anti-emetic and anti-vertigo drug, its mechanism of action remains unclear. In particular, little is known as to how diphenidol affects neuronal ion channels. Recently, we showed that diphenidol blocked neuronal voltage-gated Na^+ channels, causing spinal blockade of motor function, proprioception and nociception in rats. In this work, we investigated whether diphenidol could also affect voltage-gated K^+ and Ca^{2+} channels.

Methods: Electrophysiological experiments were performed to study ion channel activities in two neuronal cell lines, namely, neuroblastoma N2A cells and differentiated NG108-15 cells.

Results: Diphenidol inhibited voltage-gated K^+ channels and Ca^{2+} channels, but did not affect store-operated Ca^{2+} channels.

Conclusion: Diphenidol is a non-specific inhibitor of voltage-gated ion channels in neuronal cells.

Key words:

diphenidol, voltage-gated K^+ channels, voltage-gated Ca^{2+} channels, N2A cells, NG108-15 cells

Abbreviations: $[Ca^{2+}]_i$ – cytosolic Ca^{2+} concentration, Kv channels – voltage-gated K^+ channels, SOCC – store-operated Ca^{2+} channels, VGCC – voltage-gated Ca^{2+} channels

Introduction

Diphenidol has long been clinically deployed as an anti-emetic and anti-vertigo drug [5, 6, 12, 13]. In the brain, the sites of action on the chemoreceptor trigger zone in the area postrema underlies its anti-emetic effect while its anti-vertigo effects are attributed to its actions on the vestibular apparatus [2]. Nonetheless, the precise cellular/molecular actions of diphenidol in

these neurons remain unclear. There are few reports examining the direct effects of diphenidol on neurons or neuronal cell lines. In one study, diphenidol inhibited rotation-induced firing of feline medial vestibular nucleus type 1 neurons [5], but what specific neuronal receptors and ion channels diphenidol acted on were not elucidated. Diphenidol and its derivatives were shown to act as antagonists at the muscarinic receptors [15]. Recently, we showed that diphenidol could block neuronal voltage-gated Na^+ channels [10], which contributed to diphenidol-induced spinal blockade in rats, encompassing abrogation of motor function, proprioception and nociception [10]. Since voltage-gated K^+ (Kv) channels and voltage-gated Ca^{2+} channels (VGCC) are directly involved in neu-

ronal excitability, we investigated in the present study whether diphenidol could also affect these channels. We employed two neuronal cell lines, namely, N2A cells and differentiated NG108-15 cells, and reported that diphenidol inhibited Kv channels and VGCC.

Materials and Methods

Cell culture and drugs

N2A cells, NG108-15 cells and RBA-1 cells were cultured at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and penicillin-streptomycin (100 units/ml, 100 µg/ml) (Invitrogen). NG108-15 cells were induced to differentiate into more mature neurons by the addition of 0.1% fetal bovine serum, 10 µM retinoic acid and 30 µM forskolin for 3 days. Differentiation was evidenced by a change in neuronal morphology towards the generation of neurites. Diphenidol, forskolin, retinoic acid, verapamil and tetrandrine were purchased from Sigma (St. Louis, MO, USA). Cyclopiazonic acid (CPA) was obtained from Calbiochem (San Diego, CA, USA).

Electrophysiology

Electrophysiological experiments were performed as previously reported [7]. N2A cells were voltage-clamped in the whole-cell configuration. Thin-walled borosilicate glass tubes (OD 1.5 mm, ID 1.10 mm, Sutter Instrument, Novato, CA, USA) were pulled with a micropipette puller (P-87, Sutter Instrument), and then heat polished by a microforge (Narishige Instruments, Inc., Sarasota, FL, USA). For measurement of Kv currents, the intracellular solution contained (mM): 140 KCl, 1 MgCl₂, 1 EGTA, 10 HEPES, and 5 MgATP (pH 7.25 adjusted with KOH). Pipette resistance was 3–6 MΩ. The bath solution contained (mM): 140 NaCl, 4 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES (pH 7.4 adjusted with NaOH). Currents were recorded using an EPC-10 amplifier with Pulse 8.60 acquisition software and analyzed by Pulsefit 8.60 software (HEKA Elektronik, Lambrecht, Germany). Data were filtered at 2 kHz and sampled at 10 kHz. After whole-cell configuration was established, cells were

held at –70 mV and subjected to increasing depolarization pulses (–70 to +70 mV, 10-mV increments) to obtain current-voltage relation curves, or to repetitive depolarization pulses (+30 mV, every 10 s) to examine the inhibitory effects of diphenidol. The latter was added and the effects at equilibrium were quantified to yield a concentration-inhibition curve. All experiments were performed at room temperature (~25°C).

Cytosolic Ca²⁺ concentration measurement

Microfluorimetric measurement of cytosolic Ca²⁺ concentration ([Ca²⁺]_i) was performed using fura-2 AM (Invitrogen, Carlsbad, CA, USA) as a Ca²⁺-sensitive fluorescent dye [7]. Briefly, differentiated NG108-15 cells or RBA-1 astrocytes were incubated with 5 µM fura-2 AM for 1 h at 37°C and then washed and bathed in the bath solution mentioned above. Cells were alternately excited with 340 nm and 380 nm using an optical filter changer (Lambda 10-2, Sutter Instruments). Emission was collected at 500 nm and images were captured using a CCD camera (CoolSnap HQ2, Photometrics) linked to an inverted Nikon TE 2000-U microscope. Images were analyzed with an MAG Biosystems Software. All experiments were performed at room temperature (~25°C).

Statistics

Data are presented as the means ± SEM. Since each cell was treated with a single concentration of diphenidol, paired Student *t*-test was used to compare the current or fluorescence ratio before and after drug treatment. A value of *p* < 0.05 was considered to represent significant difference.

Results and Discussion

We previously reported that diphenidol inhibits voltage-gated Na⁺ channels in N2A cells [10]. In that report, we found that diphenidol blockade of voltage-gated Na⁺ channels was more potent than lidocaine blockade; but unlike lidocaine, Na⁺ channel blockade by diphenidol did not display frequency dependence. Those results led us to postulate that the anesthetic action of diphenidol on the rat spinal cord is at least partly contributed by diphenidol blockade of voltage-

Fig. 1. Diphenidol inhibited Kv currents in N2A cells. **(A–C)** In 3 different cells, Kv currents of N2A cells were triggered by +30 mV pulses and were treated with different concentrations of diphenidol to block the currents. **(D)** Concentration-inhibition curve of diphenidol. For each data point, results represent the mean $\pm$ SEM from 3–4 cells; * indicates significant ($p < 0.05$) inhibition

gated Na⁺ channels [10]. In the present work we examined if diphenidol could also affect Kv channels in N2A cells. Kv channels of N2A cells are of the delayed rectifier type [3], characteristically sensitive to tetraethylammonium and 4-aminopyridine, and composed mainly of subfamily members Kv1.1, Kv1.4 and Kv2.1 [7]. Figure 1 shows that diphenidol inhibited Kv channels in a concentration-dependent manner, with IC₅₀ of 28.2 μM.

We then proceeded to examine for possible effects of diphenidol on VGCC. We could not detect VGCC currents in N2A cells by patch clamp recording [8]. Consistently, microfluorimetric measurements using fura-2 demonstrated very small KCl depolarization-evoked [Ca²⁺]_i responses in both undifferentiated and differentiated N2A cells, indicating very low densities of VGCC [7]. The absence of VGCC currents in N2A cells may be due to the fact that the very small VGCC currents run down rapidly. The limitation of the N2A cell model prompted us to employ the NG108-15 cell model, which after differentiation, would exhibit larger [Ca²⁺]_i rises in response to KCl-elicited depolarization [7]. Nonetheless, electrophysiological recording of differentiated NG108-15 cells still detected very small VGCC currents, which might have run down very rapidly. Thus, we had to abort the electrophysiological approach and rely on the microfluorimetric strategy to examine the effects of diphenidol on Ca²⁺ homeostasis in differentiated NG108-15 cells. NG108-15 cells were induced to differentiate into more mature neurons by incubation in 0.1% fetal bovine serum, 10 μM retinoic acid and 30 μM forskolin

in DMEM medium for 3 days. In Figure 2A, KCl stimulation evoked [Ca²⁺]_i to a plateau which reflected continuous opening of VGCC; and upon addition of verapamil, a specific L-type VGCC antagonist, the [Ca²⁺]_i rise was completely inhibited, thus verifying the [Ca²⁺]_i rise to emanate from influx *via* L-type VGCC. Increasing concentrations of diphenidol induced concentration-dependent inhibition of [Ca²⁺]_i response (Fig. 2B–D, F), indicating that diphenidol inhibited L-type VGCC. Diphenidol (100 μM) itself did not affect [Ca²⁺]_i (Fig. 2E). Future work may be needed to examine if diphenidol also affects other types of VGCC.

Intracellular Ca²⁺ rise emanates not only from influx *via* VGCC but is also strongly contributed by store-operated Ca²⁺ channels (SOCC). SOCCs are gated not by voltage but by the empty state of intracellular Ca²⁺ stores. SOCCs are composed of protein subunits STIM (the sensor for Ca²⁺ content in the Ca²⁺ store) and Orai (the plasma membrane channel pore that is coupled and activated by STIM) [14]. Addition of cyclopiazonic acid (CPA), an inhibitor of sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase, causes depletion of intracellular Ca²⁺ stores, and thus activates SOCC.

We employed these insights to determine if diphenidol might also act on neuronal SOCCs. SOCC signals triggered by CPA in N2A cells and differentiated NG108-15 cells were very small (Leung, unpublished observation). We therefore employed the RBA-1 astrocyte cell line, which would reliably yield a large [Ca²⁺]_i rise to a plateau phase from SOCCs

Fig. 2. Diphenidol inhibited VGCC in differentiated NG108-15 cells. **(A)** The cell was depolarized by addition of 70 mM KCl (iso-osmotic substitution). At the plateau phase, 30 μM verapamil was added. **(B–D)** In different cells, at the plateau phase of the 70 mM KCl-stimulated Ca^{2+} signal, different concentrations of diphenidol were added. **(E)** At 100 μM, diphenidol did not affect $[\text{Ca}^{2+}]_i$. **(F)** Results of the concentration-dependence of inhibition obtained in **B–D** are summarized. For each data point, results represent the mean $\pm$ SEM of 12–14 cells from 6 separate experiments; * indicates significant ($p < 0.05$) inhibition

Fig. 3. Diphenidol did not affect SOCC in RBA-1 cells. **(A)** The cell was treated with 30 μ M CPA to cause depletion of intracellular Ca^{2+} store and activation of SOCC. At the plateau phase, 100 μ M diphenidol was added and had no effect on $[Ca^{2+}]_i$. **(B)** In another CPA-treated cell, 100 μ M tetradrine (TET) was added at the plateau phase and completely inhibited the Ca^{2+} signal. Similar results were obtained in three more experiments

when treated with CPA. The plateau phase of $[Ca^{2+}]_i$ arising from CPA-induced opening of SOCC was not affected at all by the addition of 100 μ M diphenidol (Fig. 3A). Tetradrine, an inhibitor of SOCC (9), was used as a positive control, which at 100 μ M completely inhibited the SOCC (Fig. 3B).

The data above showing that diphenidol could inhibit VGCC, along with our previous report showing that diphenidol inhibited voltage-gated Na^+ channels [10], suggest that these may be the mechanisms by which diphenidol depresses neuronal excitability to account for the anti-emetic and anti-vertigo effects of this drug. Specifically, the inhibition of voltage-gated Na^+ channels might blunt the action potential while blockade of VGCC might affect synaptic release of neurotransmitters. Repolarization of neurons requires

K_v channel activation, thus blockade of K_v channels by diphenidol would be expected to enhance neuronal excitability, which would seem to counter its effects on VGCC and Na^+ channels. We however doubt the pharmacological significance of the K_v channel blockade by diphenidol, which is likely not relevant to the anti-emetic and anti-vertigo actions of diphenidol. Future studies using primary neurons from the area postrema and the vestibular apparatus are warranted in order to directly examine the effects of diphenidol on these ion channels – voltage-gated Na^+ channels, VGCC and K_v channels, thus identifying which putative ion channel(s) in these neurons account for the anti-emetic and anti-vertigo actions of diphenidol.

Of note, diphenidol may have broader therapeutic actions on other tissues beyond the brain and spinal cord. Diphenidol was shown to decrease action potential amplitude and decreases spontaneous rates of feline cardiac atrial cells [4, 11]; these actions might contribute to the antiarrhythmic effect of this drug in ouabain-treated cats [1]. It is expected that diphenidol may also affect voltage-gated ion channels in cardiac ventricular myocytes. It would be intriguing to examine which ion channels are sensitive to diphenidol block in cardiac cells.

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