Natural Product Sciences
12(4) : 217-220 (2006)

Inhibition of Calcium Transport by (1*R*,9*S*)-β-Hydrastine Hydrochloride in PC12 Cells

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Abstract – The effects of (1*R*,9*S*)-β-hydrastine hydrochloride (BHSH) on Ca^{2+} transport in rat pheochromocytoma PC12 cells were investigated. In the presence of external Ca^{2+}, BHSH at 100 μM inhibited K^+ (56 mM)-induced dopamine release, and K^+-induced Ca^{2+} influx and a sustained rise of $[Ca^{2+}]_i$. In addition, BHSH at 100 μM reduced the sustained rise of $[Ca^{2+}]_i$ elicited by 20 mM caffeine, but not by 1 μM thapsigargin, in presence of external Ca^{2+}. These results suggest that BHSH inhibited K^+-induced dopamine release and $[Ca^{2+}]_i$ influx, and store-operated Ca^{2+} channels activated by caffeine, but not by thapsigargin, in PC12 cells.
Keywords – (1*R*,9*S*)-β-Hydrastine hydrochloride, K^+-induced dopamine release, caffeine-stimulated Ca^{2+} release, store-operated Ca^{2+} channel, PC12 cells

Introduction

(1*R*,9*S*)-β-Hydrastine is a phthalide isoquinoline alkaloid and has been isolated from the rhizomes and roots of *Hydrastis canadensis* L. (Ranunculaceae), *Berberis laurina* Billb. (Berberidaceae) and *Corydalis stricta* Steph. (Papaveraceae) (Fang *et al.*, 1981; Tang *et al.*, 1992). Recently, it is reported that (1*R*,9*S*)-β-hydrastine hydrochloride (BHSH) inhibits dopamine biosynthesis by reducing the activity of tyrosine hydroxylase (EC 1.14.16.2), which is the rate-limiting enzyme, in PC12 rat adrenal pheochromocytoma cells (Kim *et al.*, 2001; Yin *et al.*, 2004a). BHSH also inhibits L-DOPA-induced increase in dopamine content in PC12 cells (Yin *et al.*, 2004b). In addition, BHSH decreases the basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PC12 cells (Kim *et al.*, 2001; Yin *et al.*, 2004a). However, the effects of BHSH on Ca^{2+} transport are not precisely determined.

The $[Ca^{2+}]_i$ is influenced by either the fluxes of Ca^{2+} across various cellular membranes through voltage-dependent channels such as L-type, N-type, and P/Q type (Traina *et al.*, 1996; Taylor and Peers, 1999) or the release of Ca^{2+} stores within endoplasmic reticulum (ER) (Berridge, 1993). The Ca^{2+} uptake is mediated by Ca^{2+} pumps (Carafoli and Brini, 2000), which belong to sarco (endo)-plasmic reticulum Ca^{2+} ATPases (SERCA). In addition, the intracellular Ca^{2+} signaling is from store-operated Ca^{2+} channels (SOCs) in the plasma membrane (Berridge, 1993), which can be activated by the depletion of Ca^{2+} stores (Koizumi and Inoue, 1998; Taylor and Peers, 1999).

PC12 cells have been proved to mainly have L-type and N-type voltage-gated Ca^{2+} channels (Taylor and Peers, 1999). PC12 cells also possess multiple Ca^{2+} channel subtypes, including the major Ca^{2+} regulating proteins in the ER and SOCs in the plasma membrane (Koizumi and Inoue, 1998; Taylor and Peers, 1999).

In this study, therefore, to further elucidate the functions of BHSH, the effects of BHSH on intracellular Ca^{2+} transport using PC12 cells as a model system were investigated.

Experimental

Materials – BHSH, thapsigargin, caffeine, Fura-2 AM, HEPES, EGTA and sulfinpyrazone were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All sera, antibiotics and RPMI 1640 for cell culture were obtained from Gibco (Grand Island, NY, USA). All other chemicals were of the reagent grade.

Cell culture – PC12 cells were routinely grown in

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Abbreviation used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; ER, endoplasmic reticulum; PC12 cells, rat adrenal pheochromocytoma cells; SERCA: sarco (endo)-plasmic reticulum Ca^{2+} ATPase; SOC, store-operated calcium channel.

RPMI medium 1640 supplemented with 10% heat-inactivated horse serum, 5% heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin, and incubated at 37 °C in 5% CO_2/humidified air (Tischler *et al.*, 1983).

Measurement of dopamine release – PC12 cells (ca. 2×10^6 cells) were maintained for 15 min at 37 °C in Ca^{2+} solution (consisting of NaCl 135 mM, KCl 5 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 1.2 mM, glucose 10 mM and HEPES 10 mM, pH adjusted to 7.4 with NaOH) containing BHSH, and then added K^+ to 56 mM (the concentration of NaCl was reduced proportionally to maintain osmolarity) and incubated for 10 min. Dopamine content in the media was determined using an HPLC method as reported previously (Mitsui *et al.*, 1985; Kim *et al.*, 2001).

Measurement of $[Ca^{2+}]_i$ – PC12 cells (ca. 3×10^6 cells/ml) were loaded with 4 μM of Fura-2 AM at 37 °C for 30-60 min (Grynkiewicz *et al.*, 1985; Kim *et al.*, 2001). The cells were transferred into a quartz cuvette and the fluorescence intensity was measured with dual excitation wavelengths of 340 nm and 380 nm, and an emission wavelength of 510 nm (Ratio Master PTI, Brunswick, NJ, USA). Either the ratio or the levels of intracellular calcium were calculated as described (Grynkiewicz *et al.*, 1985; Harper *et al.*, 2000).

Statistical analysis – Data are presented as means ± SEM of at least four experiments. Statistical analysis was performed using ANOVA followed by Tukey's test and unpaired t-test with $p < 0.05$ being considered significant.

Results and Discussion

BHSH at 10 - 100 μM did not alter dopamine release in PC12 cells (Fig. 1A). However, BHSH at 10 - 100 μM inhibited K^+ (56 mM)-induced dopamine release in a concentration-dependent manner (Fig. 1B). BHSH at concentration up to 250 μM did not show any cytotoxicity towards PC12 cells (Yin *et al.*, 2004c).

The release of dopamine is induced by K^+ depolarization associated with a rapid and sustained increase in $[Ca^{2+}]_i$ (Koizumi and Inoue, 1998; Hirota *et al.*, 2000). In addition, Ca^{2+} transport is proved to be mediated via L-type Ca^{2+} channels in undifferentiated PC12 cells (Usowicz *et al.*, 1990). BHSH at 100 μM inhibited K^+-induced Ca^{2+} influx and the sustained rise of $[Ca^{2+}]_i$ in the presence of external Ca^{2+} in PC12 cells (Fig. 2A). Nifedipine at 2 μM, an inhibitor of L-type Ca^{2+} channels, also reduced K^+ (56 mM)-induced Ca^{2+} influx (Fig. 2B), and the further addition of 100 μM BHSH slightly decreased $[Ca^{2+}]_i$ (Fig. 2B). These results suggested that the reduction of K^+-induced dopamine release by BHSH was mainly mediated through the blockade of L-type Ca^{2+} channels in PC12 cells.

In addition, Ca^{2+} influx from SOCs in the excitable tissues can trigger the release of a neurotransmitter via exocytosis (Taylor and Peers, 1999). A SOC stimulator caffeine elicits catecholamine release from PC12 cells through Ca^{2+} influx by depleting Ca^{2+} stores in the presence of external Ca^{2+} (Avidor *et al.*, 1994). SOCs in PC12 cells can also be stimulated by a SERCA inhibitor thapsigargin.

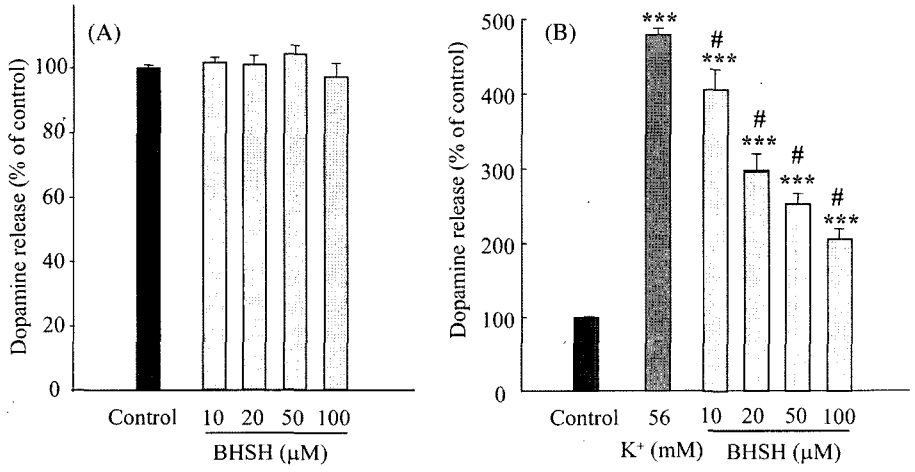

Fig. 1. Effects of (1*R*,9*S*)-β-hydrastine hydrochloride (BHSH) on basal and K^+-induced dopamine release from PC12 cells. PC12 cells were treated with BHSH (10, 20, 50 and 100 μM) for 15 min (A) and then further treated with 56 mM KCl for 10 min (B) at room temperature. Dopamine content of the control in media was 0.15 ± 0.03 nmol/mg protein, which was measured using an HPLC method. Results represent means ± SEM of 5-7 dishes. * $p < 0.001$ compared with the control, # $p < 0.05$ compared with K^+-treated sample (ANOVA followed by Tukey's test).

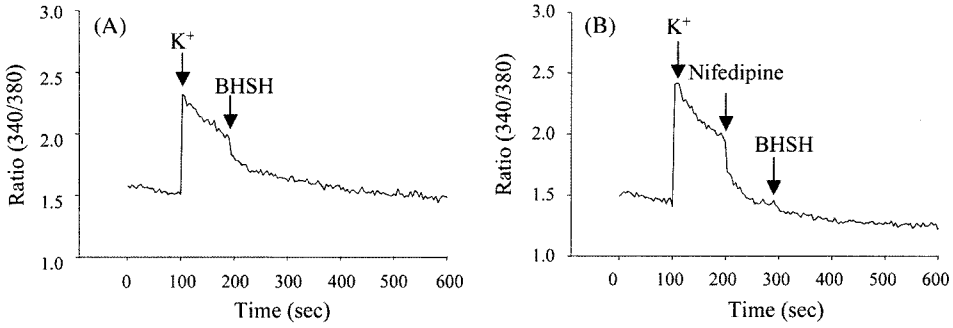

Fig. 2. Effects of BHSH on $[Ca^{2+}]_i$ in PC12 cells. PC12 cells were loaded with fura-2 AM for 30-60 min and $[Ca^{2+}]_i$ was measured as described under Experimental. The cells were treated with 56 mM K^+ (A) and application of 100 μM BHS followed with 2 μM nifedipine (B). The data shown are representative tracings from three independent experiments.

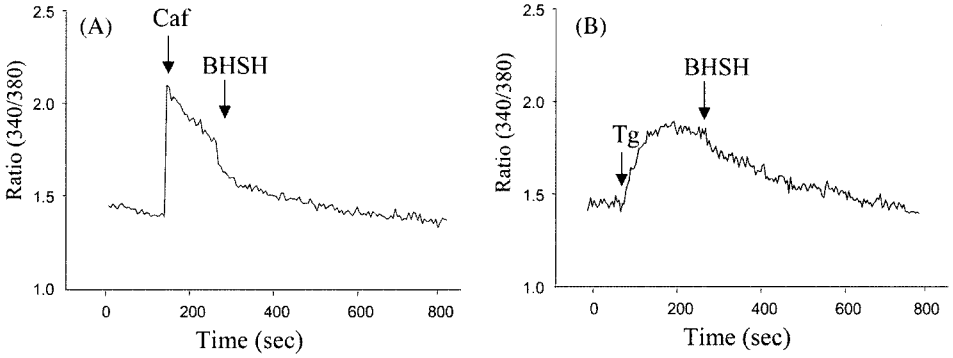

Fig. 3. Effects of BHSH on SOCs activated by 20 mM caffeine (Caf) and 1 μM thapsigargin (Tg) in the presence of external Ca^{2+} in PC12 cells. The data shown are representative tracings from three independent experiments.

Therefore, the effects of BHSH on store-operated Ca^{2+} influx using caffeine and thapsigargin in PC12 cells were investigated.

In the presence of external Ca^{2+}, caffeine stimulation caused a biphasic rise in $[Ca^{2+}]_i$ consisting of a rapid elevation of $[Ca^{2+}]_i$ due to the release of Ca^{2+} from caffeine-sensitive stores, followed by a plateau phase of slower kinetics during which $[Ca^{2+}]_i$ remains elevated (Fig. 3A) (Koizumi and Inoue, 1998). However, BHSH at 100 μM significantly reduced the sustained rise of $[Ca^{2+}]_i$ elicited by 20 mM caffeine in the presence of external Ca^{2+} in PC12 cells (Fig. 3A). A SERCA inhibitor thapsigargin also causes a slow and sustained rise of $[Ca^{2+}]_i$ due to the activation of SOCs, which is interpreted as a sign of Ca^{2+} leakage from the stores (Treiman *et al.*, 1998; Bouron, 2000). Thapsigargin (1 mM)-induced the sustained rise of $[Ca^{2+}]_i$ was slightly reduced by treatment with 100 μM BHSH, however, the values were not statistically significant (Fig. 3B).

In the presence of external Ca^{2+}, the addition of BHSH (20-100 μM) and (1*R*,9*S*)-β-hydrastine (20-100 μM) caused a decrease in $[Ca^{2+}]_i$ in a concentration-dependent manner in PC12 cells (Kim *et al.*, 2001: Yin *et al.*, 2004b). In addition, in the absence of external Ca^{2+}, BHSH at 100 μM also decreased the basal $[Ca^{2+}]_i$ and the decreased potency was similar to those obtained with in the presence of external Ca^{2+} (data not shown). Therefore, it has been suggested that BHSH had no effect on Ca^{2+} influx from extracellular milieu to cytosol in PC12 cells. Furthermore, in the absence of external Ca^{2+}, the reintroduction of 2 mM $CaCl_2$ causes a rapid and sustained elevation of $[Ca^{2+}]_i$ after the intracellular Ca^{2+} stores are depleted by both 20 mM caffeine and 1 μM thapsigargin in PC12 cells (Koizumi and Inoue, 1998; Treiman *et al.*, 1998). Previously, it has been reported that pretreatment of the cells with 100 μM (1*R*,9*S*)-β-hydrastine significantly decreased the sustained rise in $[Ca^{2+}]_i$ elicited by Ca^{2+} reintroduction after Ca^{2+} store was depleted with 20 mM caffeine, but not thapsigargin, in the absence of external Ca^{2+} (Yin *et al.*, 2003). These results suggest that BHSH has an inhibitory activity of the release of Ca^{2+} from SOCs stimulated by caffeine, but not by thapsigargin, in PC12 cells.

In conclusion, our results indicated that BHSH reduced

K^+-induced Ca^{2+} influx in PC12 cells. BHSH also inhibited the activation of caffeine-sensitive Ca^{2+} stores, but not affect Ca^{2+} leakage from Ca^{2+} stores, in PC12 cells. These results also suggest that the inhibitory properties of $[Ca^{2+}]_i$ by BHSH can regulate dopamine biosynthesis and lower intracellular cytotoxicity in PC12 cells.

Acknowledgements

The authors sincerely thank the financial support by the program of Research Center for Bioresource and Health, KOSEF and ITEP (2004-2005).

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(Accepted October 4, 2006)