Effects of dichlorobenzene on acetylcholine receptors in human neuroblastoma SH-SY5Y cells

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ABSTRACT

para-Dichlorobenzene (DCB), a deodorant and an industrial chemical, is a highly volatile compound and is known to be an indoor air contaminant. Because of its widespread use and volatility, the toxicity of DCB presents a concern to industrial workers and public. Some toxic aspects of DCB have already been focused but its effects on neuronal signal transduction have been hitherto unknown. The effects of DCB on the cytosolic calcium homeostasis are investigated in human neuroblastoma SH-SY5Y cells in this study. DCB, above 200 μ M, was found to induce a rise in cytosolic calcium concentration that could not be counteracted by nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptor (mAChR) antagonists but was partially inhibited by thapsigargin. To understand the actions of DCB on the acetylcholine receptors, we investigated its effects on the changes of cytosolic calcium concentration following nicotinic AChR stimulation with epibatidine and muscarinic AChR stimulation with methacholine in human neuroblastoma SH-SY5Y cells. DCB inhibited the cytosolic calcium concentration rise induced by epibatidine and methacholine with respective IC_{50} s of 34 and 294 μ M. The inhibitions of DCB were not the same as thapsigargin's inhibition. In the electrophysiological observations, DCB blocked the influx currents induced by epibatidine. Our findings suggest that DCB interferes with the functional activities of AChR, including its coupling influx currents and cytosolic calcium elevations.

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1. Introduction

para-Dichlorobenzene (DCB) has been widely used as a deodorant and a moth repellent, and as an intermediate for dyestuff, fungicides, pharmaceuticals, and industrial chemicals. DCB is a highly volatile compound, with a vapor pressure of 1 mmHg at 25 °C (Hayes, 1982) and is an indoor air contaminant (Wallace et al., 1987, 1988, 1989; Hartwell et al., 1992; Kostianen, 1995). The major portion of DCB used finds its way into the atmosphere (IARC, 1982). Because of its widespread use and volatility, the toxicity of DCB presents a concern to industrial workers and the public. DCB has been detected in adipose tissues (Morita and Ohi, 1975; Jan, 1983),

blood (Morita and Ohi, 1975; Hill et al., 1995) and mothers' milk (Jan, 1983) in the general population, illustrating the widespread human exposure to the compound. In animal studies, DCB causes hepatocarcinogenicity including hepatocellular carcinoma, hepatoblastoma (NTP, 1987), and kidney damage (Steinmetz et al., 1988). The primary route for the contact of DCB is inhalation, and olfactory mucosal lesions induced by DCB had been found (Bahrami et al., 1999). A major DCB metabolite, 2,6-dichlorophenol, has been detected in olfactory bulb and olfactory mucosa, and has been linked to increased glial fibrillary acidic protein in the olfactory bulb of mice (Carlsson et al., 2003). The nasal cavity contains an olfactory neuron, which links with an interneuron and is used to relay information to the brain; therefore, neuronal signal transduction is important. Moreover, the airway possesses neuronal receptors such as the nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptor (mAChR). The roles of DCB on nAChR and mAChR are also important. However, the effects of DCB on neuronal signal transduction have been hitherto unknown.

DCB has estrogenic effects and affects animal endocrine functions through its action as a xenoestrogen (Versonnen et al., 2003).

Abbreviations: $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; DCB, *para*-dichlorobenzene; EPI, epibatidine; AChR, acetylcholine receptor; nAChR, nicotinic acetylcholine receptor; mAChR, muscarinic acetylcholine receptor; VOCC, voltage-operated Ca^{2+} channel; IP₃, inositol triphosphate; CICR, Ca^{2+} -induced Ca^{2+} release.

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Estrogen not only has long-term genomic effects but also has various non-genomic actions (Chen and Farese, 1999; Kelly and Levin, 2001). These non-genomic effects are characterized by rapid time courses and the modulation of membrane receptors and membrane channels in muscle cells and neuronal cells. Estrogen acts on nAChR (Ke and Lukas, 1996) and mAChR (Cardoso et al., 2004). Using estrogen receptor-mediated estrogenic-like activities as a springboard, some xenoestrogens have been found to act at membrane receptors. AChRs are widely distributed in the nervous system (e.g., muscle, autonomic ganglia, and the central nervous system) (Sastry and Sadavongvivad, 1978) and include nAChR belonging to ligand-gated ion channel superfamily of neurotransmitter receptors and mAChRs belonging to GTP-binding protein coupled receptors. Both nAChR and mAChR play critical roles in brain and body functions (Belmonte, 2005), as well as in developmental processes (Hamassaki-Britto et al., 1994; Grant and Landis, 1991) and are the targets of steroid hormones such as estradiol (Arias, 1998) and endocrine disruptors such as phthalates (Liu and Lin, 2002).

Human SH-SY5Y neuroblastoma cells have a various characteristics of sympathetic ganglion cells and are widely used in the studies of a variety of Ca^{2+} signaling pathways, including voltage-dependent Ca^{2+} entry and receptor-mediated Ca^{2+} homeostasis (Lambert et al., 1990; Toselli et al., 1991; Passafaro et al., 1992). The SH-SY5Y cells possess various subtypes of nAChR (Lukas et al., 1993; Ke and Lukas, 1996) and mAChR (Lambert et al., 1990), therefore, these cells represent an ideal system to study not only the function of specific Ca^{2+} signaling, but also to study the activities of AChRs. Using human SH-SY5Y neuroblastoma cells, the possibility of DCB acting on AChRs was presently investigated.

2. Material and methods

2.1. Chemicals

DCB, 17α -estradiol, carbachol, epibatidine (EPI), methacholine, atropine, digitonin, hexamethonium, thapsigargin, verapamil, deoxyribonuclease I and EGTA were all obtained from Sigma Chemical Co. Fura-2 AM was obtained from Molecular Probes. DCB was dissolved in DMSO first and then diluted (at least 100 folds) into the concentration we used in loading buffer.

2.2. Cell culture

The human neuroblastoma SH-SY5Y cells, obtained from ATCC (CRL-2266), were cultured in a minimally essential medium and F12 medium (1:1), supplemented with 10% fetal bovine serum and 100 U penicillin/streptomycin, and grown in a 5% CO_2 humidified incubator at 37°C (Lambert et al., 1990). The medium was changed every 3–4 days and the cells were subcultured every 7 days. The confluent cells were collected to process the cytosolic calcium concentration ($[\text{Ca}^{2+}]_c$) measurements.

2.3. $[\text{Ca}^{2+}]_c$ measurement

The human neuroblastoma SH-SY5Y cells were loaded with fura-2 by incubation (5×10^6 cells/ml) with $10 \mu\text{M}$ fura-2 acetoxymethyl ester at 37°C for 30 min. Cells were then washed twice with loading buffer contained (in mM) NaCl 150; KCl 5, Glucose 5, MgCl_2 1, CaCl_2 2.2, and HEPES 10, pH 7.4. The cells were stimulated with agonists in the presence or absence of DCB in loading buffer or Ca^{2+} free loading buffer (containing (in mM) NaCl 150; KCl 5, Glucose 5, MgCl_2 1 and HEPES 10, pH 7.4). In high K^+ stimulations, 50 mM KCl was added to the cells. The fluorescent measurements were performed using a dual-excitation fluorometer (SPEX, CM system) at 340 and 380 nm excitation and 505 nm emission. $[\text{Ca}^{2+}]_c$ was calculated using a fluorescence ratio at 340 nm excitation to that at 380 nm (Grynkiewicz et al., 1985). R_{max} was achieved by adding 0.01% digitonin to the cuvette at the end of experiments; excess EGTA was subsequently added to obtain R_{min} . A K_d of 224 nM Ca^{2+} for fura-2 was used (Grynkiewicz et al., 1985; Vainio et al., 2000). Each data point were performed by five individual experiments in each protocol by using different cell batches and each experiment was carried out at least in duplicate.

2.4. Electrophysiological measurements

For whole-cell recording, the cell was whole-cell patch clamped as described before by Hamill et al. (1981). Patch pipettes were pulled from thin-wall capillaries with filament (Catalog 617000, A-M Systems Inc., WA, USA) using a two-stage microelectrode puller (P-97, Sutter Inc., USA), and fire-polished with a microforge

(MF-830, Narishige, Japan). When filled with pipette solution, the resistance ranged between 3 and 5 $\text{M}\Omega$. Ionic currents were measured from whole-cell patch-clamped cells using an EPC10 patch-clamp amplifier (HEKA GmbH, Germany) and controlled by Pulse (HEKA GmbH, Germany) program. To record the inward currents elicited by $10 \mu\text{M}$ of EPI, cells were incubated in medium containing different concentrations of DBP and voltage-clamped at -70 mV . The patch pipette was filled with a K^+ -containing solution (in mM): 130 K-aspartate, 20 KCl, 1 MgCl_2 , 0.1 EGTA, 3 Na_2ATP , 0.1 Na_2GTP and 20 Hepes pH 7.3. EPI was puffed onto the patched cell from a micropipette with an opening diameter of about $1 \mu\text{m}$ positioned at $10 \mu\text{m}$ away from the cell for 1 s under the control of a picospritzer (General Valve, Fairfield, NJ).

3. Results

3.1. Influence of DCB on induction of $[\text{Ca}^{2+}]_c$ in SH-SY5Y cells

DCB dose-dependently induced an increase of $[\text{Ca}^{2+}]_c$ that was sustained at a high level over a long period in human neuroblastoma SH-SY5Y cells in loading buffer containing 2.2 mM CaCl_2 (Fig. 1). DCB ($500 \mu\text{M}$) induced a net $[\text{Ca}^{2+}]_c$ increase of $191.6 \pm 29.8 \text{ nM}$ ($n = 16$). The elevation of $[\text{Ca}^{2+}]_c$ might have been due to the intracellular release of Ca^{2+} from internal stores, or extracellular Ca^{2+} influx. Hence, the extracellular Ca^{2+} was depleted to exclude the possibility of extracellular Ca^{2+} influx. EGTA was added into the Ca^{2+} free loading buffer to chelate the extra Ca^{2+} . In this case, DCB dose-dependently induced an increase in the $[\text{Ca}^{2+}]_c$, followed by a quick decay in the absence of extracellular Ca^{2+} (Fig. 1). DCB ($500 \mu\text{M}$) induced a net $[\text{Ca}^{2+}]_c$ increase of $60.67 \pm 9.33 \text{ nM}$ ($n = 8$) in the absence of extracellular Ca^{2+} . Ca^{2+} influx likely played a role in the DCB-induced $[\text{Ca}^{2+}]_c$ peak because the $[\text{Ca}^{2+}]_c$ level was much higher in the presence of extracellular Ca^{2+} compared with that in a Ca^{2+} -free buffer (Fig. 1).

Specific agonists and antagonists of mAChR, nAChR, and voltage-operated Ca^{2+} channels (VOCC) were used to determine if the DCB-induced increase of $[\text{Ca}^{2+}]_c$ is coupled to these specific receptors or channels, since human SH-SY5Y cells possess mAChRs, nAChRs, and VOCC in their plasma membrane. An elevation of $[\text{Ca}^{2+}]_c$ levels was obtained when the cells were stimulated with 0.3 mM methacholine (a muscarinic receptor agonist) or $10 \mu\text{M}$ epibatidine (a nicotinic receptor agonist) (Fig. 2). The methacholine-induced and epibatidine-induced increases of $[\text{Ca}^{2+}]_c$ were completely inhibited by $20 \mu\text{M}$ atropine or $100 \mu\text{M}$ hexamethonium, respectively. A KCl level of 100 mM, which is known to elevate membrane potential by opening the voltage-sensitive Ca^{2+} channels, caused an increase of $[\text{Ca}^{2+}]_c$ levels that was depressed by 0.1 mM verapamil, a blocker of VOCC. The increase of $[\text{Ca}^{2+}]_c$ induced by $500 \mu\text{M}$ DCB remained the same in the presence of $20 \mu\text{M}$ atropine ($104 \pm 15\%$ of control, $n = 3$), 0.1 mM hexamethonium ($105 \pm 25\%$ of control, $n = 3$), or 0.1 mM verapamil ($114 \pm 27\%$ of control, $n = 6$) (Fig. 2).

3.2. Effects of DCB on Ca^{2+} signaling coupled with nAChR, mAChR and VOCC

To investigate the effects of DCB on acetylcholine receptors, we used carbachol to stimulate AChR in human neuroblastoma SHSY5Y cells. As shown in Fig. 3, the carbachol-induced $[\text{Ca}^{2+}]_c$ increase was inhibited by DCB in a dose-dependent manner ($\text{IC}_{50} = 161 \mu\text{M}$). DCB at $50 \mu\text{M}$ significantly inhibited the carbachol-induced $[\text{Ca}^{2+}]_c$ rise ($p < 0.05$, paired t test comparing with control).

nAChRs and mAChRs differ in their signal transduction pathways: nAChR acts as a ligand-gated ion channel while intracellular Ca^{2+} release triggered by inositol triphosphate (IP_3) is associated with mAChR subtypes 3 and 5 (Zeng and Wess, 2000). To distinguish the inhibition of DCB on acetylcholine receptors, we used EPI and methacholine – specific ligands for nAChR and mAChR, respectively – in our experiments. Fig. 3 shows that DCB dose-

Fig. 1. DCB induces $[Ca^{2+}]_c$ increase in human SH-SY5Y cells in the presence (A and B) or absence (C and D) of extracellular calcium. Panel A: Cells loaded with fura-2 were treated with DCB at various concentrations in a loading buffer containing 2.2 mM $CaCl_2$. Panel B: The average net elevation of $[Ca^{2+}]_c$ induced by DCB was calculated from experiments carried as panel A in a Ca^{2+} containing loading buffer. The n symbol denotes the number of experiments using different batches of cells. Panel C: Cells loaded with fura-2 were treated with DCB at various concentrations in a Ca^{2+} free buffer. To deplete the buffer of Ca^{2+} , a Ca^{2+} free buffer was used and 0.25 mM EGTA was added to chelate extra Ca^{2+} . Panel D: The average net elevation of $[Ca^{2+}]_c$ induced by DCB was calculated from experiments similar as panel C in a Ca^{2+} free loading buffer.

Fig. 2. The elevation level of $[Ca^{2+}]_c$ induced by DCB remains in the presence of atropine, hexamethonium, or verapamil. Panel A: Cells loaded with fura-2 were stimulated with 0.3 mM methacholine in the presence (right striated bars, meth. + atr.) or absence (open bars, meth.) of 20 μ M atropine. Cells were treated with 500 μ M DCB in the presence (double striated bars, DCB + atr.) or absence (left striated bars, DCB) of 20 μ M atropine. Panel B: Cells were stimulated with 10 μ M epibatidine in the presence (right striated bars, epi. + hex.) or absence (open bars, epi.) of 0.1 mM hexamethonium. Cells were treated with 500 μ M DCB in the presence (double striated bars, DCB + hex.) or absence (left striated bars, DCB) of 0.1 mM hexamethonium. Panel C: Cells were stimulated with 50 mM high K solution in the presence (right striated bars, K + ver.) or absence (open bars, K) of 0.1 mM verapamil. Cells were treated with 500 μ M DCB in the presence (double striated bars, DCB + ver.) or absence (left striated bars, DCB) of 0.1 mM verapamil.

independently inhibited the EPI-induced $[Ca^{2+}]_c$ increase with an IC_{50} of 34 μ M. At 50 μ M, DCB had little discernable influence on basal $[Ca^{2+}]_c$ but significantly inhibited EPI-induced $[Ca^{2+}]_c$ increase (inhibitory rate $53.37 \pm 6.95\%$, $n=8$). The inhibition of 17 α -estradiol on the EPI-induced $[Ca^{2+}]_c$ increase was also found with an IC_{50} as 6.5 μ M (data not shown). Moreover, DCB dose-dependently inhibited the methacholine-induced $[Ca^{2+}]_c$ rise with an IC_{50} of 294 μ M (Fig. 4). At 100 μ M, DCB slightly inhibited the methacholine-induced $[Ca^{2+}]_c$ increase (inhibitory rate $21.14 \pm 2.08\%$ of control, $n=8$), but exerted a more marked inhibitions on nAChR.

To determine if DCB interacts with voltage-operated Ca^{2+} channel, we examined the effects of DCB on the KCl-induced $[Ca^{2+}]_c$ increase. As shown in Fig. 4, 50 μ M DCB inhibited the KCl-induced $[Ca^{2+}]_c$ increase (inhibitory rate $20.8 \pm 5.2\%$ of control, $n=4$). DCB at 400 μ M totally inhibited the KCl-induced $[Ca^{2+}]_c$ increase. A DCB IC_{50} of 165 μ M was necessary to inhibit the KCl-induced $[Ca^{2+}]_c$ increase.

3.3. Effects of DCB on Ca^{2+} signaling coupled with intracellular Ca^{2+} release

Effects of DCB on endoplasmic reticulum Ca^{2+} stores were investigated. Thapsigargin is a well-known inhibitor of endoplasmic reticulum Ca^{2+} -ATPase. The stimulation of thapsigargin induced a rise in $[Ca^{2+}]_c$ (Fig. 5A). The cells were treated with DCB at various concentrations for 10 min and then stimulated with thap-

Fig. 3. Effect of DCB on $[Ca^{2+}]_c$ changes induced by carbachol or epibatidine in human neuroblastoma SH-SY5Y cells. Cells loaded with fura-2 were stimulated with 0.3 mM carbachol ($\uparrow$ CCH, in panel A) or 10 μ M epibatidine ($\uparrow$ epi, in panel B) 100 s after the addition of 50 μ M DCB (red line) or vehicle (black line). Panels C and D show the inhibitory potency of DCB on $[Ca^{2+}]_c$ increase induced by carbachol and epibatidine, respectively. Data is present as percentage of control response $\pm$ S.E.M. where a 100% response represents the difference between the $[Ca^{2+}]_c$ increase induced by 0.3 mM carbachol or 10 μ M epibatidine. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

sigargin. The stimulation of thapsigargin could not induce further Ca^{2+} release from endoplasmic reticulum in the cells pretreated with DCB at concentrations above 400 μ M, but thapsigargin still evoked a $[Ca^{2+}]_c$ increase after pretreatment with DCB concentrations of 100 or 200 μ M (Fig. 5B–E). Moreover, DCB was still capable to induce a rise in $[Ca^{2+}]_c$ after the cells pretreated with thapsigargin for 10 min (Fig. 5F).

The effects of thapsigargin on the $[Ca^{2+}]_c$ increase induced by epibatidine and methacholine were investigated. Thapsigargin inhibited the $[Ca^{2+}]_c$ rise induced by methacholine (Fig. 6). We integrated the peak area (150 s) under the stimulation of epibatidine or methacholine and found thapsigargin, at 2.5 μ M, inhibited the peak induced by epibatidine and methacholine from 100% peak area to 74 and 3% peak area, respectively.

3.4. Effects of DCB on electrophysiological changes under the stimulation of nAChR

The earlier results suggest that DCB acts on the nAChR. To further confirm that DCB inhibited the EPI-induced Ca^{2+} signals occurred directly by its effects on the nAChR, the inward current upon stimulation of nAChR by EPI was recorded. Fig. 7A shows the representative current traces from a voltage clamped cell stimulated by 10 μ M EPI for 0.2 s in the absence or presence of 100 or

500 μ M DCB. DCB inhibited the EPI-induced inward current. Fig. 7B shows the averaged total influx current under the stimulation of nAChR by EPI. DCB significantly blocked the EPI-induced influx current. The results illustrate that DCB exerts its effect directly on the nAChR.

4. Discussion

In this study, we found several novel characteristics of DCB modulate neuronal $[Ca^{2+}]_c$ homeostasis. Firstly, DCB induces a $[Ca^{2+}]_c$ elevation and the source of Ca^{2+} including extracellular Ca^{2+} influx and intracellular Ca^{2+} release. A rise in $[Ca^{2+}]_c$ was observed after the addition of DCB in a buffer with or without Ca^{2+} (Fig. 1). Secondly, DCB inhibited the Ca^{2+} signaling coupled with the stimulation of AChRs including nAChRs and mAChRs, as evidenced by the inhibition of DCB in the $[Ca^{2+}]_c$ increase induced by carbachol, epibatidine, and methacholine. The inhibition of DCB on the activities of nAChR was also demonstrated by the electrophysiological measurements. The influx current coupled with nAChR can be blocked by DCB. Thirdly, DCB inhibited the Ca^{2+} signaling coupled with the K^+ -mediated activation of VOCC. The data reveals that DCB potentially interferes with $[Ca^{2+}]_c$ homeostasis and the functional activities of AChRs and VOCCs in human neuroblastoma SH-SY5Y cells. As far as we

Fig. 4. Effect of DCB on $[Ca^{2+}]_c$ changes induced by methacholine or high K^+ concentration in human neuroblastoma SH-SY5Y cells. Cells loaded with fura-2 were stimulated with 0.3 mM methacholine ($\uparrow$ Mch, in panel A) or 50 mM KCl solution ($\uparrow$ K, in panel B) 125 s after the addition of 50 μ M DCB (red line) or vehicle (black line). Panels C and D show the inhibitory potency of DCB on $[Ca^{2+}]_c$ increase induced by methacholine and high K^+ , respectively. Data is present as percentage of control response $\pm$ S.E.M. where a 100% response represents the difference between the $[Ca^{2+}]_c$ rise induced by 0.3 mM methacholine or 50 mM KCl. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

know, this is the first description of these three characteristics of DCB.

Precise regulation of Ca^{2+} homeostasis is essential to cellular functions and viability. We used thapsigargin as a tool to study the roles of DCB in the modulation of intracellular Ca^{2+} homeostasis. Our data shows that thapsigargin could not totally inhibit the DCB-induced $[Ca^{2+}]_c$ rise. We suggest that DCB induces Ca^{2+} release not only from thapsigargin-sensitive intracellular Ca^{2+} stores, but also from other thapsigargin-insensitive intracellular Ca^{2+} stores. However, DCB could completely deplete the Ca^{2+} stored in the endoplasmic reticulum, as shown by the lack of thapsigargin response after the pretreatment of DCB. Nonylphenol, an endocrine disruptor with estrogen-like activities, is an inhibitor of the endoplasmic reticulum Ca^{2+} -pump (Michelangelo et al., 1990). DCB has estrogen-like activities (Versonnen et al., 2003). Although estradiol has no such propensity to raise $[Ca^{2+}]_c$, DCB acts similarly to nonylphenol in releasing Ca^{2+} from the endoplasmic reticulum. Although the Ca^{2+} releasing characteristic of DCB is evidenced in this study, the possibility of DCB acting on the endoplasmic reticulum membrane needs further study.

Presently, we show that DCB can inhibit the Ca^{2+} signaling of AChRs including nAChR and mAChR. SH-SY5Y neuroblastoma cells predominantly possess mAChR subtype 3, which links with the activation of phospholipase C and the production of IP_3 which

triggers internal Ca^{2+} release (Koenig and Edwardson, 1996). The inhibition capability of DCB might come from its Ca^{2+} releasing character. In order to clarify this, we observed the inhibition capability of thapsigargin. Thapsigargin at 2.5 μ M almost depleted the Ca^{2+} stored in the endoplasmic reticulum and inhibited 97% of the methacholine-induced Ca^{2+} peak. DCB inhibited the internal thapsigargin-sensitive Ca^{2+} pools in a dose-dependent manner. DCB at 300 μ M DCB inhibited by 50% the thapsigargin-sensitive Ca^{2+} pools. The IC_{50} of DCB on methacholine-induced $[Ca^{2+}]_c$ increase was 294 μ M. We predict the Ca^{2+} -releasing character of DCB plays an important role in its inhibition of mAChR. DCB has a stronger inhibition on nAChR than on mAChR. However, thapsigargin only has a very smaller inhibition on nAChR. We predict that the inhibition of DCB on nAChR is related not only to the characteristics of DCB releasing Ca^{2+} from endoplasmic reticulum but to other characteristics of DCB.

Based on the present observations, we suggest three routes are responsible for DCB inhibition of nAChR Ca^{2+} signaling. First, DCB acts as a channel inhibitor to block the nAChR ion channel. This characteristic might arise from its estrogenic-like activities (Versonnen et al., 2003). Estradiol and other steroid hormones are able to alter neuronal excitability by augmenting or inhibiting neurotransmitter-activated responses mediated via receptor-gated ion channels (Chen and Farese, 1999; Arias, 1998). Estradiol as

Fig. 5. Effects of DCB and thapsigargin on the changes of $[Ca^{2+}]_c$. Fura-2 loaded cells were treated with vehicle (panel A), 100 μ M DCB (panel B), 200 μ M DCB (panel C), 400 μ M DCB (panel D), and 500 μ M DCB (panel E) for 10 min. Cells were then stimulated with 2.5 μ M thapsigargin in panels A–E. In panel F, the cells were stimulated with 2.5 μ M thapsigargin for 10 min, then stimulated with 500 μ M DCB.

Fig. 6. Effect of thapsigargin on $[Ca^{2+}]_c$ changes induced by epibatidine or methacholine in human neuroblastoma SH-SY5Y cells. Cells loaded with fura-2 were stimulated with 10 μ M epibatidine ($\uparrow$ EPI, in panel A) or 0.3 mM methacholine ($\uparrow$ Mch, in panel B) 270 s after the addition of 2.5 μ M thapsigargin (line b) or vehicle (line a).

Fig. 7. EPI-induced inward current was inhibited by DCB. The cell was voltage-clamped in order to record whole-cell current. Panel A: Using ejection pipette, 10 μ M EPI was applied to the clamped cell in the absence (trace a) or presence of 0.1 mM DCB (trace b) or 0.5 mM DCB (trace c). Panel B: The total current influx induced by 10 μ M EPI in loading buffer (LB) with DCB at various concentrations. Data are collected from two batches cells ($n = 12-18$). * $P < 0.01$, paired t -test comparing with control (loading buffer).

well as other steroids such as progesterone inhibit $^{86}\text{Rb}^+$ efflux induced by carbachol in both human neuronal and muscle cell lines expressing $\alpha 1\beta 1\gamma\delta$ -nAChRs and $\alpha 3\beta 4$ -nAChRs (Ke and Lukas, 1996). We found that DCB was also potent in inhibiting Ca^{2+} signaling of nAChRs. nAChRs are allosteric proteins whose functional activities are affected by functional state transitions, which can be determined by agonists and competitive antagonists that bind to acetylcholine binding sites, as well as by non-competitive inhibitors having two distinctive sites—one high- and one low-affinity (Changeux et al., 1984). Steroid compounds address a hydrophobic interaction at the low-affinity binding site (Arias, 1998). We predict that one of the mechanisms of DCB's inhibition of nAChRs is its action on the steroid binding sites of nAChRs as a steroid-compound channel blocker.

Secondly, DCB acted on VOCC as a inhibitor, evidenced by its inhibition of the high K^+ -induced $[\text{Ca}^{2+}]_i$ increase (Fig. 4). Because a high K^+ concentration bypasses the receptor and directly depolarizes the plasma membrane, the inhibitory action of DCB is not limited to the nAChR level, but also occurs at some step following plasma membrane depolarization. The non-genomic modulation of membrane channels by estrogen has previously been discussed (Zhang et al., 1994; Kim et al., 2000). Our results show that DCB inhibits VOCCs. We suggest that DCB with membrane-mediated non-genomic estrogenic characteristics is capable of altering the functional activities of nAChR channels and VOCCs; thus, the inhibition of DCB on the Ca^{2+} signaling coupled with nAChR is higher than its inhibition of mAChR.

Thirdly, the inhibition of the Ca^{2+} signaling coupled with nAChRs could possibly be through Ca^{2+} -induced Ca^{2+} release (CICR). DCB could deplete the stored Ca^{2+} in the endoplasmic reticulum so that no Ca^{2+} can be released from the stimulated endoplasmic reticulum. During stimulation, a small amount of Ca^{2+} influx can initiate CICR by opening ryanodine receptors that are sensitive to a slight elevation in $[\text{Ca}^{2+}]_i$ (Lemmens et al., 2001). Ca^{2+} influx coupled with the stimulation of nAChRs can further induce CICR from ER and the decrease of Ca^{2+} stored content in endoplasmic reticulum can inhibit the Ca^{2+} signaling coupled with nAChRs (Pan et al., 2006). We suggest that the presently observed inhibition by DCB of nAChRs is partially due to its depletion of the intracellular Ca^{2+} stores so that stimulation of nAChRs cannot evoke CICR in the presence of DCB. The involvement of all three routes allows DCB to inhibit the Ca^{2+} signaling coupled with nAChRs at the concentration lower than DCB's actions on mAChR.

DCB presently interfered with Ca^{2+} homeostasis in vitro at μM concentrations. Could this situation occur physiologically? Previous studies have determined that the plasma concentration of DCB (one day after DCB feeding) is $28\text{ }\mu\text{g/ml}$ and its metabolite dichlorophenol was $26\text{ }\mu\text{g/ml}$; the concentration of DCB in hepatic tissue was $67\text{ }\mu\text{g/g}$ and in adipose tissue was $50\text{ }\mu\text{g/g}$ (Bomhard et al., 1998). The molecular weight of DCB is 167. Thus, the concentrations of DCB in plasma, hepatic tissue, and adipose tissue, based on the above observations, are $168\text{ }\mu\text{M}$ in plasma, $401\text{ }\mu\text{M}$ in hepatic tissue, and $299\text{ }\mu\text{M}$ in adipose tissue. Since DCB could exist in high concentration in these situations, we suggest that DCB interference with Ca^{2+} homeostasis is conceivable in vitro and in vivo. Further study of DCB on neuronal activities in animal models is required to directly link human exposure to DCB and the interference of DCB on Ca^{2+} homeostasis.

Steroid hormones affect developmental processes and are involved in biological responses to homeostatic challenges. Estrogen in particular plays roles in differentiation, proliferation, homeostasis, and reproductive functions. In addition to traditional genomic effects mediated via receptors in nucleus, steroid hormones exert non-genomic membrane effects via membrane ion channels. Some neuronal receptors belong to an ion channel recep-

tor superfamily and serve as additional potential addressing sites for steroid hormones. These neuronal receptors, which appear during embryonic stages, act as putative guidelines for animal differentiation (Chen and Farese, 1999).

In this study, we describe novel characteristics of DCB, acting as AChR inhibitors and interfering Ca^{2+} homeostasis. This suggests that DCB might obstruct animal homeostasis and neuronal signaling.

Conflict of interest

None.

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