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

Ren-Ming Yan[a], Yin-Mei Chiung[b,c], Chien-Yuan Pan[d], Jenn-Hwa Liu[e], Pei-Shan Liu[a,*]

[a] Department of Microbiology, Soochow University, Shihlin, Taipei, Taiwan, ROC
[b] Division of Occupational Medicine, Institute of Occupational Safety and Health, Taipei, Taiwan, ROC
[c] Department of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan, ROC
[d] Institute of Zoology and Department of Life Science, National Taiwan University, Taipei, Taiwan, ROC
[e] Division of Gastroenterology, Kuang-Tien General Hospital, Taiwan, ROC

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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 μM, 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 μM. 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.

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; Kostiainen, 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; IP_3, inositol triphosphate; CICR, Ca^{2+}-induced Ca^{2+} release.

* Corresponding author. Tel.: +886 2 28819471x6857; fax: +886 2 28831193.
E-mail address: psliu@mail.scu.edu.tw (P.-S. Liu).

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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 ($[Ca^{2+}]_c$) measurements.

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

The human neuroblastoma SH-SY5Y cells were loaded with fura-2 by incubation (5×10^6 cells/ml) with 10 μM 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. $[Ca^{2+}]_c$ 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 MΩ. 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 μM 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 μm positioned at 10 μm 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 $[Ca^{2+}]_c$ in SH-SY5Y cells

DCB dose-dependently induced an increase of $[Ca^{2+}]_c$ 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 μM) induced a net $[Ca^{2+}]_c$ increase of 191.6 ± 29.8 nM (n = 16). The elevation of $[Ca^{2+}]_c$ 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 $[Ca^{2+}]_c$, followed by a quick decay in the absence of extracellular Ca^{2+} (Fig. 1). DCB (500 μM) induced a net $[Ca^{2+}]_c$ increase of 60.67 ± 9.33 nM (n = 8) in the absence of extracellular Ca^{2+}. Ca^{2+} influx likely played a role in the DCB-induced $[Ca^{2+}]_c$ peak because the $[Ca^{2+}]_c$ 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 $[Ca^{2+}]_c$ 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 $[Ca^{2+}]_c$ levels was obtained when the cells were stimulated with 0.3 mM methacholine (a muscarinic receptor agonist) or 10 μM epibatidine (a nicotinic receptor agonist) (Fig. 2). The methacholine-induced and epibatidine-induced increases of $[Ca^{2+}]_c$ were completely inhibited by 20 μM atropine or 100 μM 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 $[Ca^{2+}]_c$ levels that was depressed by 0.1 mM verapamil, a blocker of VOCC. The increase of $[Ca^{2+}]_c$ induced by 500 μM DCB remained the same in the presence of 20 μM atropine (104 ± 15% of control, n = 3), 0.1 mM hexamethonium (105 ± 25% of control, n = 3), or 0.1 mM verapamil (114 ± 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 $[Ca^{2+}]_c$ increase was inhibited by DCB in a dose-dependent manner (IC_{50} = 161 μM). DCB at 50 μM significantly inhibited the carbachol-induced $[Ca^{2+}]_c$ 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-