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Research Article

Inhibition of Voltage-Gated Calcium Channels After Subchronic and Repeated Exposure of PC12 Cells to Different Classes of Insecticides

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ABSTRACT

We previously demonstrated that acute inhibition of voltage-gated calcium channels (VGCCs) is a common mode of action for (sub)micromolar concentrations of chemicals, including insecticides. However, because human exposure to chemicals is usually chronic and repeated, we investigated if selected insecticides from different chemical classes (organochlorines, organophosphates, pyrethroids, carbamates, and neonicotinoids) also disturb calcium homeostasis after subchronic (24 h) exposure and after a subsequent (repeated) acute exposure. Effects on calcium homeostasis were investigated with single-cell fluorescence (Fura-2) imaging of PC12 cells. Cells were depolarized with high-K^+ saline to study effects of subchronic or repeated exposure on VGCC-mediated Ca^{2+} influx. The results demonstrate that except for carbaryl and imidacloprid, all selected insecticides inhibited depolarization (K^+)-evoked Ca^{2+} influx after subchronic exposure (IC_{50}'s: approximately 1–10 μM) in PC12 cells. These inhibitory effects were not or only slowly reversible. Moreover, repeated exposure augmented the inhibition of the K^+-evoked increase in intracellular calcium concentration induced by subchronic exposure to cypermethrin, chlorpyrifos, chlorpyrifos-oxon, and endosulfan (IC_{50}'s: approximately 0.1–4 μM). In rat primary cortical cultures, acute and repeated chlorpyrifos exposure also augmented inhibition of VGCCs compared with subchronic exposure. In conclusion, compared with subchronic exposure, repeated exposure increases the potency of insecticides to inhibit VGCCs. However, the potency of insecticides to inhibit VGCCs upon repeated exposure was comparable with the inhibition previously observed following acute exposure, with the exception of chlorpyrifos. The data suggest that an acute exposure paradigm is sufficient for screening chemicals for effects on VGCCs and that PC12 cells are a sensitive model for detection of effects on VGCCs.

Key words: subchronic exposure; repeated exposure; *in vitro* neurotoxicology; calcium homeostasis; voltage-gated calcium channels; insecticides

ABBREVIATIONS:

AChE, acetylcholine esterase;
$[Ca^{2+}]_i$, intracellular calcium concentration;
IC_{20}, concentration that induces an inhibitory effect of 20%;
IC_{50}, concentration that induces an inhibitory effect of 50%;
nAChR, nicotinic acetylcholine receptor;
VGCCs, voltage-gated calcium channels;
VGSCs, voltage-gated sodium channels.

Insecticides are abundantly used because of their neurotoxicity to insects, but their mode(s) of action are often not restricted to insects. The widespread use of insecticides may therefore be of

concern for (developmental) neurotoxicity in mammals, including humans (Mackenzie Ross *et al.*, 2013). Insecticides are commonly classified according to their structure and best-known neurotoxic mode of action. Organochlorine insecticides are generally banned but very persistent and still present in the environment (Mrema *et al.*, 2013). Organochlorines are subclassified in 2 main groups: 1,1,1-Trichloro-2,2-bis(4-chlorophenyl)ethane (DDT)-type insecticides that target voltage-gated sodium channels (VGSCs) and chlorinated alicyclic insecticides that target γ-Aminobutyric acid (GABA) and glycine receptors (Coats, 1990). Pyrethroid insecticides delay the inactivation of VGSCs and thus increase the Na^+ influx, which consequently depolarizes the cell membrane resulting in overexcitation (Soderlund, 2012). Organophosphate insecticides are best known for their irreversible inhibition of acetylcholine esterase (AChE), which results in increased levels of ACh and subsequent overexcitation (Eaton *et al.*, 2008). Bioactivation of organophosphates via the formation of oxon metabolites strongly increases their neurotoxic potency via AChE inhibition (Eaton *et al.*, 2008). Carbamate insecticides also inhibit AChE, but these insecticides inhibit AChE activity reversibly and do not require bioactivation (Moser *et al.*, 2010). Neonicotinoid insecticides exert neurotoxic overexcitation via activation of nicotinic ACh receptors (nAChRs; Matsuda *et al.*, 2009).

Traditional *in vivo* tests for the investigation of neurotoxicity of such insecticides and other chemicals are expensive, laborious, may not be sufficiently sensitive and often lack information on mechanisms of toxicity (Coecke *et al.*, 2006). Therefore, there is an increasing interest for the development of *in vitro* screening methods to test chemicals for (developmental) neurotoxicity. Voltage-gated calcium channels (VGCCs) are likely a suitable target for *in vitro* neurotoxicity screening as—together with calcium permeable neurotransmitter receptors, intracellular calcium stores, and calcium-binding proteins—VGCCs tightly control the intracellular calcium concentration ($[Ca^{2+}]_i$; Westerink, 2006). This strict control of $[Ca^{2+}]_i$ is essential for a range of cellular processes including proper neuronal function (Simms and Zamponi, 2014; Westerink, 2006), development (Leclerc *et al.*, 2011), and survival (Zündorf and Reiser, 2011).

PC12 cells are well characterized neuronotypical cells that are known to express L-, N-, and P/Q-type VGCCs (Dingemans *et al.*, 2009). PC12 cells are therefore a suitable cell model for mechanistic neurophysiological and neurotoxicological (screening) studies related to these channels (Westerink, 2013; Westerink and Ewing, 2008). The effects of acute *in vitro* exposure to pesticides on VGCCs in PC12 cells are already well-studied, and it is known that acute inhibition of VGCCs is a common mode of action of organochlorine, pyrethroid, and organophosphate insecticides (Heusinkveld and Westerink, 2012; Meijer *et al.*, 2014a, 2014b) and conazole fungicides (Heusinkveld *et al.*, 2013). Moreover, several other types of chemicals have been demonstrated to acutely inhibit VGCCs, such as brominated and halogen-free flame retardants (Dingemans *et al.*, 2009, 2010; Hendriks *et al.*, 2014), polychlorinated biphenyls (PCBs; Langeveld *et al.*, 2012), and drugs of abuse (Hondebrink *et al.*, 2011, 2012).

It is, however, largely unclear if these effects of insecticides on VGCCs persist and if subchronic or repeated exposure to insecticides increases the potency of insecticides for inhibition of VGCCs. Subchronic and/or repeated exposures are more realistic exposure scenarios because exposure to insecticides usually occurs chronically and repeatedly via occupational (eg, agriculture) settings or consumption of food. Therefore, this study investigated the effects of subchronic (24 h) and repeated (24 h pre-exposure followed by a second, acute exposure) exposure to endosulfan, cypermethrin, chlorpyrifos, chlorpyrifos-oxon, carbaryl, and imidacloprid on calcium homeostasis *in vitro*.

MATERIALS AND METHODS

Chemicals. Fura-2 AM was obtained from Molecular Probes (Invitrogen, Breda, The Netherlands). Chlorpyrifos-oxon (purity 93.5%) was obtained from AccuStandard (New Haven). Cypermethrin (purity 95.1%), chlorpyrifos (purity 99.9%), endosulfan (α:β 2:1; purity 99.9%), imidacloprid (purity 99.9%), carbaryl (purity 99.5%), and all other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless otherwise noted. Saline solutions (containing in mM: 125 NaCl, 5.5 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3, adjusted with NaOH) were prepared with deionized water (Milli-Q; resistivity > 18 MΩ·cm). Stock solutions were prepared in dimethyl sulfoxide (DMSO), and final solutions (solvent concentration 0.1% DMSO) were prepared just prior to the experiments.

Cell culture. Rat pheochromocytoma (PC12) cells (Greene and Tischler, 1976) were cultured for up to 10 passages in RPMI1640 (Invitrogen) supplemented with 10% horse serum, 5% fetal bovine serum, and 2% penicillin/streptomycin (ICN Biomedicals, Zoetermeer, The Netherlands) as described previously (Meijer *et al.*, 2014a,b). Medium was refreshed every 2–3 days.

Rat primary cortical cells were isolated from brain cortices of newborn (PND1) rats (Wistar; HsdCpb:WU; Harlan Laboratories B.V., Horst, The Netherlands). Briefly, rats were decapitated, and brains were rapidly dissected in phosphate buffered saline on ice. Tissue was dissociated to single cells by use of a cell strainer (diameter 100 μM) and suspended in Neurobasal-A medium supplemented with 25 g/l sucrose, 450 μM glutamine, 30 μM glutamate, 1% penicillin/streptomycin, and 10% FBS (pH 7.4). A droplet of approximately 400 μl cell suspension was added to 35 mm glass-bottom dishes (to obtain a cell density of 400 000 cells/dish), and cells were allowed to adhere for approximately 3 h prior to the addition of extra medium to obtain a total of 2.5 ml medium/dish. After day 1 in culture, medium was replaced with comparable medium, but with 2% B27 instead of FBS. After day 4 in culture, the medium was replaced with Neurobasal-A medium supplemented with 25 g/l sucrose, 450 μM glutamine, 1% penicillin/streptomycin, and 10% FBS (glutamate-free medium).

All cell culture material was coated with poly-L-lysine (50 μg/ml). Cells were cultured in a humidified incubator at 37°C and 5% CO_2.

For cell viability experiments, PC12 cells were seeded in 48-wells plates (0.3×10^6 cells/well) and allowed to attach overnight. Cells were exposed to 0.1% DMSO (vehicle control) or insecticide (0.1–100 μM) in phenol red- and serum-free RPMI1640 medium for 24 h.

For single-cell fluorescent microscopy Ca^{2+} imaging experiments, undifferentiated PC12 cells were subcultured in glass-bottom dishes (MatTek, Ashland, Massachusetts) at approximately 0.5×10^6 cells/dish (approximately 75% confluency) and were allowed to attach for at least 2.5 h. Rat primary cortical cells were cultured in glass-bottom dishes for 7–11 days. Then, medium of PC12 cells was replaced with serum-free RPMI1640 medium, and medium of primary cortical cells was replaced for 50% with glutamate-free medium that contained 0.1% DMSO (vehicle control) or insecticides (1 nM to 10 μM).

Cell viability (alamar Blue and CFDA-AM). Cell viability was quantified with a combined alamar Blue (aB)/CFDA-AM assay as described previously (Heusinkveld *et al.*, 2013) with minor modifications. The aB assay determines mitochondrial activity as a measure of cell viability based on the ability of the cells to reduce resazurin to resorufin. In the same experiment, membrane integrity was assessed by CFDA-AM, which is cleaved by nonspecific esterases in living cells to the fluorescent compound CFDA. After 24 h exposure to the different pesticides (0.1–100 μM), aB/CFDA-AM was added to the wells to obtain a final concentration of 12 and 4 μM aB and CFDA-AM, respectively. Then, cells were incubated in the dark with the aB/CFDA-AM solution for 30 min at 37°C. Fluorescence was measured with an Infinite M200 microplate reader (10 W Xenon flash light source; Tecan Trading AG, Männedorf, Switzerland) at excitation wavelength 540 nm, emission 590 nm (aB) and excitation wavelength 493 nm, emission 541 nm (CFDA).

$[Ca^{2+}]_i$ measurements. Changes in $[Ca^{2+}]_i$ were measured with the Ca^{2+}-sensitive fluorescent ratio dye Fura-2 AM. PC12 cells, 24 h pre-exposed to insecticides, were loaded with 5 μM Fura-2 AM for 20 min in saline, followed by 15 min de-esterification of Fura-2 AM in saline at room temperature. Cells were placed on the stage of an Axiovert 35 M inverted microscope (×40 oil-immersion objective, NA 1.0: Zeiss, Göttingen, Germany) equipped with a TILL Photonics Polychrome IV (Xenon Short Arc lamp, 150 W; TILL Photonics GmBH, Gräfelfing, Germany) and continuously superfused with saline by use of a Valvelink 8.2 (Automate Scientific, California). Fluorescence, excited by 340 and 380 nm wavelengths (F_{340} and F_{380}), was collected every 3–6 s at 510 nm with an Image SensiCam digital camera (TILL Photonics GmBH). All experiments were performed at room temperature. Every experiment consisted of a 5-min baseline recording and a subsequent depolarization of the cells by superfusion with saline containing 100 mM K^+ (containing in mM: 5.5 NaCl, 100 KCl, 2 $CaCl_2$, 0.8 $MgCl_2$, 10 HEPES, 24 glucose, and 36.5 sucrose at pH 7.3, adjusted with NaOH) for 24 s. Then, superfusion was reset to saline for approximately 28 min prior to a second depolarization with 100 mM K^+-containing saline (see Fig. 1A). In a separate set of experiments (for repeated exposure), superfusion was changed 8 min after the first depolarization to saline containing insecticides for 20 min (acute exposure). Following this 20 min repeated acute exposure, cells were depolarized for a second time with K^+- and insecticide-containing saline (see Fig. 1B).

At the end of each recording, cells were permeabilized with 5 μM ionomycin to determine the maximum ratio (R_{max}), after which all Ca^{2+} was chelated with 17 mM ethylenediamine

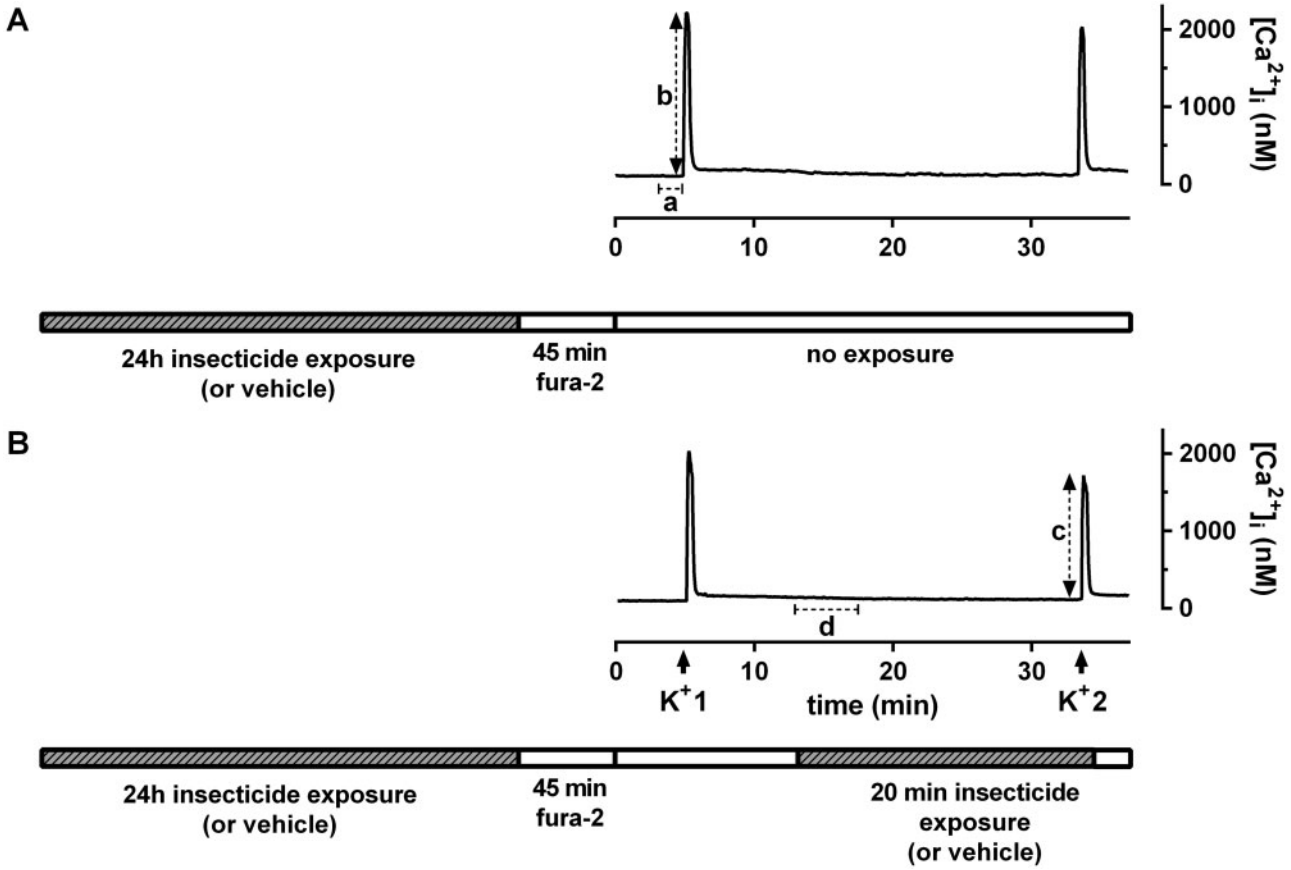

FIG. 1. Example traces of control experiments. PC12 cells were pre-exposed to insecticides (or vehicle control, 0.1% DMSO) for 24 h as indicated by the dashed box before the example recordings. Then, insecticide containing saline was removed to load cells with Fura-2 AM for the recording of $[Ca^{2+}]_i$. In the case of subchronic experiments, cells were not exposed during the $[Ca^{2+}]_i$ recording and during depolarization as indicated by the empty box below the example recording (A). To investigate the effects of a repeated exposure, cells were exposed for a second time for 20 min before a second depolarization (K^+2) was induced as indicated by the dashed box below the example recording (B). During the recording of $[Ca^{2+}]_i$, cells were depolarized with high K^+-containing saline after approximately 5 min (K^+1) and 33 min (K^+2). To quantify effects on basal $[Ca^{2+}]_i$ after subchronic exposure the average basal $[Ca^{2+}]_i$ of 3–5 min of the recording was used (indicated by a in Fig. 1A). To calculate effects on basal $[Ca^{2+}]_i$ for repeated exposure approximately 13.3–18.3 min was used (indicated by d in Fig. 1B). Effects on the depolarization-evoked increase in $[Ca^{2+}]_i$ were expressed as a net increase in $[Ca^{2+}]_i$ as indicated by b (for subchronic exposure; A) and c (for repeated exposure; B).

tetraacetic acid to determine the minimum ratio (R_{min}). The obtained R_{max} and R_{min} values were used to calculate $[Ca^{2+}]_i$ (described below).

Data analysis and statistics. Cell viability data were analyzed by use of MS-Excel. Raw data were background-corrected and normalized to plate-matched DMSO controls. At least 3 independent experiments (*N*) were performed to obtain at least 12 wells (*n*) per condition. Data are expressed as mean ± SEM of *n* wells. Cell viability data were compared by use of an one-way ANOVA and a *post hoc* Bonferroni test. Data were considered statistically significant if $p < .05$.

Calcium-imaging data were processed with TILLVisION software (version 4.01) and further analyzed with a custom-made MS-Excel macro that calculates background-corrected F_{340}/F_{380} ratio values and $[Ca^{2+}]_i$. To calculate free cytosolic $[Ca^{2+}]_i$ from background-corrected F_{340}/F_{380} ratio values, we used a modified Grynkiewicz's equation $[Ca^{2+}]_i = K_{d^*} \times (R - R_{min})/(R_{max} - R)$, where K_{d^*} is the dissociation constant of Fura-2 determined in the experimental setup (Meijer *et al.*, 2014a,b).

Insecticide-induced effects on basal $[Ca^{2+}]_i$ after repeated exposure were expressed as the average increase in $[Ca^{2+}]_i$ (± SEM from *n* cells) during the first 5 min of re-exposure of responding cells (Fig. 1B). Responding cells are cells that displayed an increase in $[Ca^{2+}]_i$ during exposure ≥ average + SD of basal $[Ca^{2+}]_i$. Effects on the depolarization-evoked increase in $[Ca^{2+}]_i$ were expressed as a net increase (see Fig. 1 for illustration). Data were expressed as mean ± SEM (from *n* cells), normalized to the control, unless otherwise noted. The data were expressed in *n*, rather than *N* (independent experiments), to enable the study of single-cell calcium kinetics and oscillations. Moreover, the variation within an independent experiment (within a dish [*n*]; CV of control repeated exposure: 0.44) was larger compared with the variation between the experiments (between dishes [*N*]; CV of control repeated exposure: 0.17), which indicates that cells are independent units derived from the same population and justifies the use of *n* as statistical unit. Cells that showed effects over 2 times SD above or below average were considered as outliers and were excluded for further analysis (approximately 13%). Per test condition at least 4 independent experiments (*N*) were performed to obtain ≥ 25 cells (*n*) after outlier exclusion. For the calculation of concentrations that induces an inhibitory effect of 20% or 50% (IC_{20} or IC_{50}), a non-linear regression curve with Hill-slope was fitted by use of GraphPad Prism software (version 6, GraphPad Software, La Jolla, California). Continuous data were compared with an unpaired *t* test. Data were considered statistically significant if $p < .05$.

RESULTS

Cytotoxicity Concentration-Effect Range Finding: Effects of Subchronic (24 h) Exposure to Insecticides on Cell Viability in PC12 Cells

Carbaryl, imidacloprid, endosulfan, chlorpyrifos, and cypermethrin did not significantly affect mitochondrial activity in PC12 cells at concentrations 0.1–100 μM after 24 h of exposure as indicated by the aB assay (data not shown). Only chlorpyrifos-oxon was able to significantly reduce the mitochondrial activity to 67 ± 5% of control at the highest concentration tested (100 μM; $n = 12$, $N = 3$; data not shown). Similarly, membrane integrity was not significantly affected by imidacloprid, endosulfan, and chlorpyrifos as indicated by the CFDA assay in PC12 cells (Fig. 2). Carbaryl modestly reduced membrane integrity to 88 ± 2% of control at 100 μM ($n = 12$, $N = 3$). At 100 μM, membrane integrity compared with control was also reduced by chlorpyrifos-oxon (58 ± 5%; $n = 12$, $N = 3$) and cypermethrin (54 ± 3%; $n = 16$, $N = 4$; Fig. 2). Because cell viability is only affected by some insecticides at 100 μM, the maximum concentration in subsequent experiments was kept ≤ 10 μM.

Effects of 24 h of Subchronic Exposure to Insecticides on Basal $[Ca^{2+}]_i$ and Depolarization-Evoked Increase in $[Ca^{2+}]_i$ in PC12 Cells

In control (0.1% DMSO) PC12 cells, basal $[Ca^{2+}]_i$ was low and stable (106 ± 2 nM; $n = 212$, $N = 15$; Fig. 1A). Basal $[Ca^{2+}]_i$ following subchronic (24 h) exposure to insecticides was largely unchanged (ranging from 95 ± 5 nM [10 μM cypermethrin; $n = 65$, $N = 4$] to 131 ± 5 nM [10 μM endosulfan; $n = 59$, $N = 5$]; Fig. 3).

The effects of 24 h subchronic exposure to insecticides on Ca^{2+} influx via VGCCs were investigated by depolarization of the cell membrane 5 min after the start of the recording (for illustration see b in Fig. 1A). In control cells (24 h subchronic exposure to 0.1% DMSO), depolarization induced a net increase

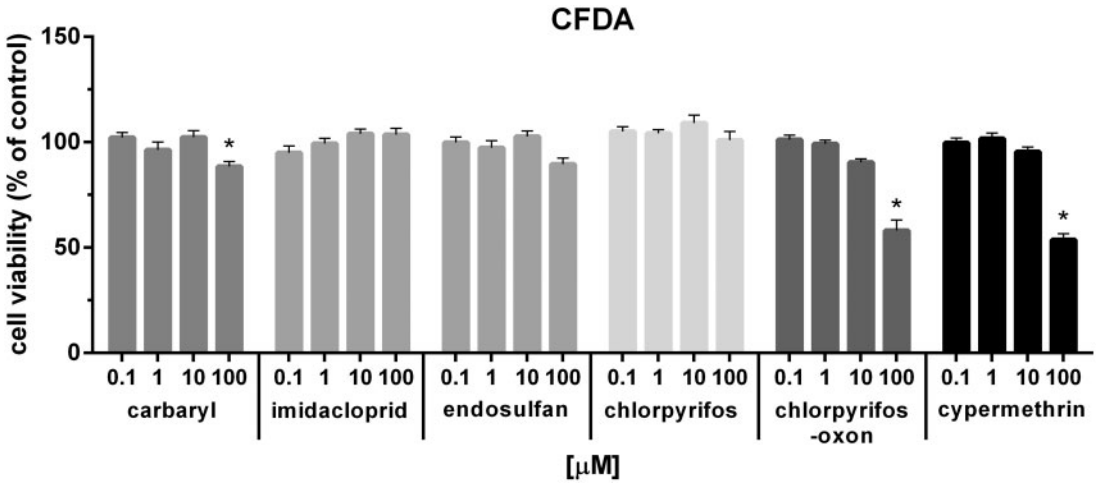

FIG. 2. Cell viability following 24 h of exposure to insecticides from different chemical classes. Effects on membrane integrity were investigated with a CFDA-AM assay to study the effects of insecticides on cell viability. Data, presented as mean + SEM of $n = 12$–16 obtained from $N = 3$–4, was normalized to DMSO control (%). An asterisk indicates a significant difference from the control ($p < .05$).

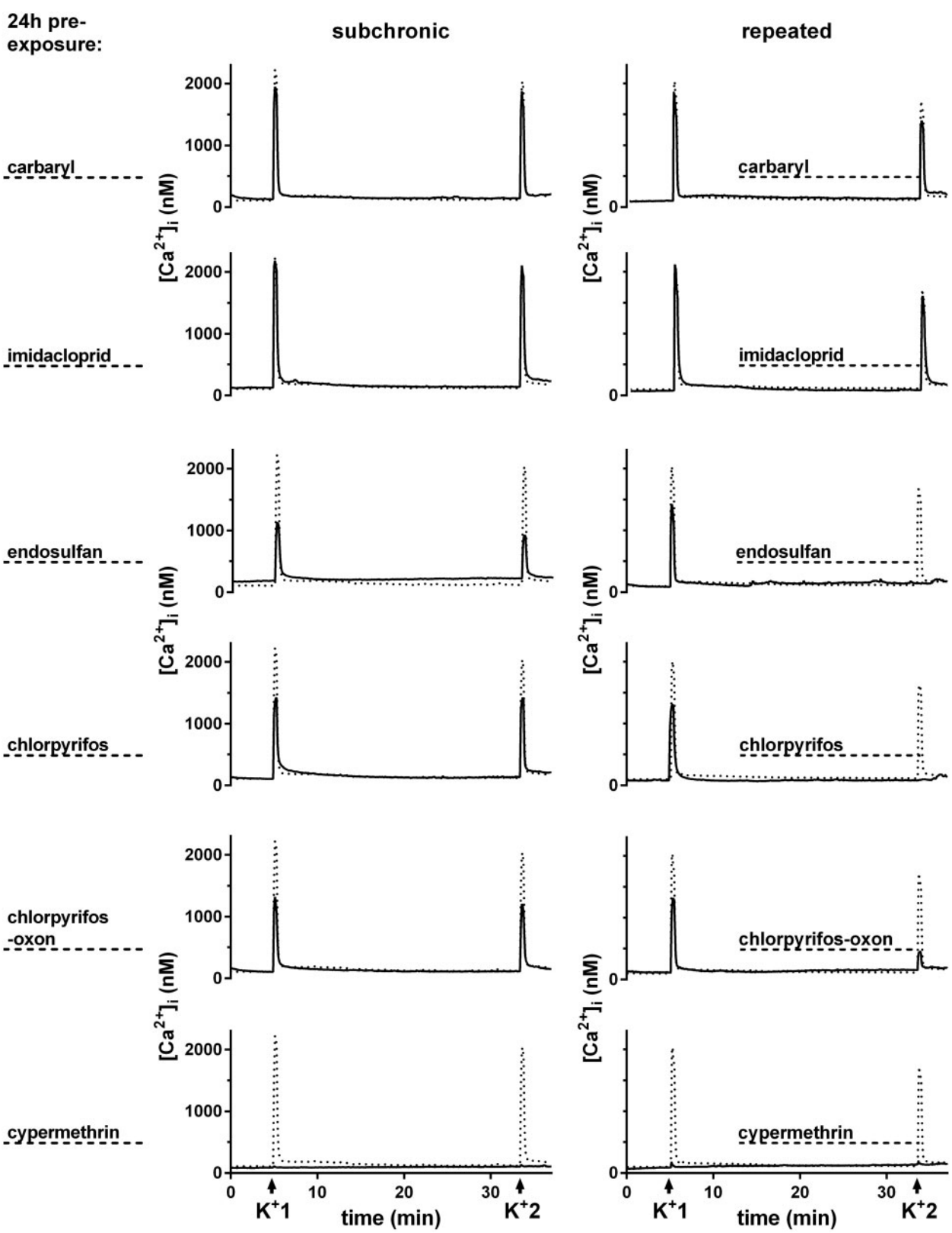

FIG. 3. Representative recordings of $[Ca^{2+}]_i$ from individual PC12 cells exposed subchronic (left) and repeatedly (right) to insecticides (10 μM). PC12 cells were exposed for 24 h to carbaryl, imidacloprid, endosulfan, chlorpyrifos, chlorpyrifos-oxon, and cypermethrin prior to the $[Ca^{2+}]_i$ recordings as indicated by the dashed line on the left. In a separate set of experiments (right), cells were exposed for a second time (repeated exposure) as indicated by the dashed line in the recording to the same insecticide after 24 h of pre-exposure. The dotted trace in the recording shows a representative trace of a control cell. Cells were depolarized at 5 and 33.3 min as indicated by K^+1 and K^+2.

in $[Ca^{2+}]_i$ of 2.1 ± 0.08 μM ($n = 212$, $N = 15$; Fig. 1A). Carbaryl (≤ 10 μM) was not able to significantly reduce the depolarization-evoked increase in $[Ca^{2+}]_i$ (89 ± 6% at 1 μM [$n = 57$, $N = 4$] and 92 ± 6% at 10 μM [$n = 61$, $N = 6$] compared with control; Fig. 3). Similarly, imidacloprid did not affect the depolarization-evoked increase in $[Ca^{2+}]_i$ (96 ± 5% at 10 μM; $n = 58$, $N = 4$; Fig. 3). In contrast, if depolarization was induced in cells subchronically exposed to 10 μM endosulfan, the net increase in $[Ca^{2+}]_i$ amounted only to 1.0 ± 0.08 μM, which is 46 ± 4% compared with control ($n = 59$, $N = 5$; Figs. 3 and 4A). At 1 μM, endosulfan did not significantly affect the depolarization-evoked increase in $[Ca^{2+}]_i$ (107 ± 7% compared with control; $n = 69$, $N = 4$; Fig. 4A). Chlorpyrifos did not significantly affect the depolarization-evoked increase in $[Ca^{2+}]_i$ at 0.1 μM (96 ± 6%; $n = 57$, $N = 4$; Fig. 4B). However, at 1 μM, chlorpyrifos significantly reduced the net increase in $[Ca^{2+}]_i$ to 78 ± 6% of control ($n = 59$, $N = 4$; Fig. 4B), and to 55 ± 4% of control at 10 μM ($n = 59$, $N = 5$; Figs. 3 and 4B). For chlorpyrifos-oxon, no significant reduction in the depolarization-evoked increase in $[Ca^{2+}]_i$ was observed at 0.01 μM (92 ± 5% compared with control; $n = 54$, $N = 5$; Fig. 4C). At 0.1 μM, chlorpyrifos-oxon significantly reduced the depolarization-evoked increase in $[Ca^{2+}]_i$ to 74 ± 4% ($n = 61$, $N = 5$; Fig. 4C), 62 ± 6% ($n = 62$, $N = 5$; Fig. 4C) at 1 μM and 55 ± 3% at 10 μM ($n = 64$, $N = 6$; Figs. 3 and 4C) compared with control. Cypermethrin did not significantly affect the depolarization-evoked increase in $[Ca^{2+}]_i$ at 0.1 μM (101 ± 6% compared with control; $n = 64$, $N = 5$; Fig. 4D), but reduced the depolarization-evoked increase in $[Ca^{2+}]_i$ significantly at 1 μM to 45 ± 4% ($n = 70$, $N = 5$; Fig. 4D) and at 10 μM to 1 ± 0.2% compared with control ($n = 65$, $N = 4$; Figs. 3 and 4D).

To investigate if the inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ by insecticides is reversible, cells were depolarized approximately 28 min after the first depolarization-evoked increase in $[Ca^{2+}]_i$ for a second time. In control experiments, this second depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 1.9 ± 0.07 μM ($n = 212$, $N = 15$; Fig. 1A; Table 1). At 10 μM endosulfan, the second depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 0.9 ± 0.07 μM ($n = 59$, $N = 5$; Fig. 3; Table 1), which was 46 ± 4% compared with control (Table 1). This second depolarization-evoked increase in $[Ca^{2+}]_i$ was comparable with the first depolarization-evoked increase in $[Ca^{2+}]_i$, which was also 46 ± 4% compared with control (Table 1). At 10 μM cypermethrin, both the first and the second depolarization-evoked increases in $[Ca^{2+}]_i$ were completely inhibited (Fig. 3; Table 1). Chlorpyrifos and chlorpyrifos-oxon also demonstrated comparable net increases in $[Ca^{2+}]_i$ during the first and second induced depolarization (Table 1). Carbaryl and imidacloprid were not able to change the second depolarization-evoked increase in $[Ca^{2+}]_i$ compared with control and these insecticides thus also demonstrated comparable net increases in $[Ca^{2+}]_i$ during the first and second depolarization (Fig. 3; Table 1).

Effects of Repeated Exposure (24 h Pre-exposure Followed by an Acute Exposure) to Insecticides on Basal $[Ca^{2+}]_i$ and Depolarization-Evoked Increase in $[Ca^{2+}]_i$ in PC12 Cells

To investigate the effects of repeated exposure to insecticides, cells pre-exposed to insecticides for 24 h were re-exposed acutely for 20 min starting 8 min after the first depolarization-evoked increase in $[Ca^{2+}]_i$ (see Fig. 1B for illustration). No changes in basal $[Ca^{2+}]_i$ were observed with the exception of 10 μM endosulfan, which induced an increase in basal $[Ca^{2+}]_i$ of 71 ± 13 nm in responding cells (41%; 25 of 61 cells; Fig. 3).

The first depolarization-evoked increase in $[Ca^{2+}]_i$ in repeated exposure experiments was, as expected, comparable with the 24 h subchronically exposed cells (Fig. 3). In control cells (0.1% DMSO), the first net depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 2.0 ± 0.05 μM and the second depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 1.4 ± 0.04 μM ($n = 293$, $N = 23$; Fig. 1B). Carbaryl induced a small but significant inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ after repeated exposure at 10 μM (87 ± 6% compared with control; $n = 62$, $N = 5$; Fig. 3). When cells were exposed repeatedly to 10 μM imidacloprid, no significant change in the depolarization-evoked increase was observed (105 ± 6% compared with control; $n = 65$, $N = 5$; Fig. 3). In contrast, repeated exposure to endosulfan concentration-dependently inhibited the depolarization-evoked increase in $[Ca^{2+}]_i$ at 0.1 μM (65 ± 6%; $n = 71$, $N = 5$; Fig. 4E) and resulted in a nearly complete inhibition at 10 μM (2 ± 0.3%; $n = 61$, $N = 6$; Figs. 3 and 4E). Chlorpyrifos also concentration dependently reduced the depolarization-evoked increase in $[Ca^{2+}]_i$ at 0.1 μM (66 ± 5%; $n = 65$, $N = 5$; Fig. 4F) and induced a nearly complete inhibition at 10 μM (3 ± 0.4%; $n = 61$, $N = 5$; Figs. 3 and 4F). Chlorpyrifos-oxon induced a significant inhibition at 0.1 μM (83 ± 3%; $n = 62$, $N = 5$; Fig. 4G) and inhibited the depolarization-evoked increase to 38 ± 3% of control at 10 μM ($n = 62$, $N = 6$; Figs. 3 and 4G). Cypermethrin inhibited the depolarization-evoked increase in $[Ca^{2+}]_i$ concentration dependently at 1 nM (86 ± 6%; $n = 83$, $N = 5$; Fig. 4H) and inhibited the depolarization-evoked increase to 5 ± 0.6% at 10 μM ($n = 74$, $N = 5$; Figs. 3 and 4H).

Effects of Exposure to Insecticides on Basal $[Ca^{2+}]_i$ and Depolarization-Evoked Increase in $[Ca^{2+}]_i$ in Rat Primary Cortical Cells

In control rat primary cortical cells, basal $[Ca^{2+}]_i$ was low and stable following acute exposure to 0.1% DMSO (113 ± 3 nM; $n = 101$, $N = 17$; data not shown). Acute exposure to 10 μM chlorpyrifos induced an increase in basal $[Ca^{2+}]_i$ of 107 ± 15 nM in responding cells (90%; 41 of 45 cells; data not shown). Interestingly, upon exposure of cells to endosulfan or cypermethrin, continuous oscillations in basal $[Ca^{2+}]_i$ were observed (Fig. 5). No changes in basal $[Ca^{2+}]_i$ were observed for 10 μM chlorpyrifos-oxon upon acute exposure (data not shown).

Due to the observed continuous oscillations upon exposure to endosulfan or cypermethrin, inhibition of VGCCs could not be accurately determined as the oscillations could influence the sensitivity to the K^+-induced depolarization and hampers reliable quantification of the inhibitory effects. Therefore, effects on the depolarization-evoked increase in $[Ca^{2+}]_i$ were only qualified for chlorpyrifos and chlorpyrifos oxon.

Upon depolarization of control (0.1% DMSO) primary cells with high K^+-containing saline, $[Ca^{2+}]_i$ increased rapidly and transiently to 1.5 ± 0.08 μM ($n = 101$, $N = 17$; data not shown). Following 20 min of exposure to 0.1% DMSO, the depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 1.0 ± 0.05 μM ($n = 101$, $N = 17$; data not shown). Following acute exposure to chlorpyrifos, the depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 116 ± 7% of control at 0.1 μM ($n = 40$, $N = 6$), and was significantly reduced to 62 ± 4% of control at 1 μM ($n = 56$, $N = 5$) and to 81 ± 7% of control at 10 μM ($n = 45$, $N = 7$; Fig. 6). Upon acute exposure to chlorpyrifos-oxon, the depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 90 ± 8% of control at 0.1 μM ($n = 25$, $N = 4$), and was significantly reduced to 44 ± 5% of control at 1 μM ($n = 47$, $N = 6$) and to 67 ± 7% of control at 10 μM ($n = 43$, $N = 4$; data not shown).

To compare the effects of acute, subchronic and repeated exposure scenarios in PC12 cells with effects in primary cortical cells, primary cortical cells were also subchronically and

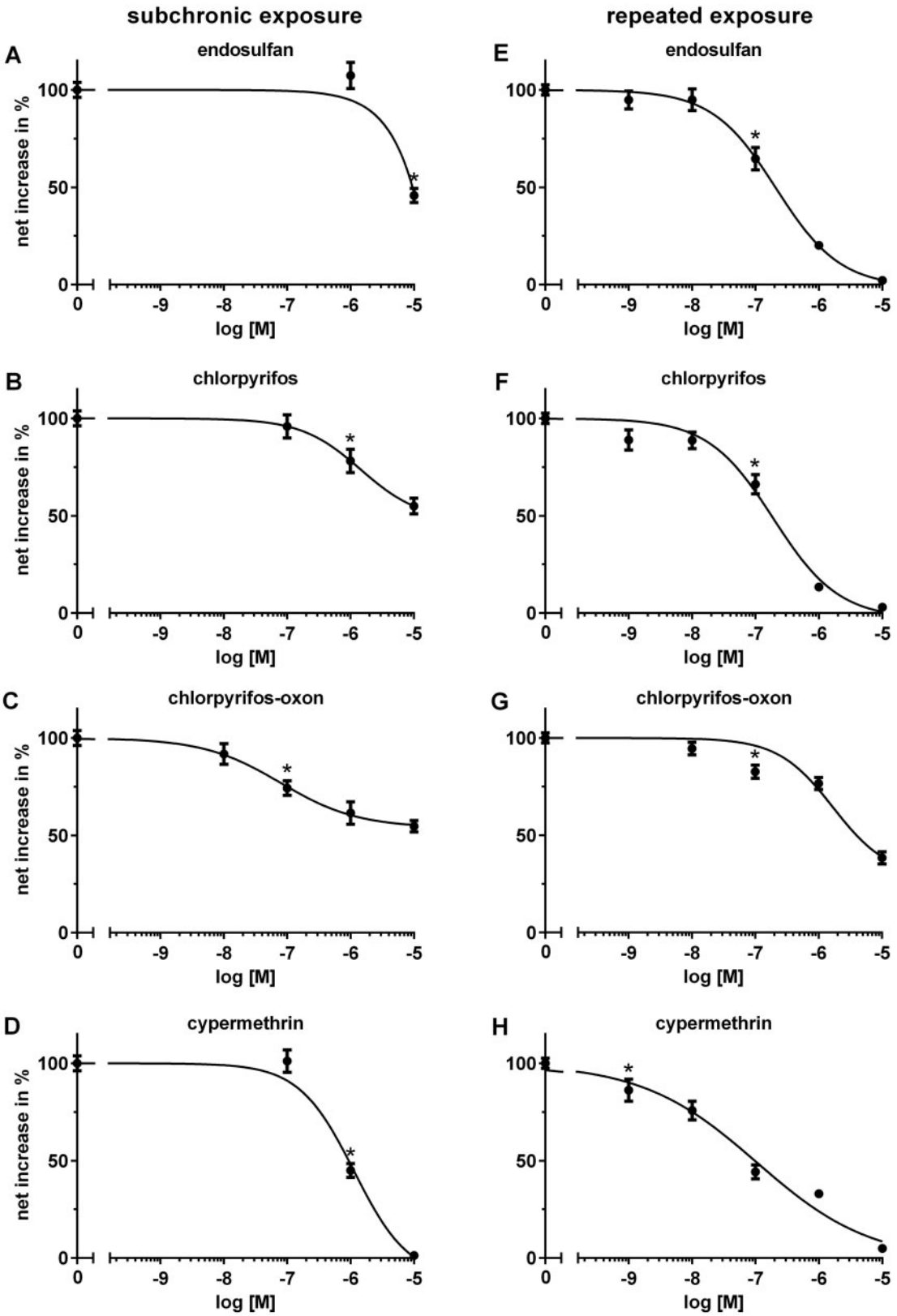

FIG. 4. Concentration-response curves of inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ by insecticides after 24 h subchronic exposure (left) and after subsequent repeated exposure (right). The selected insecticides inhibit the depolarization-evoked increase in $[Ca^{2+}]_i$ concentration dependently after 24 h of subchronic exposure (A–D) and after subsequent repeated exposure (E–H) with the exception of carbaryl and imidacloprid (data not shown). Data represent mean ± SEM ($n = 54$–293, $N = 4$–23). An asterisk indicates the lowest concentration that is significantly different from the control ($p < .05$).

TABLE 1. Net and Relative Increases in $[Ca^{2+}]_i$ (Expressed as Mean ± SEM [of *n*]) Upon Depolarization of Cells Subchronically Exposed to Insecticides for 24 h

Insecticide (10 μM)	Net increase first depolarization (μM)	Net increase second depolarization (μM)	Net increase first depolarization compared with control (%)	Net increase second depolarization compared with control (%)	*n*/*N*
Control (0.1% DMSO)	2.1 ± 0.08	1.9 ± 0.07	100 ± 4	100 ± 4	212/5
Carbaryl	2.0 ± 0.13	1.9 ± 0.12	92 ± 6	99 ± 6	61/6
Imidacloprid	2.0 ± 0.11	2.1 ± 0.12	96 ± 5	107 ± 6	58/4
Endosulfan	1.0 ± 0.08	0.9 ± 0.07	46 ± 4	46 ± 4	59/5
Chlorpyrifos	1.2 ± 0.09	1.1 ± 0.07	55 ± 4	58 ± 4	59/5
Chlorpyrifos-oxon	1.2 ± 0.06	1.2 ± 0.06	55 ± 3	65 ± 3	64/6
Cypermethrin	0.03 ± 0.00	0.05 ± 0.01	1 ± 0.2	3 ± 0.3	65/4

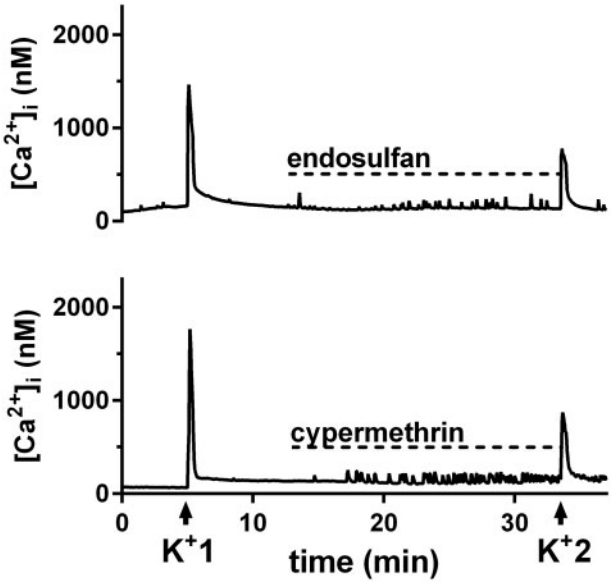

FIG. 5. Representative recordings of $[Ca^{2+}]_i$ from individual primary cortical cells exposed acute to endosulfan (10 μM) or cypermethrin (1 μM). Primary cortical cells were exposed for 20 min (acute) to endosulfan or cypermethrin as indicated by the dashed line in the recordings. Cells were depolarized at 5 and 33.3 min as indicated by K^+1 and K^+2.

repeatedly exposed to chlorpyrifos. In control primary cortical cells, basal $[Ca^{2+}]_i$ was low and stable following subchronic and repeated exposure to 0.1% DMSO (107 ± 4 nM, $n = 82$, $N = 7$; 125 ± 3 nM, $n = 58$, $N = 5$, respectively; data not shown). In control cells subchronically exposed to 0.1% DMSO, depolarization-evoked increase in $[Ca^{2+}]_i$ amounted to 0.8 ± 0.05 μM ($n = 82$, $N = 7$; data not shown). In control repeated exposure experiments, depolarization-evoked increase in $[Ca^{2+}]_i$ after the second exposure amounted to 0.7 ± 0.04 μM ($n = 58$, $N = 5$; data not shown). The depolarization-evoked increase in $[Ca^{2+}]_i$ after subchronic exposure to chlorpyrifos was largely unchanged (89 ± 9% at 1 μM; $n = 43$, $N = 6$; and 104 ± 10% at 10 μM; $n = 46$, $N = 4$; Fig. 6). However, following a second, repeated exposure, chlorpyrifos did reduce the depolarization-evoked increase in $[Ca^{2+}]_i$ significantly to 74 ± 6% at 1 μM ($n = 46$, $N = 5$) and to 61 ± 3% at 10 μM ($n = 41$, $N = 5$; Fig. 6).

DISCUSSION

Previously, we have demonstrated that insecticides from different classes acutely inhibit VGCCs. In this study, we demonstrate that insecticides also inhibit VGCCs after subchronic and repeated exposure which are more relevant exposure scenarios for neurotoxicity testing because exposure to insecticides is generally (sub)chronically and repeatedly. Also, studies have previously shown that prolonged insecticide exposure may change the potency of insecticides for neurotoxicity (eg, Cao *et al.*, 2014; Slotkin and Seidler, 2008). This study demonstrates that the potency of most insecticides for inhibition of VGCCs is comparable for acute and repeated exposure and also that the potency of insecticides for inhibition of VGCCs is lower in subchronic exposure conditions. In addition, the inhibition of VGCCs appeared not or slowly reversible.

The observation of not or slowly reversible inhibition of VGCCs in subchronic exposure conditions suggests that a single subchronic (24 h) exposure could have prolonged consequences for VGCCs function. Prolonged effects of insecticides on neurotoxicological targets have been reported before *in vitro*, eg, a decrease in $GABA_A$ receptor function by long-term (6 days) dieldrin exposure (Babot *et al.*, 2007). Also, *in vivo* studies indicated that subacute and repeated exposure to chlorpyrifos could result in persistent effects on neurotoxicological endpoints, eg, neurobiochemistry and neurobehavior were permanently changed by chlorpyrifos in animals (eg, Chakraborti *et al.*, 1993, Moser *et al.*, 2005). We therefore hypothesized that the not or slowly reversible inhibition of VGCCs could affect the response upon subsequent repeated exposure, in particular for persistent and bioaccumulative chemicals like endosulfan. Our experiments demonstrate that repeated exposure indeed augmented the insecticide-induced inhibition of VGCC. However, our experiments with repeated exposure conditions also reveal that cypermethrin, chlorpyrifos-oxon, and endosulfan are basically equipotent in inhibiting VGCCs in a repeated exposure condition compared with acute exposure (Table 2). Inhibitory concentrations of insecticides that induced a 20% or 50% inhibition (IC_{20} and IC_{50} respectively) in acute and repeated exposure conditions were generally in the same order of magnitude. The exception to this is chlorpyrifos, which was clearly more potent in the repeated exposure scenario compared with the acute exposure condition in PC12 cells (Table 2). Nevertheless, the results suggest that the difference in potency of insecticides for inhibition of VGCCs between the 2 exposure conditions was generally limited and that the effects induced by a preceding subchronic exposure hardly influence the degree of inhibition of VGCCs by a repeated, acute exposure.

The observed higher potency for chlorpyrifos after repeated exposure compared with acute exposure in PC12 cells are likely the result of adaptive mechanisms. As described, studies on subacute and repeated exposure to chlorpyrifos have shown

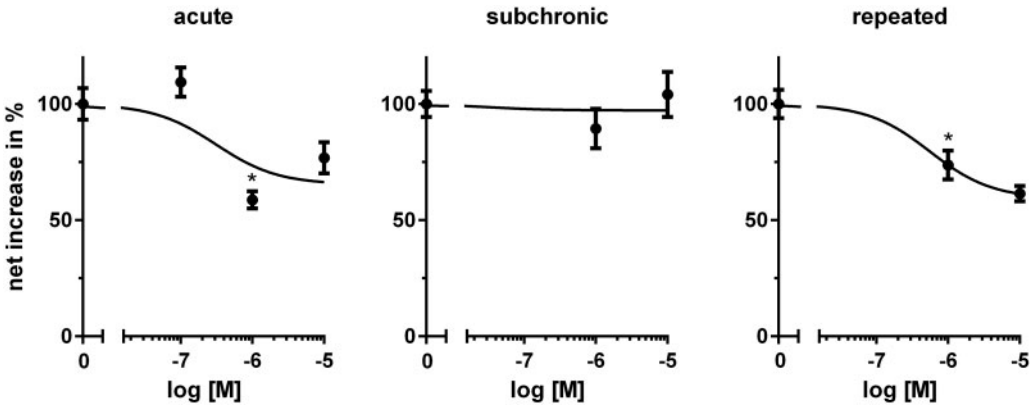

FIG. 6. Concentration-response curves of inhibition of the depolarization-evoked increase in $[Ca^{2+}]_i$ by chlorpyrifos after acute (left), 24 h subchronic exposure (middle), and after subsequent repeated exposure (right) in primary cortical cells. Chlorpyrifos inhibits the depolarization-evoked increase in $[Ca^{2+}]_i$ concentration dependently after acute and repeated exposure. Data represent mean ± SEM ($n = 40–101$, $N = 4–17$). An asterisk indicates the lowest concentration that is significantly different from the control ($p < .05$).

TABLE 2. Summary of Inhibitory Concentrations of Insecticides (in nM) That Induced a 20% or 50% Inhibition of VGCCs After Subchronic (24 h), Repeated or Acute Exposure and Inhibitory Concentrations of Insecticides That Induced a 50% Inhibition of Their Presumed Primary Modes of Action

Insecticides	Subchronic		Repeated		Acute[a,b,c]		Primary mode of action[a,d,e,f,g]
	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{20}	IC_{50}	IC_{50}
Carbaryl	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	990
Imidacloprid	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	$>10^4$	1000 (LOEC)
Endosulfan	3798	9440	42	206	215	362	400 and 1000
Chlorpyrifos	859	$>10^4$	35	179	188	1331	$>50^4$
Chlorpyrifos-oxon	54	$>10^4$	659	3977	231	$>10^4$	9
Cypermethrin	250	931	6	97	6	92	10^4
Legend color code (nM)	$\geq 10^4$						
	≥1000 to $<10^4$						
	≥100 to <1000						
	≥10 to <100						
	≥1 to <10						

[a]Meijer *et al.* (2014a): VGCCs inhibition and AChE inhibition.
[b]Meijer *et al.* (2014b): VGCCs inhibition.
[c]Data were recalculated from treatment ratios to net Ca^{2+} for comparison.
[d]Kimura-Kuroda *et al.* (2012): nAChR agonist.
[e]Vale *et al.* (2003): GABA-R inhibition.
[f]Huang and Casida (1996): GABA-R inhibition.
[g]Meacham *et al.* (2008): modification VGSCs currents.

persistent and permanent changes on neurotoxicological endpoints and we have demonstrated that the inhibition in VGCCs was not or slowly reversible. This suggests that adaptive mechanisms, such as gene expression, protein function, phosphorylation, or ubiquitination of VGCCs and/or changes in VGCCs turnover (Lipscombe *et al.*, 2013), may play a role in the availability, functionality, and/or binding affinity of VGCCs. However, it remains to be determined which adaptive mechanisms are involved and why particularly for chlorpyrifos an increased inhibition of VGCCs was observed after repeated exposure compared to acute exposure.

Previously, we have compared the effective concentrations of the presumed primary modes of action of organophosphates, organochlorines, and pyrethroids with effective concentrations for acute inhibition of VGCCs by chlorpyrifos, endosulfan, and cypermethrin (Meijer *et al.*, 2014b). This comparison suggested that effective concentrations for acute inhibition of VGCCs by insecticides were lower compared with effective concentrations of the presumed primary modes of action, with the exception of chlorpyrifos-oxon (Meijer *et al.*, 2014a,b). In case of repeated exposure, effective concentrations for inhibition of VGCCs by insecticides are thus also lower compared with the effective concentrations of the presumed primary modes of action of insecticides, with the exception of chlorpyrifos-oxon (Table 2). In case of subchronic exposure, effective concentrations of chlorpyrifos, chlorpyrifos-oxon, and endosulfan were comparable or higher compared with the effective concentrations of the primary modes of action (Table 2). Although inhibition of VGCCs thus appears a sensitive endpoints, it must however be noted that for the different studies different experimental models were used. These different models may differ in sensitivity due to the expression of different ion channels and/or receptors and presence/absence of potential feedback mechanisms (eg, *Xenopus* oocytes). Also, different exposure scenarios were used

in these experimental models that, in combination with the different properties of the model, may have influenced the effective concentrations.

Notably, earlier studies on VGCCs demonstrated that beside a variety of insecticides (organophosphates, organochlorines, pyrethroids: Heusinkveld and Westerink, 2012; Hildebrand *et al.*, 2004; Liu *et al.*, 1994; Meijer *et al.*, 2014a,b; Neal *et al.*, 2010; Yan *et al.*, 2011), fungicides (Heusinkveld *et al.*, 2013), PCBs (Langeveld *et al.*, 2012), and brominated and halogen-free flame retardants (Dingemans *et al.*, 2009, 2010; Hendriks *et al.*, 2014) and other neurotoxicants can acutely inhibit VGCCs. Because VGCCs are essential for the regulation of calcium signaling, which is crucial for proper neuronal cell function (Simms and Zamponi, 2014; Westerink, 2006), development (Leclerc *et al.*, 2011), and survival (Zündorf and Reiser, 2011), the investigation of chemical-induced inhibition of VGCCs can be useful for screening chemicals for neurotoxicity. Obviously, chemicals that specifically affect postsynaptic targets, such as the imidacloprid-induced modulation of nAChRs (Matsuda *et al.*, 2009), may remain undetected. Consequently, a neurotoxicity screening battery should consist of a range of endpoints to include additional neurotoxicological targets.

For this study, PC12 cells were used because they express L-, N-, and P/Q-type VGCCs (Dingemans *et al.*, 2009). The observed complete inhibition of VGCCs by insecticides thus indicates that the insecticides inhibit all VGCCs types present in PC12 cells. Calcium channels in PC12 cells are comparable with neurons, and PC12 cells are therefore a suitable model for screening for effects on VGCCs as a measure of neurotoxicity (Westerink, 2013). Also, we show that primary rat cortical cells are a less sensitive cell model for the investigation of effects specifically on VGCC inhibition. Primary rat cortical cells constitute a physiologically relevant model and these heterogeneous cultures form spontaneously active neuronal networks. However, effects on VGCCs can be thus obscured due to interaction with additional targets. Many chemicals have additional targets, such as VGSCs in case of cypermethrin and GABA receptors in case of endosulfan, which may cause, eg, continues oscillations in basal $[Ca^{2+}]_i$ that could interfere with the depolarization-evoked increase in $[Ca^{2+}]_{i,}$. For chlorpyrifos exposure, no continues oscillations in primary cortical cells were observed and effects of chlorpyrifos on VGCCs could be determined. These data show that chlorpyrifos induces a comparable rank order potency for the different exposure scenarios in PC12 cells and primary cortical cells. However, the level of inhibition in cortical cells was lower compared with the inhibition observed in PC12 cells. This may be due to the use of serum in the medium of primary cortical cells, potentially decreasing the free insecticide concentrations, but may also be explained by interactions between different types of neurons that express different ion channels and receptors. Taken together, the current data indicate that primary cortical neurons may not be sufficiently sensitive to reliably detect effects on VGCCs by insecticides. However, for the measurement of, eg, electrical activity, spontaneously active primary cultures, such as primary cortical neurons, are preferred. Therefore, for each endpoint, the most appropriate cell model should be selected and consequently, within a battery of screening methods for neurotoxicity various cell types and lines should be used (Coecke *et al.*, 2007; Westerink, 2013).

In conclusion, our data (together with other studies) demonstrate that VGCCs are an important target for screening insecticides (and other chemicals) for neurotoxicity. Inhibition of VGCCs could be part of a battery of functional *in vitro* neurotoxicity tests. Furthermore, our data indicate that investigation of effects of acute exposure to chemicals is generally sufficient to detect insecticide (or chemical)-induced inhibition of VGCCs. However, some differences in potency of insecticides for inhibition of VGCCs were observed between the exposure scenarios, which argues for the inclusion of repeated exposure conditions for specific studies.

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