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Research Paper

Protective Effect of the Natural Product, Chaetoglobosin K, on Lindane- and Dieldrin-induced Changes in Astroglia: Identification of Activated Signaling Pathways

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Purpose. The purpose of the present study was to identify the biochemical mechanism(s) of the preventative and reversal effects of Chaetoglobosin K (ChK), a bioactive natural product, on inhibition of gap junction-mediated communication and connexin phosphorylation by the tumor promoting organochlorine compounds, lindane, and dieldrin.
Materials and methods. A fluorescent dye transfer assay was used to quantify gap junction-mediated communication and sensitivity to lindane and dieldrin. Analyses of connexin 43, PKC, ERK, GSK-3β, Raf, and Akt kinase phosphorylation were performed by Western blotting.
Results. Pre-incubation of astroglial cells with 10 μM ChK prevented inhibition of dye transfer by lindane and dieldrin, which correlates with stabilization of the connexin 43 P2 isoform, and addition of ChK after lindane or dieldrin reversed the inhibitory effect, which correlated with re-appearance of the P2 isoform. Using phosphorylation site-specific antibodies, we demonstrated that lindane, dieldrin, and ChK all activated p44/42 ERK, but only ChK activated Akt kinase. ChK also activated a downstream effector of Akt, GSK-3β, and activation of both kinases was inhibited by Wortmannin. Wortmannin also blocked ChK's ability to prevent dieldrin-induced inhibition of gap junction-mediated communication between RG-2 cells.
Conclusion. ChK's protective effects, both preventative and reversal, on lindane and dieldrin inhibition of gap junction-mediated communication are associated with stabilization and reappearance of the connexin 43 P2 phosphoform and may be mediated by the Akt pathway.

KEY WORDS: chaetoglobosin K; connexin; gap-junction; organochlorine.

INTRODUCTION

Natural product compounds play a significant role in the development of new drugs, particularly in anti-cancer and anti-hypertensive therapeutic domains (1,2). Chaetoglobosin K (ChK) is a natural product compound isolated from *Diplodia macrospora* (3) that has previously been shown to suppress the tumorigenic phenotype in transformed fibroblasts (4), and inhibit growth of *ras*-transformed liver epithelial cells (5). It has also been shown to prevent inhibitory effects of tumor promoting organochlorine compounds on gap junction-mediated cell–cell communication in astroglial and epithelial cells (6). Intercellular communication mediated through gap junctions is thought to play a critical role in cell growth and differentiation (7–9). Down-regulated gap junction-mediated communication has been reported in different types of tumor cells, and these data support the hypothesis that the loss of gap junction-mediated communication is associated with neoplastic transformation of cells (10–14).

A large number of organochlorine compounds present in the environment inhibit gap junction membrane channels. Many of these compounds have been shown to be liver toxins and/or tumor promoters (10,15). There is also increasing concern about their effects in the brain. Astroglia, the predominant cell type in the brain (16), are coupled through gap junctions, and inhibition of astroglial cell–cell communication by organochlorine compounds may play a role in their neurotoxicity (17). Lindane and dieldrin are organochlorine compounds of particular concern because of their persistence in the environment and continuous detection in the food supply and drinking water. Both compounds have been shown to inhibit gap junction-mediated communication between a variety of types of cells including astroglia (6,18, 19). Lindane, once widely used to kill lice and agricultural pests, has been progressively eliminated from many applications in Europe or USA since the mid-1970s, but is still used in many countries.

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ABBREVIATIONS: ChK, Chaetoglobosin K; Cx43, Connexin 43; Dld, Dieldrin; Lnd, Lindane; NBT/BCIP, Nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate; PBS, Phosphate-buffered saline; PMSF, Phelylmethylsul fonylfluoride.

It is absorbed through respiratory, digestive, or cutaneous routes, damages liver, kidney, neural and immune systems and induces birth defects, cancer, and death (20). Dieldrin has also been banned from use by many countries due to its persistence and accumulation in the environment and tumor promoting potential. In addition, this compound has been reported to have neurotoxic effects, including toxicity for dopaminergic neurons (17), inhibition of GABA receptor binding (21), and inhibition of gap junction-mediated communication between neurons *in vitro* (22).

The signal transduction pathways and key signaling molecules involved in lindane- and dieldrin-induced disruption of gap junction-mediated communication and prevention by ChK have not been clearly identified. In this study, we demonstrate that ChK not only prevents but also reverses the inhibition of gap junction-mediated communication by lindane and dieldrin in astroglial cells, which correlates with stabilization of the connexin 43 P2 phosphoform in the case of prevention, and reappearance of the P2 isoform in the case of reversal. We further identify signaling pathways activated by ChK, lindane and dieldrin in these cells.

MATERIALS AND METHODS

Materials

Alpha-MEM culture media and L-glutamine were purchased from Fisher Scientific. Lucifer yellow, Neutral Red, PMSF, and fetal bovine serum were purchased from Sigma-Aldrich. Dieldrin and lindane were purchased from Accustandard. Connexin 43-specific monoclonal antibodies were obtained from Chemicon International or Zymed. Akt, phospho-Akt, p44/42 ERK, phospho- p44/42 ERK, PKC-β, and phospho-PKC-β, and c-Raf antibodies were purchased from Cell Signaling Technologies, Inc. Biotin conjugated anti-mouse antibody and alkaline phosphatase-conjugated streptavidin were purchased from ICN. Acrylamide, bis-acrylamide, Tween-20, non-fat dry milk and NBT/BCIP were purchased from Biorad. Chaetoglobosin K, (ChK) was purified from semi-solid fermentation of the fungal phytopathogen *D. macrospora* and was greater than 97% pure. The empirical formula is $C_{34}H_{40}O_5N_2$ and the formula weight is 556.2934 (3).

Cell Cultures

A rat astroglial cell line derived from embryonic rat cerebral cortex and designated RG-2, was grown in alpha-MEM media supplemented with 2 mM L-glutamine and 5% fetal bovine serum at 37°C in a 5% CO_2/95% air atmosphere. In all experiments, media was changed to serum-free alpha-MEM 20–24 h before treatments. Cells were subcultured by trypsinization and used when 90–100% confluent at passages 10–30. For dye transfer assays, cells were plated in 35 mm^2 dishes, for phosphorylation/immunoblot assays of connexin 43 in 75 cm^2 flasks, and for phosphorylation/immunoblot analysis of all other proteins in 25 cm^2 flasks.

Treatment of Cells with Test Compounds

Test chemicals were dissolved in dimethylsulphoxide (DMSO) at 1,000× concentrations to be used in experiments and added at 1 μl/ml for the indicated times. Controls consisted of treatments with identical amounts of DMSO. Lindane (50 μM) and dieldrin (10 μM) were used in all experiments, and ChK was used at 2, 5, or 10 μM as indicated. In all experiments in which cells were pre-incubated with ChK, pre-incubation time was 15 min before addition of lindane or dieldrin.

Protein Assay

Protein concentrations were determined using the Bio-rad DC protein assay in a 48 well format on samples solubilized in buffer containing 1% SDS. BSA was used as a standard and absorbances read at 750 nm using a Tecan Platereader.

Neutral Red Cell Viability/Cytotoxicity Assay

Cells grown in 48-well culture plates were treated with increasing concentrations of lindane dissolved in DMSO for 24 h and incubated for 3 h at 37°C with 0.15% Neutral Red dye added to the culture media in the presence of the test compound (modification of Borenfreund and Puerner, 1985) (23). Solvent vehicle-treated cells were used as controls. Cells were washed free of external dye once with Ca^{2+}/Mg^{2+} PBS and twice with PBS, followed by addition of 1 ml of extraction solution containing 50% ethanol, 1% acetic acid. Absorbances of samples run in quadruplicate were read at 540 nm on a Tecan plate reader. Neutral Red uptake of assay of lindane (Fig. 1) indicated no cytotoxicity of this compound on RG-2 exposed for 24 h to concentration as high as 75 μM.

Dye Transfer Assay of Gap Junction-mediated Cellular Communication

Cells grown in 35 mm^2 dishes were washed once with PBS containing 0.5 mM CaCl, 0.5 mM MgCl (Ca^{2+}/Mg^{2+} PBS) and twice in PBS. One milliliter of 0.5 mg/ml Lucifer Yellow in PBS was added and several areas of the cell monolayer scored with a surgical blade to allow entry of

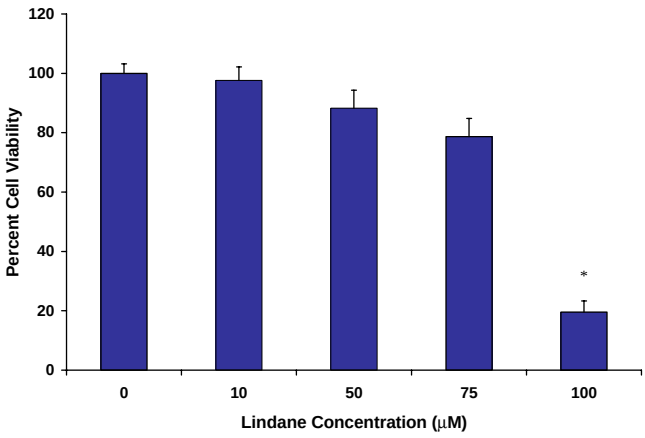

Fig. 1. Neutral Red cytotoxicity assay of lindane on RG-2 cells. Cells grown in 48-well culture plates were exposed for 24 h to different concentrations of lindane (0–100 μM) and incubated for 3 h at 37°C with 0.15% Neutral Red dye. Vehicle (DMSO)-treated cells were used as controls. Data are presented as the mean±SD for each group (n=4). *, Statistically significant (P<0.05) compared to controls.

Lucifer Yellow. After 90 s, dye was removed and cells washed once with Ca^{2+}/Mg^{2+} PBS followed by twice with PBS and fixed with 4% paraformaldehyde in PBS. Fluorescence was observed using a Leitz inverted microscope using a 10-× objective lens. Several areas of each test plate were photographed and the number of fluorescent cells counted in a defined area using a template. Since cells along the score line were fluorescent, regardless of their ability to communicate, the average number of cells in fully inhibited (negative control) samples was subtracted from the total number of cells counted in the various treatment groups to give a better estimate of the number of communication positive cells. Statistical analyses were performed using StatistiXL. Data was analyzed by ANOVA, followed by Tukey's post-hoc test. $p<0.05$ was used to indicate a statistically significant difference.

Immunoblot Analysis of Connexin 43

Membrane-enriched protein fractions were extracted from RG-2 cells grown in 75 cm^2 flasks by lysis in 0.375 ml of 10 mM Tris, 10 mM iodoacetamide, 1 mM PMSF, pH 7.5 as previously described (6). Samples were alkalinized by addition of 0.55 ml of 40 mM NaOH, chilled on ice, and sonicated for 2, 15 s pulses on a Bronson sonicator at 35% maximal power using a micro tip. Samples were centrifuged for 30 min in a microfuge (14,000 RPM), membrane enriched pellets washed with 10 mM Tris, 1 mM PMSF, pH 7.5 and resuspended in the Tris/PMSF buffer by sonication. Aliquots were removed for protein assay and the remainder solubilized in Laemmli sample buffer (24). Following electrophoresis on 12.5% acrylamide, 1 mm thick minigels, proteins were transferred to PVDF membranes in transfer buffer containing 0.02% SDS overnight at 20 V. Membranes were blocked for 2 h at room temperature in 40 mM Tris, 4% nonfat dry milk, 0.1% Tween-20, then incubated in the same buffer containing connexin 43-specific antibodies diluted 1:3,000 for 12–24 h at 4°C on a shaker. Membranes were washed in block buffer, incubated for 1–2 h with biotinylated anti-mouse antibodies diluted 1:200 in block buffer, washed, then incubated for 1 h with streptavidin-conjugated alkaline phosphatase and after washing, immunopositive bands were visualized using the NBT/BCIP detection system.

Immunoblot Analysis of p44/42 MAPK, Phospho-p44/42 MAPK, Akt, Phospho-Akt, PKC, Phospho-PKC, Phospho-c-Raf, and Phospho-GSK-3β

For total protein extraction, cells were grown to 90–95% confluency in 25 cm^2 flasks and treated according to the designed experiment. Cultures were washed with phosphate-buffered saline (PBS,) solubilized in lysis buffer (250 μM of 2% SDS and 1 mM PMSF, and 1:1,000 dilution of protease inhibitor mix), and the cells were scraped from the culture dishes with a cell scraper. Whole cell lysates were sonicated for 2× 15 s pulses on a Bronson sonicator at 35% maximal power using a microtip, separated by 12% SDS-PAGE, and electroblotted onto PVDF membranes overnight at 20 V. Membranes were blocked for 1–2 h at room temperature in blocking buffer (40 mM Tris, 4% nonfat dry milk, 0.1% Tween-20), then incubated separately in the same buffer containing p44/42 MARK or phospho-p44/42 MARK, Akt, phospho-Akt, PKC, phospho-PKC, and phospho-c-Raf, and phospho-GSK-3β polyclonal antibodies diluted 1:2,000 for 2 h on a shaker at room temperature. Immunopositive bands were visualized using alkaline phosphatase antirabbit secondary antibody with the NBT/BCIP detection system. Molecular size was estimated with molecular mass markers (7.1–209 kDa).

RESULTS

Effect of Different Concentrations of Lindane on Cell Viability

We first evaluated the lindane toxicity threshold by monitoring the viability of RG-2 cells exposed for 24 h to different concentrations of lindane (0–100 μM). Concentrations of lindane between 10 and 75 μM did not exert a significant effect on RG-2 cell viability (Fig. 1). In contrast, cell exposure to higher concentration of lindane (100 μM) significantly reduced the number of cells ($P<0.05$). Results of Neutral Red assays of ChK and dieldrin have been previously reported showing evidence of cytotoxicity to RG-2 cell starting at concentration above 15 μM for ChK and indicating no cytotoxicity on RG-2 cells exposed for 24 h to concentration as high as 50 μM for dieldrin (6, 25).

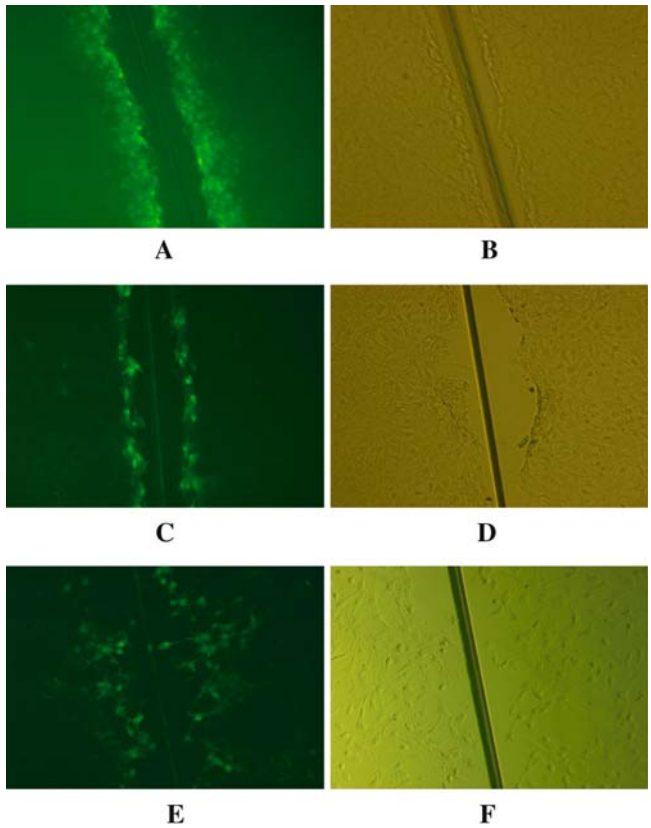

Fig. 2. Fluorescent dye transfer assay of gap junction-mediated intercellular communication. RG-2 cells were grown to 90–100% confluence on 35 mm dishes. Fluorescent dye transfer assay was performed as described in "MATERIALS AND METHODS." **A** Control dishes were treated with an equal volume of vehicle (DMSO); **C** cells treated with 50 μM Lindane; **E** Cells were preincubated with 10 μM ChK for 15 min followed by treatment with 50 μM Lindane for 30 min. (**B**, **D**, **F**) phase-contrast images corresponding to (**A**, **C**, **E**).

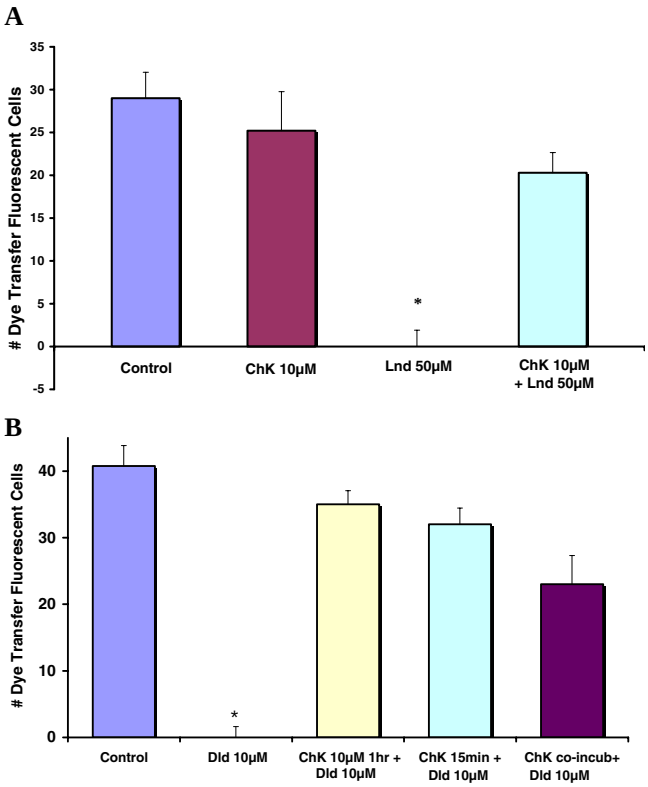

Fig. 3. **A** Preventative effect of ChK on lindane inhibition of gap junctional communication. RG-2 cells were grown to 90–100% confluence on 35 mm dishes. Cells were preincubated with 10 μM ChK for 15 min followed by treatment with 50 μM lindane for 30 min. Control dishes were treated with an equal volume of vehicle. Incubation with 10 μM ChK for 45 min showed no inhibition. Fluorescent dye transfer assay was performed as described in "MATERIALS AND METHODS." Values from separate lindane dishes were subtracted as background fluorescent cells for each group. Data are presented as the mean±SD for each group (n=5). *, Statistically significant (P<0.05) compared to controls. **B** Effect of ChK at different pre-incubation time on dieldrin inhibition of GJIC (1 h vs. 15 min vs. co-incubation). RG-2 cells were grown to 90–100% confluence on 35 mm dishes. Cells were preincubated with 10 μM ChK for 1 h or 15 min followed by treatment with 10 μM dieldrin for 30 min or co-incubation. Control dishes were treated with an equal volume of vehicle. Fluorescent dye transfer assay was performed as described in "MATERIALS AND METHODS." Values from separate dieldrin dishes were subtracted as background fluorescent cells for each group. Data are presented as the mean±SD for each group (n=5). *, Statistically significant (P<0.05) compared to controls.

Inhibition of Gap Junction-mediated Communication by Lindane and Dieldrin and Restoration by ChK in Astroglial Cells

Figure 2 shows the inhibitory effect of lindane and preventative effect of ChK on gap junction-mediated communication assayed by fluorescence dye transfer. Lucifer-yellow dye loaded into untreated RG-2 cells along the score line spread well into neighboring cells in the absence of inhibitor (Fig. 2A), normal communication, and poorly in cells treated 50 μM lindane for 1 h (Fig. 2C). Pre-incubation of RG-2 cells with ChK prevented the lindane-induced inhibition of fluorescent dye transfer (Fig. 2E). Figure 2B, D, F represent phase-contrast images corresponding to Fig. 2A, C, E. Data previously obtained using dieldrin show a similar preventative effect by ChK (6).

Figure 3A demonstrates quantitative analysis of the percentage of coupled cells in control, ChK, lindane and ChK–lindane treated cells showing that pre-incubation with 10 μM ChK for 15 min, followed by 30 min incubation with 50 μM lindane resulted in a significantly greater number of dye-transfer fluorescent cells than cells treated for 30 min with 50 μM lindane alone.

To extend our studies with dieldrin, an experiment showing time-dependent effect of ChK on gap junction-mediated communication was conducted. Figure 3B represents quantitative analysis of the percentage of coupled cells in control, dieldrin, and ChK–dieldrin treated cells. This graph shows that increasing the preincubation time to 1 h only slightly increased the preventative effect of ChK

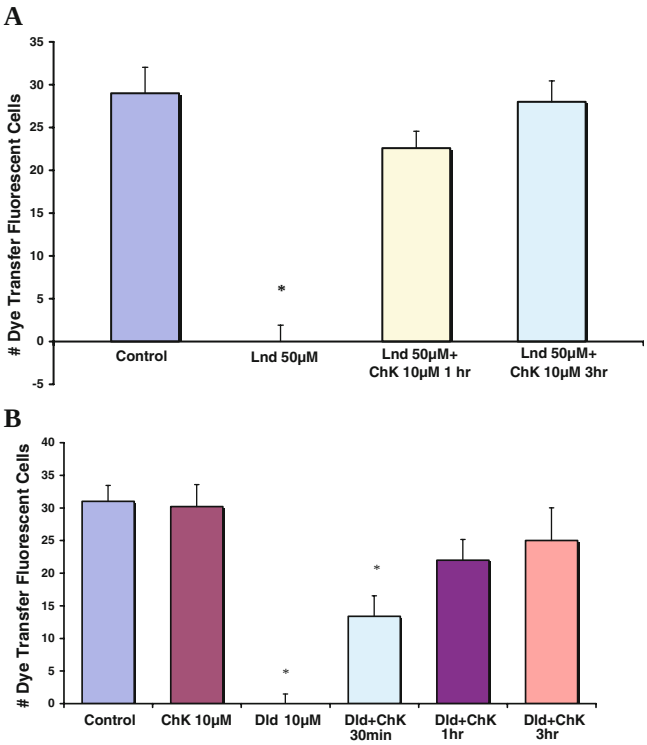

Fig. 4. **A** Reversal effect of ChK on lindane inhibition of gap junctional communication. RG-2 cells were grown to 90–100% confluence on 35 mm dishes. Cells were preincubated with lindane for 1 h followed by treatment with 10 μM ChK for 1 or 3 h. Control dishes were treated with an equal volume of vehicle. Fluorescent dye transfer assay was performed as described in "MATERIALS AND METHODS." Values from separate lindane dishes were subtracted as background fluorescent cells for each group. Data are presented as the mean±SD for each group (n=5). *, Statistically significant (P<0.05) compared to controls. **B** Reversal effect of ChK on dieldrin inhibition of gap junctional communication. RG-2 cells were grown to 90–100% confluence on 35 mm dishes. Cells were preincubated with dieldrin for 15 min followed by treatment with 10 μM ChK for 30 min, 1 or 3 h. Control dishes were treated with an equal volume of vehicle. Fluorescent dye transfer assay was performed as described in "MATERIALS AND METHODS." Values from separate dieldrin dishes were subtracted as background fluorescent cells for each group. Data are presented as the mean±SD for each group (n=5). *, Statistically significant (P<0.05) compared to controls.

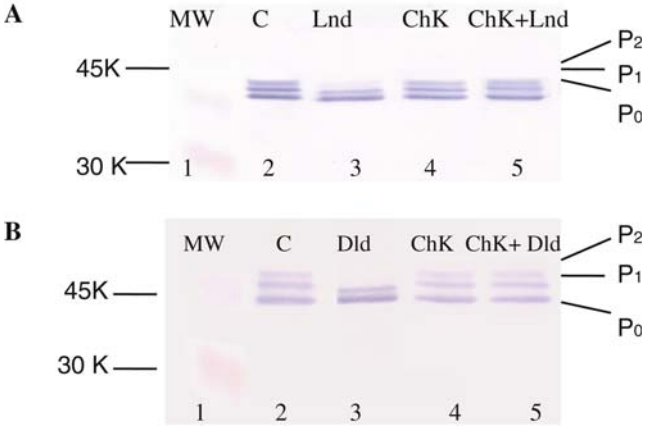

Fig. 5. **A** Preventative effect of ChK on lindane-induced connexin 43 hypophosphorylation: stabilization of the connexin 43 P_2 phosphoform. RG-2 cells were grown to 90% confluence in 75 cm^2 flasks, preincubated with 10 μM ChK for 15 min, followed by 30 min treatment with 50 μM lindane (lane 5). The 41-kDa molecular mass marker is shown in lane 1. Control dishes were treated with an equal volume of vehicle (lane 2) or with 50 μM lindane (lane 3), showing loss of P_2 phosphoform. Incubation with 10 μM ChK for 45 min showed no inhibition (lane 4). Western blot analysis was performed as described in "MATERIALS AND METHODS." **B** Preventative effect of ChK on dieldrin-induced connexin 43 hypophosphorylation: stabilization of the connexin 43 P_2 phosphoform. RG-2 cells were grown to 90% confluence in 75 cm^2 flasks, preincubated with 10 μM ChK for 15 min, followed by 30 min treatment with 10 μM dieldrin (lane 5). The 41-kDa molecular mass marker is shown in lane 1. Control dishes were treated with an equal volume of vehicle (lane 2) or with 10 μM dieldrin (lane 3), showing loss of P_2 phosphoform. Incubation with 10 μM ChK for 45 min showed no inhibition (lane 4). Western blot analysis was performed as described in "MATERIALS AND METHODS."

compared to 15 min incubation, whereas co-incubation of ChK and dieldrin resulted in reduced preventative effect of ChK. Treatment of RG-2 cells with 10 μM ChK alone for up to 45 min showed no effect on dye transfer compared to control cells (Fig. 3A).

Having determined that ChK has a preventive effect, further experiments were performed to determine the reversal potential of ChK on lindane and dieldrin inhibition of gap junction-mediated communication. RG-2 cells were pretreated with 50 μM lindane for 1 h to reach full inhibition, followed by treatment with 10 μM ChK for 1 or 3 h (Fig. 4A). In Fig. 4B, cells were pretreated with 10 μM dieldrin for 15 min, then treated with 10 μM ChK for 30 min, 1, and 3 h. These data show that over time, gap junction-mediated communication increased with increasing ChK treatment time and support the idea that ChK reverses inhibitory effects of lindane and dieldrin on gap junction-mediated communication.

ChK Prevents and Reverses Lindane- and Dieldrin-induced Changes in Connexin 43 Phosphorylation

To determine whether effects of lindane, dieldrin, and ChK on gap junction-mediated cell communication correlated with changes in phosphorylation of connexin 43, membrane-enriched fractions were isolated from treated and vehicle-treated RG-2 cells and analyzed by Western blotting. Membrane-enriched fractions from vehicle-treated RG-2 cells (Fig. 5A and B, lane 2) exhibited the characteristic Po/P1/P2 connexin 43 pattern seen previously in rat liver epithelial cell cultures and other cell types (26–28). In previous studies, we identified Po as the unphosphorylated form of connexin 43 and P1 and P2 two phosphoforms (28). Western blot analysis of connexin 43 from lindane-treated cells (Fig. 5A lane 3) showed loss of the P2 phosphoform. This loss of P2 was prevented by pre-treatment of cells with ChK for 15 min. before addition of lindane (Fig. 5A, lane 5) or dieldrin (Fig. 5B, lane 5). Treatment of cells with ChK alone (Fig. 5A and B, lane 4) did not substantially alter the phosphorylation of connexin 43 compared to vehicle control cells.

Two separate experiments were performed to determine whether ChK was able to reverse the P_2 phosphoform loss induced by lindane and dieldrin (Fig. 6). In Fig. 6A, lane 2 and B lanes 2 and 3, we observed P_0, P_1, P_2 connexin 43 immunopositive bands in vehicle-treated RG-2 cells. Cells

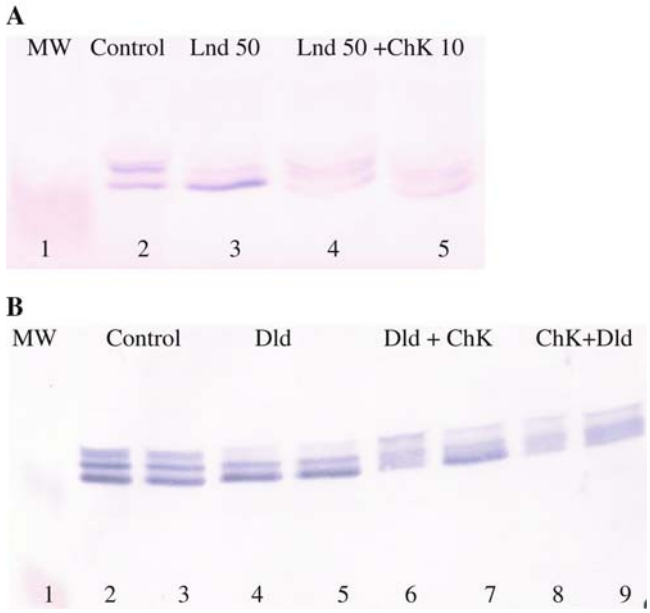

Fig. 6. **A** Reversal potential of ChK on Cx43 hypophosphorylation induced by lindane. RG-2 cells were grown to 90% confluence in 75 cm^2 flasks. The 41-kDa molecular mass marker is shown in lane 1. Cells were incubated with 50 μM lindane for 1 h and then treated with 10 μM ChK for 1 h (lanes 4 and 5, duplicates) showing that treatment with ChK *after* treatment with 50 μM lindane resulted in reappearance of the P_2 phosphoform compared to lanes with lindane alone (lane 2). Western blot analysis was performed as described in "MATERIALS AND METHODS." **B** Reversal potential of ChK on Cx43 hypophosphorylation induced by dieldrin. RG-2 cells were grown to 90% confluence in 75 cm^2 flasks. The 41-kDa molecular mass marker is shown in lane 1. Cells were incubated with 10 μM dieldrin for 15 min and then treated with 10 μM ChK for 30 min (lanes 6 and 7, duplicates) showing that treatment with ChK *after* treatment with dieldrin resulted in reappearance of the P_2 phosphoform compared to lanes with dieldrin alone (lanes 4 and 5, duplicates). Preincubation RG-2 cell with 10 μM ChK for 15 min followed by 30 min treatment with 10 μM dieldrin (lanes 8 and 9, duplicates) also resulted in a greater amount of the P_2 band compared to RG-2 cells treated with dieldrin alone (lanes 4 and 5, duplicates), showing the preventive effect of ChK. Western blot analysis was performed as described in "MATERIALS AND METHODS."

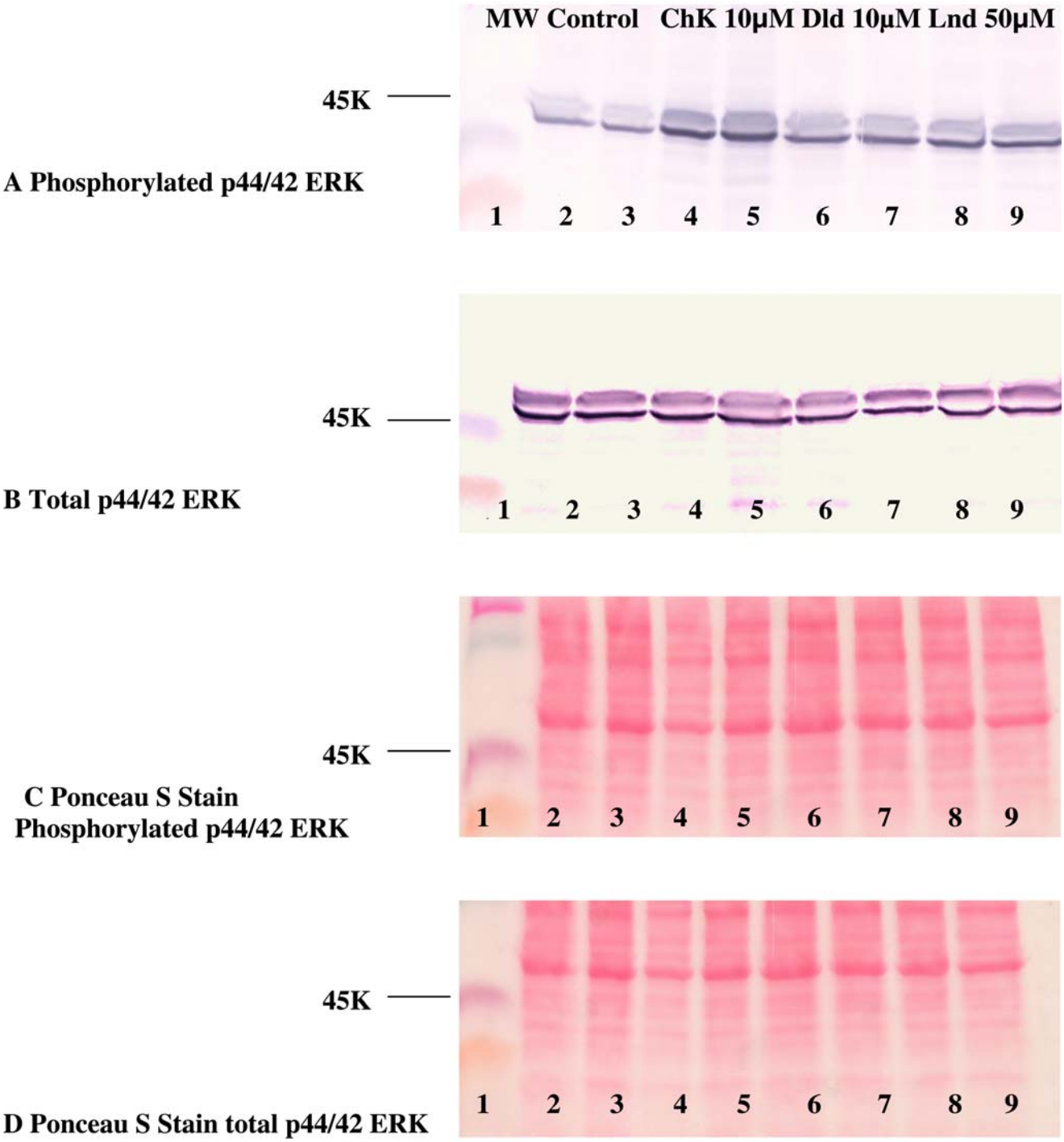

Fig. 7. Effect of ChK, lindane, and dieldrin on p44/42 ERK phosphorylation. RG-2 cells were grown to 90% confluence in 25 cm^2 flasks, treated with vehicle (DMSO), ChK, lindane, or dieldrin for 1 h, and extracted for Western blot analysis of: **A** Phosphorylated p44/42 ERK or **B** Total p44/42 ERK as described in "MATERIALS AND METHODS." **C** and **D** are Ponceau staining blots corresponding to **A** and **B**, respectively. Treatment groups were molecular weight markers (lane 1), vehicle (DMSO) for 1 h (lanes 2 and 3, duplicates), ChK 10 μM (lanes 4 and 5), dieldrin 10 μM (lanes 6 and 7), lindane 50 μM (lanes 8 and 9).

were incubated with 50 μM lindane for 2 h and then treated with 10 μM ChK for 2 h (Fig. 6A, lanes 4 and 5, duplicates) or with 10 μM dieldrin for 15 min and then treated with 10 μM ChK for 30 min (Fig. 6B, lanes 6 and 7, duplicates). These experiments showed that treatment with ChK *after* treatment with 50 μM lindane or 10 μM dieldrin resulted in reappearance of the P_2 phosphoform compared to lanes with lindane (Fig. 6A, lane 3) or dieldrin (Fig. 6B, lanes 4 and 5, duplicates) alone. Preincubation of RG-2 cells with 10 μM ChK for 15 min, followed by 30 min treatment with 10 μM dieldrin (Fig. 6B, lanes 8 and 9, duplicates) also resulted in a greater amount of the P_2 band compared to RG-2 cells treated with dieldrin alone (Fig. 6B, lanes 4 and 5, duplicates) again showing the preventative effect of ChK.

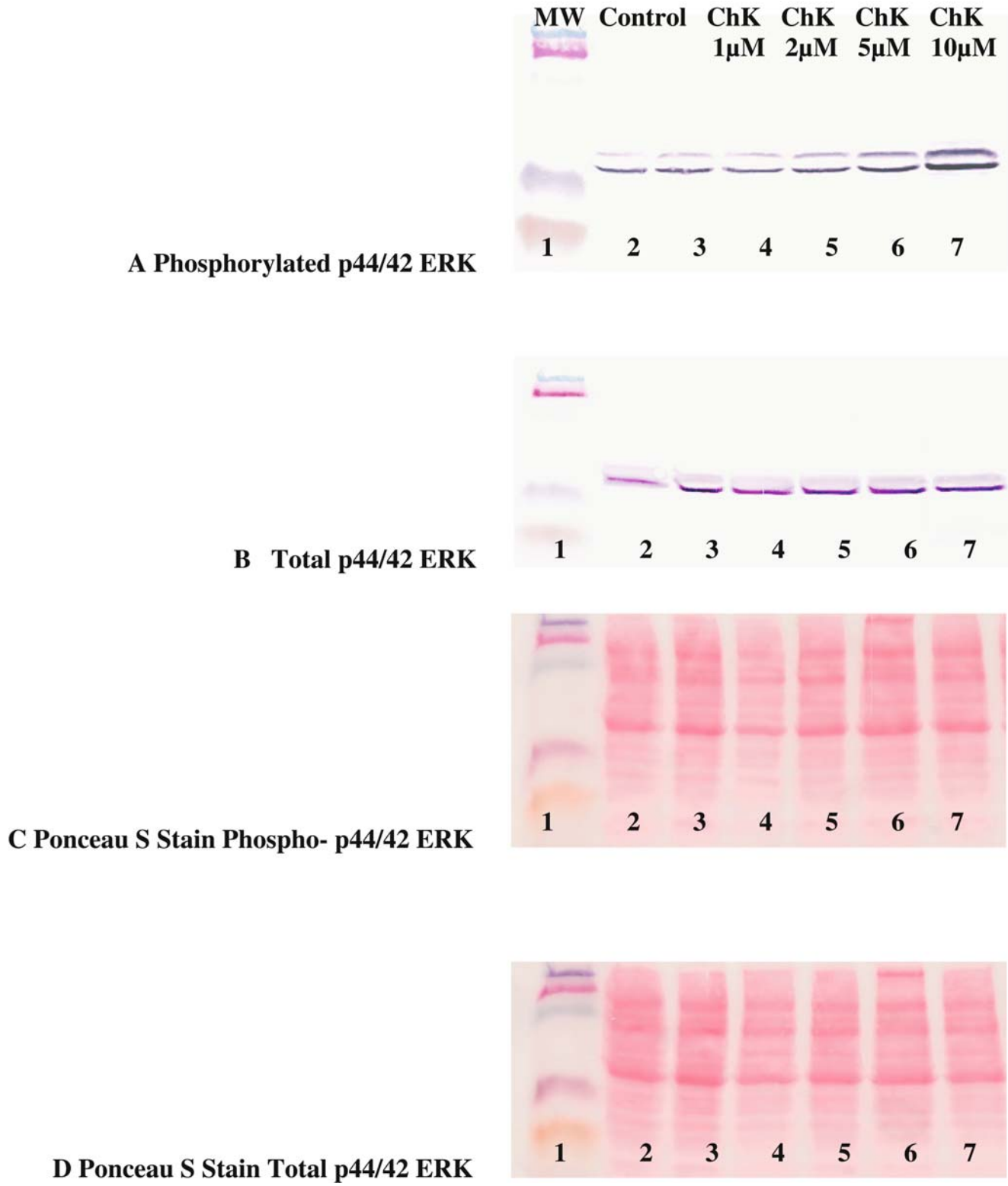

Fig. 8. Effect of ChK on p44/42 ERK phosphorylation at varying concentrations. RG-2 cells were grown to 90% confluence in 25 cm^2 flasks, treated with vehicle (lanes 2, 3) or with ChK at varying concentrations 1 μM (lane 4), 2 μM (lane 5), 5 μM (lane 6), and 10 μM (lane 7) for 1 h showing increased phosphorylation, and extracted for Western blot analysis of: **A** Phosphorylated p44/42 ERK or **B** Total p44/42 ERK as described in "MATERIALS AND METHODS." **C** and **D** are Ponceau staining blots corresponding to **A** and **B**, respectively.

Intracellular Signaling Pathways Affected by Lindane, Dieldrin, and Chaetoglobosin K

To determine signaling pathways affected by lindane, dieldrin, and ChK, experiments were performed using phosphorylation site-specific antibodies. Figure 7A shows that a 1 h treatment with any of the three compounds increased the level of phosphorylation of a key activation site on p44/42 ERK (lanes 4 and 5, ChK, lanes 6 and 7, dieldrin, and lanes 8 and 9, lindane, compared to lanes 2 and 3, vehicle-treated control). ChK (lanes 4 and 5) appeared to induce the greatest increase in phospho-ERK at the given concentrations. Levels of total ERK (Fig. 7B) were not altered by treatment with ChK, lindane, or dieldrin (lanes 3–9). Ponceau staining (Fig. 7C) of the identical blot shown in Fig. 7a showed that the changes in phospho-ERK were not due to loading differences between lanes. Figure 8 shows that the effect of ChK on p44/42 ERK phosphorylation was concentration-dependent (lanes 4–7 compared to control lanes 2 and 3). In contrast, treatment of cells for 1 h with ChK, dieldrin or lindane had no effect on the level of phosphorylation of PKC at a key activation site (Fig. 9).

Figure 10A shows that 1-h treatment with ChK (lanes 4 and 5) increased phosphorylation of Akt kinase on a key activation site. However, dieldrin (lanes 6 and 7) or lindane (lanes 8 and 9) did not substantially alter the level of phosphorylation of Akt kinase. Total Akt kinase levels (Fig. 10B) were unaltered by any of the compounds. In contrast, analysis of phospho-Raf (ser259) in identical samples run on separate gels (Fig. 10D) showed that ChK treatment of cells caused decreased phosphorylation of phospho-Raf at this site (lanes 4 and 5) compared to control (lanes 2 and 3), dieldrin (lanes 6 and 7), or lindane (lanes 8 and 9) treatments.

Further, we examined the effect of the PI3 kinase/Akt pathway inhibitor, Wortmannin, on the activation of Akt kinase and a downstream effector of Akt, GSK-3β in RG-2 cells to see whether this blocks ChK's increased phosphorylation. (Fig. 11). RG-cells were treated with vehicle (DMSO, lanes 2 and 3), dieldrin (lane 4), lindane (lane 5), 5 μM ChK (lanes 6 and 7), 10 μM ChK (lanes 8 and 9), 10 μM ChK and 0.5 μM Wortmannin (lane 10) for 1 h, and extracted for Western blot analysis of: (A) Phosphorylated Akt, (B) total Akt, and (C) Phosphorylated GSK-3β as described in "MATERIALS AND METHODS." These results show that ChK increased Akt and GSK-3β phosphorylation, while Wortmannin blocked the increased phosphorylation induced by ChK alone.

To determine whether Wortmannin modulated ChK's protective effect on gap junctional communication, cells were treated with vehicle, dieldrin alone, ChK with dieldrin, Wortmannin alone, or ChK with dieldrin plus Wortmannin (Fig. 12). The results show that Wortmannin blocked ChK's ability to prevent dieldrin-induced inhibition of gap junctional communication.

DISCUSSION

The results presented in this study demonstrate for the first time that lindane, a persistent organochlorine pesticide and tumor promoter, inhibits gap junction-mediated communication between RG-2 cells, and that Chaetoglobosin K prevents and reverses this inhibition of communication. This agrees with our previous results demonstrating prevention by ChK of inhibitory effects of dieldrin and another organochlorine compound, endosulfan, on gap junctional communication in astroglial cells (6) and extends these studies to show that ChK also reverses the effect of dieldrin. In the present study, we found that the preventative effect of ChK correlated with the stabilization of the connexin43 P2 phosphoform and the reversal effect of ChK correlated with reappearance of the connexin43 P2 phosphoform.

Guan and Ruch (18) showed that lindane decreased levels of the Cx43 P2 phosphoform in liver epithelial cells and provided evidence that the loss of P2 was due primarily to proteolytic degradation of P2. Our results presented here are consistent with these previous findings but do not distinguish proteolytic degradation from P2 dephosphorylation. Unpublished observations suggest that at least some P2 degradation occurs when RG-2 cells are treated greater than 4 h with lindane. Thus, ChK may protect against proteolytic degradation of connexin 43 as well as dephosphorylation.

MAP kinases are key signaling molecules involved in the control of cell proliferation (34). We considered the possibility that the ChK-organochlorine effects are due to the activation of intracellular signaling pathways that have been previously linked to regulation of gap-junction mediated communication, specifically, ERK, PKC, and Akt kinases (12,29–32). Activation of the Ras/Raf/mitogen-activated protein kinase kinase/mitogen-activated protein (MAP) kinase signaling cascade is initiated by activation of growth factor receptor and is regulated many cellular events,

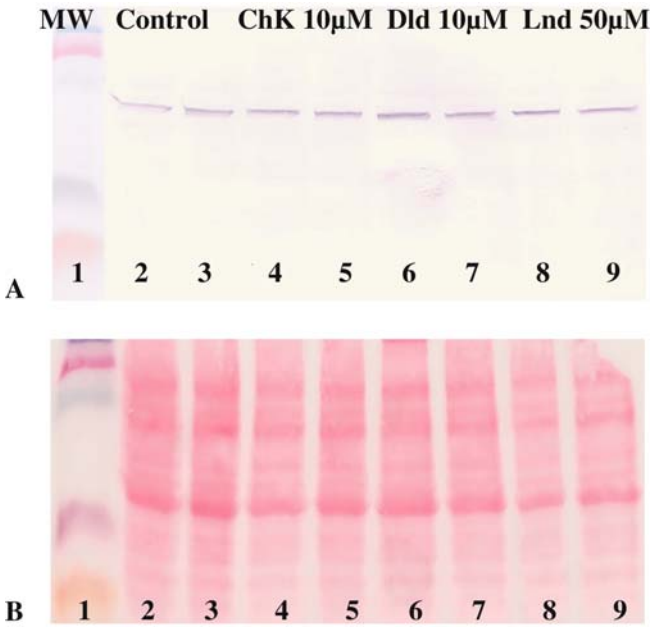

Fig. 9. Effect of ChK, dieldrin and lindane on PKC activation. RG-2 cells were grown to 90% confluence in 25 cm^2 flasks, treated with vehicle (DMSO), ChK, lindane, or dieldrin for 1 h, and extracted for Western blot analysis of **A** Phosphorylated PKC as described in "MATERIALS AND METHODS." **B** Ponceau staining blot corresponding to **A**. Treatment groups were: molecular weight markers (lane 1), vehicle (DMSO) (lanes 2 and 3, duplicates), ChK 10 μM (lanes 4 and 5), dieldrin 10 μM (lanes 6 and 7), lindane 50 μM (lanes 8 and 9) for 1 h.

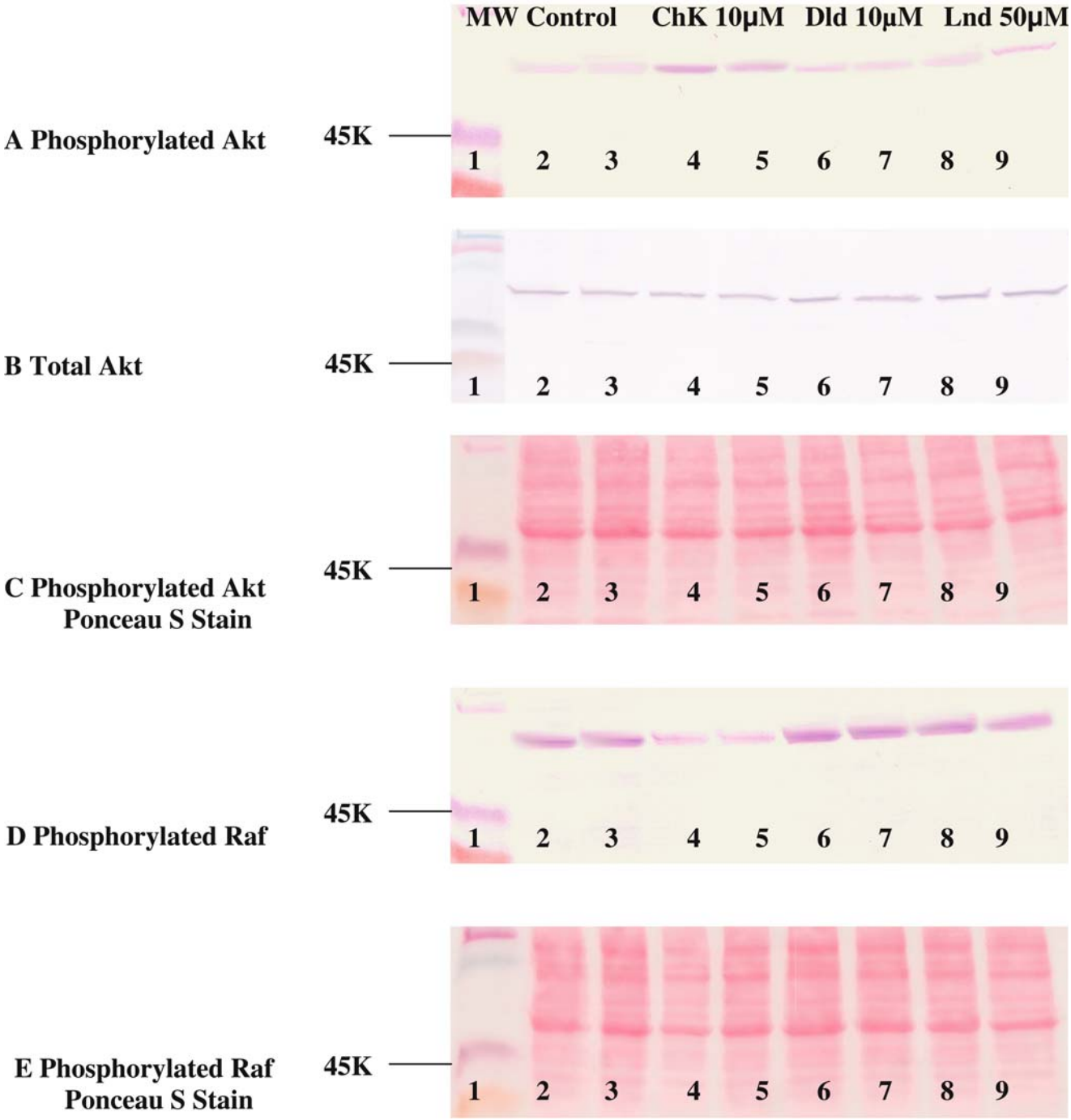

Fig. 10. Effect of ChK, lindane, and dieldrin on Akt and c-Raf phosphorylation. RG-2 cells were grown to 90% confluence in 25 cm^2 flasks, treated with vehicle (DMSO), ChK, lindane, or dieldrin for 1 h, and extracted for Western blot analysis of: **A** Phosphorylated Akt, **B** Total Akt, and **D** Phosphorylated c-Raf. **C** and **E** are Ponceau staining blots corresponding to **A** and **D**, respectively. Treatment groups were: molecular weight markers (lane 1), vehicle (DMSO) for 1 h (lanes 2 and 3, duplicates), ChK 10 μM (lanes 4 and 5), dieldrin 10 μM (lanes 6 and 7), lindane 50 μM (lanes 8 and 9).

including cell cycle control. Furthermore, studies suggest that a target of activated MAP kinase (ERK 1/2) might be the connexin-43 gap junction protein (33).

To gain insight into their possible involvement in the mediation of ChK-organochlorine-induced effects, activation of the MAPK pathway was assessed by Western blotting with antibodies directed against the phosphorylated and active forms of p44/42 ERK. In the studies reported here, these key intracellular signaling pathways were examined using phosphorylation site-specific antibodies, to determine whether they are activated or inhibited by lindane, dieldrin, and/or ChK. Our results show that a 1-h treatment with either ChK, lindane, or dieldrin increased the level of phosphorylation of a key activation site on p44/42 ERK (Fig. 7A). Levels of total ERK were not altered by treatment with ChK, lindane, or dieldrin. Activation the ERK1/2 signal transduction pathway

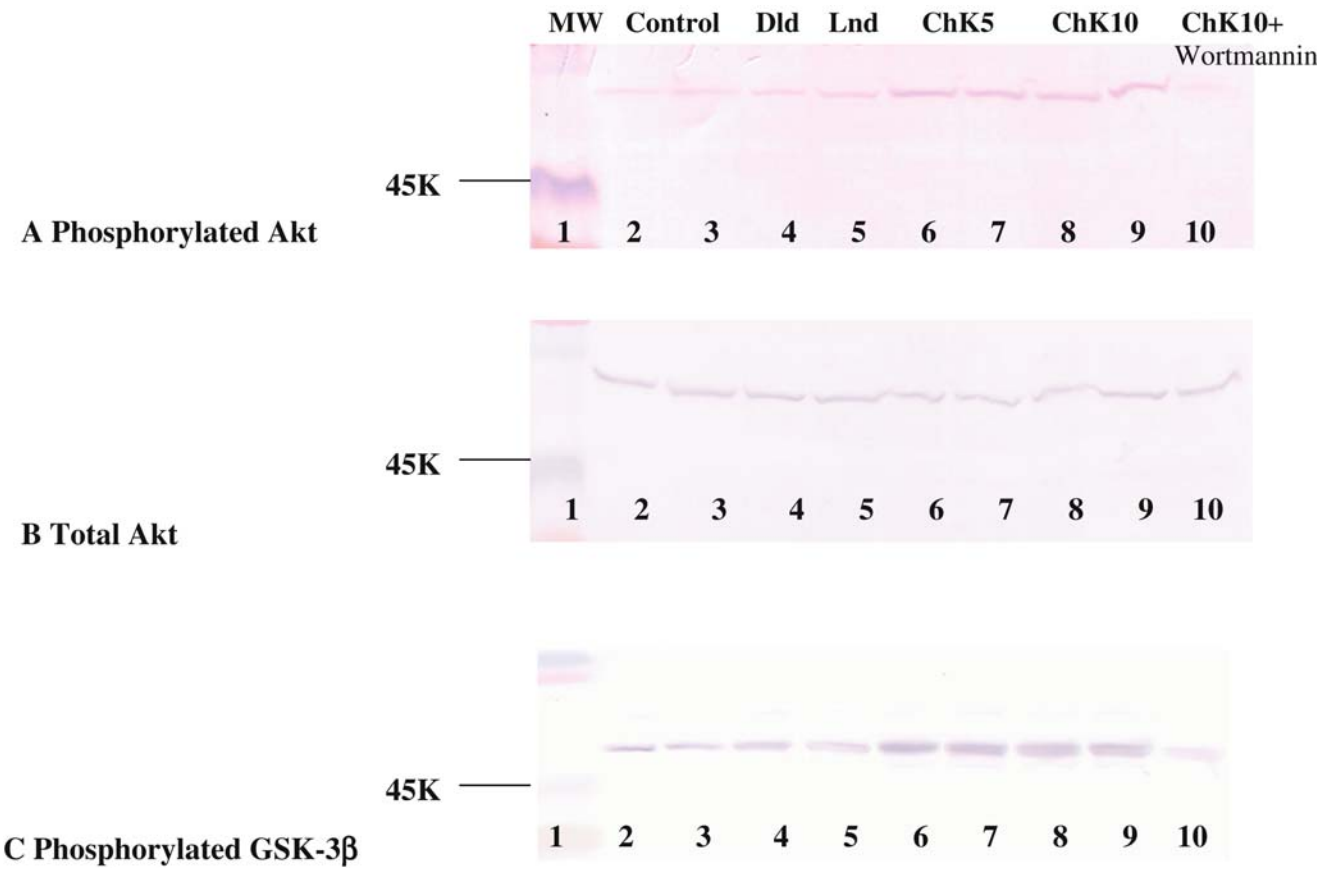

Fig. 11. Effect of ChK, Wortmannin, lindane, and dieldrin on Akt phosphorylation. RG-2 cells were grown to 90% confluence in 25 cm^2 flasks, treated with vehicle (DMSO), lindane, dieldrin, ChK or ChK and Wortmannin for 1 h, and extracted for Western blot analysis of: **A** Phosphorylated Akt, **B** Total Akt, and **C** Phosphorylated GSK-3β as described in "MATERIALS AND METHODS." Treatment groups were: molecular weight markers (lane 1), vehicle (DMSO) for 1 h (lanes 2 and 3, duplicates), dieldrin 10 μM (lane 4), lindane 50 μM (lane 5), ChK 5 μM (lanes 6 and 7, duplicates), ChK 10 μM (lanes 8 and 9, duplicates), ChK 10 μM and Wortmannin 0.5 μM (lane 10).

by all three compounds suggested the presence of an alternative mechanism(s) that accounts for the protective effect of ChK. We hypothesized that the preventative effect of ChK on organochlorine-induced changes might be due to activation of one or more other kinases, for instance PKC or Akt kinase.

Results of experiments monitoring the effect of ChK on PKC activation show that treatment of cells for 1 h with ChK, dieldrin or lindane had no effect on the level of phosphorylation of PKC at a key activation site (Fig. 9). This suggests that PKC does not mediate the inhibitory effect of lindane or dieldrin or the preventive effect of ChK.

To investigate whether the protective effect of ChK involves activation/inhibition of the Akt kinase pathway, Western blotting using phospho-Akt and total Akt primary antibodies was performed and the effect of ChK on Akt phosphorylation/inhibition compared with effects of dieldrin and lindane on Akt activation/inhibition. Our results show that a 1-h treatment with ChK increased phosphorylation of Akt kinase on a key activation site. However, dieldrin or lindane did not substantially alter the level of phosphorylation of Akt kinase (Fig. 10A). Total Akt kinase levels (Fig. 10B) were unaltered by any of the compounds. The inhibitory effect of the PI3 kinase/Akt pathway inhibitor, Wortmannin, on ChK-induced phosphorylation of Akt kinase (Fig. 11A) on a key activation site, as well as on a downstream effector of Akt, GSK-3β, (Fig. 11C) further support the idea that ChK acts on the Akt kinase pathway in RG-2 cells. Our key finding that Wortmannin blocked the preventative effect ChK on dieldrin-induced inhibition of gap junctional communication (Fig. 12) suggests that the Akt pathway is involved in ChK's protective effect in RG-2 cells.

Akt has previously been reported to phosphorylate c-Raf on the inhibitory site Ser259 (35). Our finding that ChK decreases c-Raf phosphorylation on the inhibitory Ser259 site, in spite of its activation of Akt, suggests that ChK has an additional target independent of that which causes activation of Akt. The decreased phosphorylation on the inhibitory Ser259 site by ChK would be expected to cause increased activity of c-Raf, and therefore increased activity of downstream effectors. Indeed, we reported increased phosphorylation at a key activation site of p44/p42 ERK, which is downstream of c-Raf, and this may be due at least in part to the effects of ChK on c-Raf phosphorylation. The mechanism of how dieldrin and lindane alter p44/p42 ERK remains to be determined, but in any case, does not appear to involve inhibitory effects on c-Raf phosphorylation on Ser259.

Activated c-Raf has been shown to play an anti-apoptotic role through interactions with Bcl-2 (36) and I-kB (37,38), among other effectors. However, while ChK appears to activate c-Raf, it has been reported to induce apoptosis in

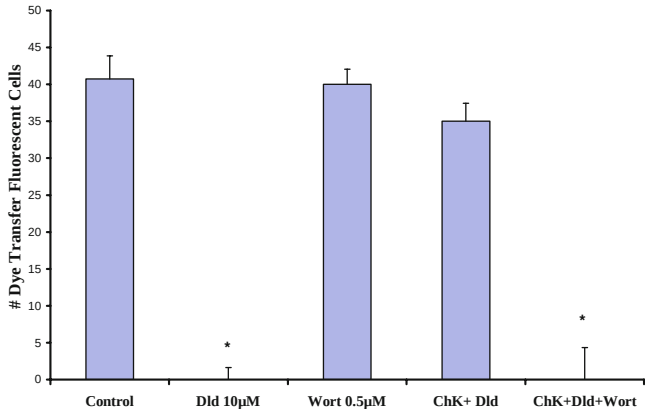

Fig. 12. Inhibitory effect of Wortmannin on ChK's preventative effect on gap junctional communication. RG-2 cells were grown to 90–100% confluence in 35 mm dishes and incubated with vehicle alone, Wortmannin alone, dieldrin alone, preincubated with 10 M ChK for 15 min followed by treatment with 10 M dieldrin for 30 min, or preincubated with 10 M ChK and 0.5 M Wortmannin for 15 min followed by treatment with 10 M dieldrin for 30 min. Fluorescent dye transfer assay was performed as described in "MATERIALS AND METHODS." Values from separate dieldrin dishes were subtracted as background fluorescent cells for each group. Data are presented as the mean±SD for each group (n=5). *, Statistically significant (P<0.05) compared to controls.

ras-transformed fibroblasts (4) and *ras*-transformed epithelial cells (unpublished observations). In addition, gap junction-mediated cell–cell communication and/or connexins have been correlated with increased apoptosis (39–41). In light of the observation that ChK stabilizes/restores gap junction-mediated cell–cell communication in the presence of tumor promoting compounds, it remains to be determined whether treatment of RG-2 astroglial cells with ChK for varying lengths of time is anti-apoptotic due to c-Raf activation or pro-apoptotic due to its effects on gap junction-mediated communication and, possibly, signaling pathways that are affected in *ras*-transformed cells.

We previously reported that ChK down-regulates the Akt pathway in *ras*-transformed epithelial cells (5). In those studies, 2–10 μM ChK induced decreased Akt phosphorylation at 4–72 h treatment times. No detectable effect was seen at less than 2 h. In RG-2 cells, we observed increased Akt phosphorylation at 1–2 h using the same site-specific antibody. This suggests that ChK has two different targets in the *ras*-transformed cells with down-regulated gap junction-mediated communication and in the RG-2 cells with normal functional gap junctions. This observation agrees with major lines of evidence supporting a link between aberrant gap junctional intercellular communication and cancer: (1) aberrant gap junctional intercellular communication in tumor cells, (2) down-regulation of gap junctional intercellular communication by cancer-causing agents or genes, and (3) up-regulation of gap junctional intercellular communication by inhibitors of carcinogenesis (13). Tumor suppressor activity has also been associated with connexin expression (42). Another possibility is that ChK has a common target in the transformed and non-transformed cells, but different downstream effectors are activated.

CONCLUSION

The natural product ChK showed protective effects, both preventative and reversal, against lindane and dieldrin disruption of gap junction mediated cell–cell communication in astroglial cells. The present study provides the first evidence that lindane inhibits gap junctional intercellular communication between astroglia at non-cytotoxic concentrations, and that ChK can prevent and reverse this inhibition of both lindane and dieldrin. We demonstrate that ChK's protective effect, both preventative and reversal, correlates with stabilization or reappearance of the connexin 43 P2 phosphoform. We provide here the first evidence that ChK, lindane and dieldrin induced the activation of ERK1/2 whereas no induction of PKC was observed. Our main finding of these studies is that only ChK, and not lindane or dieldrin, activates Akt kinase and its downstream effector GSK-3β. Results of our further experiments with the PI3 kinase/Akt pathway inhibitor, Wortmannin, support our main conclusion that activation of the Akt pathway by ChK is involved in ChK's protective effect. The data obtained from these studies contribute to the understanding and potential treatment of neurotoxic, carcinogenic, and other biological effects of organochlorine compounds.

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