The 2,2′,4,4′,5,5′-Hexachlorobiphenyl–Enhanced Degradation of Connexin 43 Involves Both Proteasomal and Lysosomal Activities

Pavlína Šimečková,* Jan Vondráček,**† Zdeněk Andrysík,*† Jiřina Zatloukalová,*† Pavel Krčmář,* Alois Kozubík,† and Miroslav Machala*,†

*Department of Chemistry and Toxicology, Veterinary Research Institute, 62100 Brno, Czech Republic; and †Department of Cytokinetics, Institute of Biophysics, 62165 Brno, Czech Republic

Received May 27, 2008; accepted September 18, 2008

One of the toxic effects of non-dioxin–like polychlorinated biphenyls (NDL-PCBs) is the acute inhibition of gap junctional intercellular communication (GJIC), an event possibly associated with tumor promotion. The model NDL-PCB—2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153)—induces a sustained GJIC inhibition in rat liver epithelial WB-F344 cells. As this effect might be related to deregulation of connexin 43 (Cx43) synthesis, trafficking, or degradation, we investigated the impact of PCB 153 on these events. Although PCB 153 had no effect on Cx43 mRNA levels, it induced a gradual loss of Cx43 protein and significantly decreased the amount of gap junction plaques in plasma membrane. PCB 153 contributed to extracellular signal–regulated kinases 1 and 2 (ERK1/2)–dependent accumulation of hyperphosphorylated Cx43-P3 form, thus indicating that ERK1/2 activation by PCB 153 might contribute to its effects on Cx43 internalization or degradation. Inhibition of either proteasomes or lysosomes with their specific inhibitors largely restored total Cx43 protein levels, thus suggesting that both proteasomes and lysosomes may participate in the PCB 153–enhanced Cx43 internalization and degradation. However, neither the proteasomal nor the lysosomal inhibitors restored normal GJIC or number/size of gap junction plaques. Finally, PCB 153 also interfered with restoration of gap junction plaques following the inhibition of Cx43 transport to plasma membrane. Taken together, multiple modes of action seem to contribute to downregulation of Cx43 in PCB 153–treated rat liver epithelial cells. The enhanced degradation of Cx43, together with persistent inhibition of GJIC, might contribute to tumor-promoting effects of NDL-PCBs.

Key Words: PCBs; connexin; tumor promotion; gap junctions; lysosomes; proteasome.

Polychlorinated biphenyls (PCBs) are environmental pollutants that have become ubiquitously distributed throughout environment and food web due to their widespread industrial use. PCBs can be classified based on their structure and the structure-related toxic modes of action. Non-ortho-substituted coplanar PCBs, sometimes termed as dioxin-like PCBs, have been shown to elicit a set of adverse effects associated with the activation of the aryl hydrocarbon receptor (AhR), resulting in liver damage, thymus atrophy, skin lesions, a wasting syndrome, and tumor promotion (Robertson and Hansen, 2001; Safe, 1994). The ortho-substituted PCBs, which tend to form noncoplanar structures of the biphenyl molecule that does not significantly activate AhR, exhibit a different spectrum of toxic modes of action, which have been also linked to neurotoxicity, immunotoxicity, endocrine disruption, or tumor promotion (Glauert et al., 2001; Hansen, 1998; Pessah et al., 2006; van der Plas et al., 2000). A widespread distribution and persistence of non-dioxin–like polychlorinated biphenyls (NDL-PCBs) has led to numerous studies on their potential role in carcinogenesis (Silberhorn et al., 1990). Various mixtures of PCBs have been demonstrated to cause liver cancer in rodents, and purified NDL-PCBs have been shown to act as tumor promoters (Glauert et al., 2001). However, the precise mechanisms underlying various modes of toxic action of the NDL-PCBs are still not fully described.

The inhibition of gap junctional intercellular communication (GJIC) has been suggested to provide a useful tool for detection of tumor-promoting agents (Rosenkranz et al., 2000; Yamasaki, 1996). The removal of an initiated cell from the growth suppression by neighboring cells inflicted by the intercellular transfer of signal messengers via gap junctions might play an important role in cancer development (Trosko and Upham, 2005). The available data seem to suggest that NDL-PCBs can inhibit GJIC in various types of cells (Hemming et al., 1991; Kang et al., 1996; Machala et al., 2003; Ruch and Klaunig, 1986; Swierenga et al., 1990). The inhibitory activity of NDL-PCBs seems to involve alterations of lipid signaling (Machala et al., 2003; Umannová et al., 2008); however, this toxic mode of action of NDL-PCBs is still not understood in detail. In contrast to other tumor promoters or environmental toxicants, which induce a rapid downregulation of GJIC followed by its gradual recovery, the model NDL-PCB—2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153)—has been found to induce a sustained GJIC inhibition in rat liver epithelial WB-F344 cells.
The life cycle of Cx43 is a complex, highly regulated, and a very dynamic process (Berthoud et al., 2004). Following translation, Cx43 proteins are translated into endoplasmic reticulum (ER) membrane, where they oligomerize to form hexameric connexons that are delivered through Golgi apparatus to plasma membrane (Segretain and Falk, 2004). They subsequently join with connexons in adjacent cells and form tightly packed groups of channels, known as the gap junction plaques (Segretain and Falk, 2004). Cx43 has a rapid turnover rate with half-life of 1–4 h in most tissues (Berthoud et al., 2004). The older channels are removed from the center, by invagination of both connexon into one of the adjacent cells, followed by lysosomal degradation (Berthoud et al., 2004; Laird, 2005). However, both lysosomal and proteasomal pathways can play an important role in the degradation of Cx43, with internalized gap junctions being degraded via lysosomes, whereas active proteasomal degradation may destabilize phosphorylated gap junctions at the plasma membrane or participate in ER-assisted degradation of Cx43 (Berthoud et al., 2004; Qin et al., 2003; Segretain and Falk, 2004). Both phosphorylation and ubiquitination of Cx43 are important steps in regulation of Cx43 endocytosis and degradation (Laird, 2005; Leithe and Rivedal, 2004a,b, 2007). Tumor promoters, such as 12-o-tetradecanoylphorbol 13-acetate (TPA), or growth factors, such as epidermal growth factor, have been shown to promote internalization and degradation of Cx43 as a part of their GJIC inhibitory effects (Leithe and Rivedal, 2004a,b; Rivedal and Leithe, 2005). However, nothing is currently known about the effects on NDL-PCBs on processes leading to Cx43 degradation.

The principal aim of the present study was thus to analyze the impact of PCB 153 (the most abundant NDL-PCB) on the levels and cellular distribution of Cx43. Using selective inhibitors of lysosomal and proteasomal pathways, protein trafficking, and the inhibitor of extracellular signal–regulated kinases 1 and 2 (ERK1/2) activation, we then investigated the role of the respective mechanisms in the PCB 153–induced downregulation of Cx43. Our results seem to suggest that PCB 153 induces a gradual loss of Cx43 in WB-F344 cells, proceeding via lysosomal pathway, with active participation of both ERK1/2-induced Cx43 phosphorylation and proteasome in regulation of Cx43 degradation. Moreover, PCB 153 seems also to interfere with restoration of gap junction plaques following inhibition of Cx43 trafficking to plasma membrane.

MATERIALS AND METHODS

Chemicals. PCB 153 was purchased from Ehrenstorfer (Augsburg, Germany). Stock solutions were prepared in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and stored in the dark. Polyvinylidene difluoride (PVDF) membrane Hybond-P and chemiluminescence ECL Plus detection reagents were purchased from GE Healthcare (Buckinghamshire, UK). Leupeptin was from AppliChem (Darmstadt, Germany). MG132, lactacystin, chloroquine, TPA, brefeldin A, and all other chemicals were provided by Sigma-Aldrich (Prague, Czech Republic).

Cells and treatment. Nontumorigenic, diploid rat liver WB-F344 epithelial cells (Tsao et al., 1984) were kindly provided by Dr. James E. Trosko (Michigan State University, East Lansing, MI). Cells were maintained in Dulbecco’s modified Eagle’s medium, supplemented with 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 24mM NaHCO3, and 5% fetal bovine serum. All tissue culture reagents were from Sigma-Aldrich. Cells were incubated in a humidified atmosphere of 5% CO2 at 37°C, and they were routinely maintained in 75 cm2 flasks and subcultured twice a week. For detection of effects of PCB 153 on GJIC inhibition, Cx43 degradation, and mitogen-activated protein kinase (MAPK) activation, cells were treated with 40μM PCB 153 for time intervals indicated in legends to figures. This concentration, leading to 80% inhibition of GJIC in WB-F344 cells, was based on our previous study (Machala et al., 2003). The specific treatments with inhibitors are indicated below.

Cells were treated with inhibitors of proteasomal (10μM MG132 or 10μM lactacystin) and lysosomal degradation (100μM chloroquine and 100μM leupeptin) for 30 min preincubation with 10μM U0126. For the experiments addressing restoration of gap junction plaques, we exposed confluent cells grown on coverslips to 100 ng/ml brefeldin A, an inhibitor of protein transport to plasma membrane, for 3 h. After washing the cells three times with warm PBS, serum-free medium with or without PCB 153 was added for another 3 h.

GJIC inhibition assay. Confluent cells, grown in 24-well plates, were exposed to 40μM PCB 153 or 20nM TPA for 1, 24, and 48 h. After the exposure, a modified protocol of scrape-loading/dye transfer technique (Bláha et al., 2002) was used to assess in vitro modulations of GJIC. The cells were washed twice by PBS solution, fluorescent dye was added (lucifer yellow, 0.05% w/v in PBS), and the cells were scraped using a surgical steel blade. After 2 min of the dye diffusion between the adjacent cells via gap junctions, the cells were washed by PBS and fixed with 4% (v/v) formaldehyde. The dye transfer from the scrape line was measured with an epifluorescent microscope. At least three independent experiments were carried out in duplicates; at least three scrapes per well were evaluated using Lucia image analysis software (Laboratory Imaging, Prague, Czech Republic). The GJIC inhibition was expressed as a percentage of GJIC in cells treated with vehicle (DMSO).

Western blotting. Cells were grown to reach confluence, medium was then changed, and cells were exposed to 40μM PCB 153 for indicated time. The 1-h treatment with 20nM TPA or 0.2% DMSO was used as a positive control and vehicle control, respectively. The cells were harvested in 90 μl of the lysis buffer (1% SDS, 10% glycerol, 100mM Tris pH 7.4, 1mM NaF, 1mM NaVO3, 1mM PMSF). The samples were then sonicated, and the protein concentration was determined using the bicinchoninic acid method. The samples were then diluted to equal protein levels and boiled for 4 min. Following SDS-polyacrylamide gel electrophoresis (PAGE) in 10% gels, proteins were transferred onto a PVDF membrane using a semidry blotter. The proteins were detected using the following primary antibodies: polyclonal rabbit anti-Cx43 antibody (Sigma-Aldrich; cat. no. C6219), anti-phospho-Cx43 antibody (Ser 279/282) (Santa Cruz Biotechnology, Santa Cruz, CA; cat. no. 120900), anti-phospho-p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technology, Beverly, MA; cat. no. 9101), or anti β-actin (Sigma-Aldrich; cat. no. A1978). The immunoreactive bands were visualized using chemiluminescence detection with ECL Plus reagent. The densitometric analysis of selected Western blots was performed using Scanner 3 equipped with WinCATS software (CAMAG, Muttenz, Switzerland).

Real-time reverse transcription-PCR. The level of Cx43 mRNA was determined by quantitative real-time reverse transcription (RT)-PCR. Cells
PCB 153–ENHANCED DEGRADATION OF CX43

RESULTS

PCB 153 Reduced the Level of Cx43 Protein and Amount of Gap Junction Plaques in WB-F344 Cells

As shown in Figure 1A, unlike TPA, PCB 153 induced a sustained downregulation of GJIC in WB-F344 cells. As the restoration of GJIC in TPA-treated cells has been related to renewal of membrane pool of phosphorylated Cx43 (Leithe and Rivedal, 2004b; Rivedal and Leithe, 2005), we investigated the impact of PCB 153 on Cx43 amount and phosphorylation pattern. When WB-F344 cell lysates were separated on SDS-PAGE, Cx43 protein migrated as three separate bands according to phosphorylation state. According to previous studies, the faster migrating band (P0) represents intracellular nonphosphorylated Cx43. Two slower migrating phosphorylated forms are termed P1 and P2, with the second one being reportedly located in gap junctions (Musil and Goodenough, 1991). We found that PCB 153 reduced levels of both P1- and P2-form of Cx43 in WB-F344 (Fig. 1B). In contrast to TPA, PCB 153 did not induce the rapid hyperphosphorylation of Cx43, observed as the P3-Cx43 band.

Since these observations seemed to imply that PCB 153 induced a preferential loss of gap junction–localized Cx43, we next used immunofluorescence staining of Cx43 in fixed WB-F344 cells, in order to examine whether the alterations in Cx43 phosphorylation correspond with changes in Cx43 localization. A significant part of Cx43 protein in untreated cells was found in plasma membrane forming gap junction plaques, with some perinuclear staining, corresponding to ER and Golgi apparatus Cx43 pool (Fig. 2). The exposure to PCB 153 caused a progressive reduction of both size and number of gap junction plaques (Fig. 2). The maximum effect was reached already after 6-h treatment, when we also observed an increase of the intracellular Cx43 amount. This corresponded with the effects of PCB 153 on phosphorylated forms of Cx43 detected by Western blotting. Nevertheless, there was still a certain amount of gap junction plaques present in plasma membrane even after 24 h, which corresponded with the approximately 20% level of GJIC relative to control (Fig. 1A).

In order to find out whether the decrease of Cx43 protein might be also due to reduced transcription, we used the
real-time RT-PCR to determine the levels of Cx43 mRNA. As shown in Figure 3, treatment with PCB 153 did not reduce the Cx43 mRNA amount in WB-F344 cells as compared to cells treated with DMSO (vehicle control). This confirmed that the observed decrease of Cx43 occurs at the protein level.

Effects of Proteasomal and MEK1/2 Inhibitors on Degradation of Cx43

Cx43 phosphorylation and ubiquitination have been linked to its degradation (Laird, 2005; Leithe and Rivedal, 2004a,b, 2007) following the treatment of cells with either growth factors or TPA. Our previous studies have suggested that PCB 153 is a potent inducer of activation of ERK1/2 (Machala et al., 2003; Umannová et al., 2008). However, no hyperphosphorylation of Cx43 was observed in cells treated with PCB 153 (Fig. 1B). Therefore, we next investigated ERK1/2-specific phosphorylation using antibody specifically recognizing Cx43 phosphorylated at Ser 279/282. However, as shown in Figure 4A, although PCB 153 efficiently activated ERK1/2 at both 1 and 6 h time intervals, in contrast to TPA, it induced only a faint increase of Ser 279/282 phosphorylation (Fig. 4A).

In order to examine more closely the role of ERK1/2 and proteasome in the degradation of Cx43, we used specific chemical inhibitors of MEK1/2 (upstream ERK1/2–activating kinases) and proteasome, U0126 and MG132, respectively (Fig. 4B). As summarized in Figure 4C, the results of densitometric analysis of Western blots showed that total levels of Cx43 were similar in control cells and cells pretreated with either MG132 or MG132 + U0126, prior to PCB 153 treatment, suggesting that both types of treatment preserved total Cx43 levels. U0126 alone was not sufficient to prevent Cx43 degradation, and it had no effect on Cx43 levels itself. Nevertheless, there were significant differences among effects of inhibitors regarding the levels of individual Cx43 forms. As shown in Figure 4B, MG132 alone or in combination with PCB 153 induced accumulation of hyperphosphorylated P3 band, whereas hypophosphorylated P0-form almost disappeared. When U0126 was applied simultaneously with MG132, the accumulation of hyperphosphorylated band was prevented, suggesting that it depends on ERK1/2 activity. As the results of Western blotting also suggested that PCB 153 further potentiated accumulation of Cx43-P3 form in cells pretreated with MG132, we performed densitometric analysis of P3 bands. As shown in Figure 4D, significantly more Cx43 was present as hyperphosphorylated Cx43, which is only observable after inhibition of proteasome and which might be enhanced through the PCB 153–induced ERK1/2 activation. Therefore, PCB 153 might contribute to increased internalization of Cx43, which would be also dependent on proteasomal activity. Similar data were obtained also with another proteasome inhibitor, lactacystin (data not shown).

Lysosomal Pathway Is Involved in PCB 153-Induced Degradation of Cx43

Nevertheless, it has been suggested that proteasome plays only a partial role in Cx43 degradation, regulating, e.g., Cx43 internalization (Berthoud et al., 2004; Leithe and Rivedal, 2004a,b). The internalized protein would then be degraded via lysosomal pathway. Therefore, in the next step, we studied the role of lysosomal degradation in the PCB 153–induced decrease of Cx43 protein. Inhibition of lysosomal pathway...
by leupeptin or chloroquine completely suppressed Cx43 degradation, which is also supported by the results of densitometric analysis of Western blots (Fig. 5). These results seem to indicate that both proteasomes and lysosomes are involved in enhanced degradation of Cx43 in cells treated with PCB 153.

**Inhibitors of Proteasomal and Lysosomal Activities Differentially Modulated Gap Junction Plaques and GJIC in WB-F344 Cells**

As the above data suggested that proteasomal and lysosomal inhibitors may differentially affect Cx43 forms in WB-F344 cells, we next examined their impact on Cx43 levels and cellular distribution in both control- and PCB 153–treated cells, using immunofluorescent staining of Cx43 in fixed WB-F344 cells. MG132 induced accumulation of Cx43 within cell membrane in both control- and PCB 153–treated cells (Fig. 6). These results, similar to pretreatment with another inhibitor of proteasomal degradation lactacystin (data not shown), suggested that proteasomal inhibitors might counteract the PCB 153–enhanced internalization of Cx43. The accumulation of hyperphosphorylated Cx43-P3 form in WB-F344 cells treated with both MG132 and PCB 153 (see Fig. 4B) seems also to provide further support for this hypothesis, suggesting that the membrane-accumulated Cx43 indeed corresponded to the Cx43-P3 form. Moreover, in cells cotreated with both the proteasomal inhibitors MG132 and U0126, the MEK1/2 inhibitor which prevented the increased formation of P3-form (Fig. 4B), we observed a control-like pattern of gap junction plaques (Fig. 6), thus suggesting that ERK1/2-dependent phosphorylation may contribute to the PCB 153–enhanced internalization and degradation of Cx43. However, U0126 alone was not sufficient to prevent the PCB 153–induced degradation of gap junction plaques, thus indicating that additional mechanisms might be involved in deleterious effects of PCB 153 on Cx43 protein and gap junction plaques in membranes of rat liver epithelial cells.
In contrast to proteasomal inhibitors, pretreatment with lysosomal inhibitors leupeptin (Fig. 6) or chloroquine (data not shown) in control cells led to accumulation of intracellular Cx43. Moreover, leupeptin prevented the PCB 153–assisted Cx43 degradation; however, despite a strong intracellular staining of Cx43, the pretreatment with leupeptin failed to restore the gap junction plaques in cells treated with PCB 153 (Fig. 6).

As the pretreatment with the above inhibitors prevented the PCB 153–induced degradation of Cx43 and in some cases also contributed to restoration of gap junction plaques, we next examined their impact on GJIC in both control- and PCB 153–treated cells. However, none of these inhibitors restored GJIC in PCB 153–treated cells (Table 1). Moreover, inhibitors of proteasomal degradation alone significantly reduced the GJIC.
PCB 153 May Interfere with Restoration of Gap Junction Plaques in WB-F344 Cells

Finally, as a recent study suggested that Cx43 could be degraded not only from its membrane pool but also directly after transportation from early secretory compartments to lysosomes (Qin et al., 2003), we next investigated whether PCB 153 may interfere with restoration of gap junction plaques in cells pretreated with brefeldin A, an inhibitor of transport of membrane proteins to plasma membrane. When the protein transport to cell surface was inhibited, most of the gap junction plaques disappeared from the membrane (Fig. 7B). The plaques were restored when cells were allowed to recover for 3 h in medium without brefeldin A (Fig. 7C). Using this approach, we found that PCB 153 disrupted the restoration of gap junction plaques and Cx43 remained localized intracellularly (Fig. 7D). Thus, PCB 153 might interfere also with processes involved in intracellular transport of Cx43 or contribute to its direct lysosomal degradation.

DISCUSSION

Gap junctions play important roles in growth regulation and neoplastic transformation (Mesnil et al., 2005). Many toxic agents have been reported to inhibit GJIC both in vitro and in vivo, thus suggesting that inhibition of GJIC might contribute to their carcinogenicity (Rosenkranz et al., 2000; Yamasaki, 1996). However, the mechanisms of actions of many of these agents on GJIC and connexin functions are still only partly understood. While some of these toxic compounds, such as polyaromatic compounds, induce only a transient inhibition of GJIC (Bláha et al., 2002; Upham et al., 2008), others, such as pesticides or PCBs, may induce a sustained downregulation of intercellular communication (Bager et al., 1997; Guan and Ruch, 1996; Machala et al., 2003). Our previous studies in WB-F344 rat liver epithelial cells have shown that both NDL-PCBs and their hydroxylated metabolites inhibit GJIC and that this is a phenomenon persisting for several days (Machala et al., 2003, 2004), suggesting that it may involve changes in Cx43 life cycle. Therefore, in the present study, we investigated effects of PCB 153, the most abundant environmental NDL-PCB, on phosphorylation and degradation of Cx43, using established inhibitors of ERK1/2 activation, proteasomal and lysosomal degradation, or Cx43 transportation to plasma membrane.

The mechanisms underlying Cx43 degradation have received a considerable attention because its half-life is relatively short (1–4 h) and modulation of its turnover rate may significantly modify GJIC in response to model inhibitory

**TABLE 1**

Effects of Selected Proteasomal and Lysosomal Inhibitors on GJIC in WB-F344 Cells (Expressed as Percentage of Control)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inhibitors</th>
<th>GJIC (expressed as % of control ± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>—</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>PCB 153</td>
<td>—</td>
<td>26 ± 6**</td>
</tr>
<tr>
<td>DMSO</td>
<td>MG132</td>
<td>40 ± 11**</td>
</tr>
<tr>
<td>PCB 153</td>
<td>MG132</td>
<td>6 ± 3**</td>
</tr>
<tr>
<td>DMSO</td>
<td>U0126</td>
<td>85 ± 13</td>
</tr>
<tr>
<td>PCB 153</td>
<td>U0126</td>
<td>30 ± 10**</td>
</tr>
<tr>
<td>DMSO</td>
<td>MG132 + U0126</td>
<td>67 ± 11**</td>
</tr>
<tr>
<td>PCB 153</td>
<td>MG132 + U0126</td>
<td>5 ± 3**</td>
</tr>
<tr>
<td>DMSO</td>
<td>Leupeptin</td>
<td>92 ± 10</td>
</tr>
<tr>
<td>PCB 153</td>
<td>Leupeptin</td>
<td>22 ± 14**</td>
</tr>
</tbody>
</table>

*All data represent means of at least three independent experiments.
**A significant decrease of GJIC in comparison with control cells (DMSO) (p < 0.01), as determined by two-way ANOVA followed by Dunnett’s post hoc test versus a control group. The effects of MG132 (10μM), U0126 (10μM), leupeptin (100μM), and PCB 153 (40μM) on GJIC in WB-F344 cells were determined by the dye transfer/scrape-loading assay. Cells were treated with PCB 153 (40μM) or DMSO (0.2%; control) for 6 h; the respective inhibitors were added to cell culture 30 min prior to treatment. See ‘‘Materials and Methods’’ section for details of treatment and other abbreviations.
stimuli, such as growth factors or phorbol esters, as reviewed in Berthoud et al. (2004), Laird (2005), Leithe and Rivedal (2007), and Segretain and Falk (2004). The regulation of connexin degradation is also an important mechanism regulating gap junction assembly and function (Musil et al., 2000). In the present study, we found that PCB 153 did not modify Cx43 transcription but instead induced a preferential degradation of phosphorylated P1- and P2-forms of Cx43, the latter one representing the mature form of Cx43-forming gap junctions (Musil and Goodenough, 1991). The results of Western blotting analysis matched those of immunofluorescence detection of gap junction plaques as PCB 153 apparently reduced both size and number of gap junction plaques. These effects were similar to the impact of other toxic compounds on gap junctions in WB-F344 cells, thus suggesting that both PCBs and, e.g., chlorinated pesticides might share at least some mechanisms leading to GJIC downregulation (Guan and Ruch, 1996; Hakulinen et al., 2006).

Both proteasomal and lysosomal pathways have been suggested to take part in degradation of connexins (Berthoud et al., 2004; Laing et al., 1997; Leithe and Rivedal, 2007). In the present study, the proteasomal inhibitors both restored the total Cx43 levels in cells treated with PCB 153 and induced accumulation of hyperphosphorylated Cx43-P3 form in control- and PCB 153–treated cells. Previously, a similar accumulation of phosphorylated Cx43 at the expense of hypophosphorylated P0-form, which is thought to represent the intracellular pool of Cx43 (Berthoud et al., 2004; Segretain and Falk, 2004), has been observed, e.g., in human breast carcinoma cells overexpressing Cx43 (Qin et al., 2003). As shown in Figure 4, PCB 153 enhanced the ERK1/2–dependent formation of the hyperphosphorylated form of Cx43. This seemed to indicate that PCB 153 might enhance Cx43 internalization and degradation via ERK1/2 activation. However, unlike TPA, PCB 153 did not induce a major phosphorylation of Cx43 at Ser 279/282 residues or accumulation of Cx43-P3 form in PCB 153–treated cells. The principal reason for the absence of hyperphosphorylated Cx43-P3 forms in cells treated with PCB 153 might be its rapid internalization and degradation. Therefore, this form of Cx43 might only be observed in cells treated with proteasomal inhibitors (e.g., MG132) which may prevent Cx43 internalization (Laing et al., 1997; Leithe and Rivedal, 2004b; Qin et al., 2003). Indeed, the results shown in both Figures 4 and 6 seem to support this hypothesis—application of MG132 significantly increased both the P3-form levels and the membrane Cx43 staining, which was prevented by U0126 inhibitor. Therefore, despite having no role in acute inhibition of GJIC by NDL-PCBs (Machala et al., 2003), ERK1/2 activation might still contribute to NDL-PCB–induced Cx43 degradation. Our data also suggested that although proteasomal inhibitor restored total Cx43 levels, a preferential accumulation of Cx43-P3 form did not lead to restoration of GJIC, which is in line with the evidence that phosphorylation of Cx43 by ERK1/2 blocks gap junction channels (reviewed in Lampe and Lau, 2004). Both phosphorylation and ubiquitination of Cx43 have been suggested to play a role of a signal in internalization and degradation of Cx43 in rat liver epithelial cells (Leithe and Rivedal, 2004a,b; Leykauf et al., 2006). Active proteasomal degradation may also destabilize phosphorylated gap junctions at the plasma membrane (Segretain and Falk, 2004). Nevertheless, the precise role of proteasome in these events still remains unclear as it has been also suggested that the proteasomal inhibitors may alter also ubiquitination pattern of Cx43 (Leithe and Rivedal, 2004a), thus preventing the ubiquitination signaling for Cx43 internalization and subsequent degradation (Leykauf et al., 2006).

Following their internalization, gap junctions (connexosomes) may be fused with and degraded in lysosomes. It has been suggested that Nedd4, an ubiquitin–protein ligase, controls the ubiquitination of Cx43 protein, prior to its endocytosis (Leykauf et al., 2006). Like other membrane proteins, Cx43 contains sorting signal, which might mediate both Cx43 internalization and its targeting to lysosomes (Bonifacino and Traub, 2003; Thomas et al., 2003). Proteasomal and ERK1/2 inhibitors may thus prevent signaling leading to lysosomal degradation of Cx43 in WB-F344 cells. When using lysosomal inhibitors, we observed that both leupeptin and chloroquine prevented degradation of Cx43 in response to PCB 153 treatment, similar to, e.g., chlorinated pesticides (Guan and Ruch, 1996). However, this Cx43 was predominantly intracellular (Fig. 6) and the application of lysosomal inhibitors thus failed to restore GJIC function as shown in Table 1. This, together with the results summarized above, seems to suggest that both proteasomes and lysosomes are involved in the effects of PCB 153 on Cx43 degradation in WB-F344 cells, which is, in the former case, probably related to internalization and sorting signaling.

Finally, it has been recently suggested that Cx43 may bypass transport to membrane and become directly degraded in lysosomes (Qin et al., 2003). In order to analyze possible impact of PCB 153 on Cx43 trafficking to the surface of the cells, we employed WB-F344 cells, where Cx43 transport was blocked by the fungal antibiotic brefeldin A (Laird et al., 1995; VanSlyke and Musil, 2000). We found that PCB 153 inhibited restoration of gap junction plaques following the inhibition of transport of synthesized Cx43 to plasma membrane. This seems to indicate that PCB 153 might also interfere with the proper transport of Cx43 to cell membrane, which might be related to direct lysosomal degradation of ER/Golgi compartment–trapped Cx43. These results might perhaps help to explain why the MEK1/2 inhibitor U0126 failed to prevent the PCB 153–induced Cx43 degradation.

Taken together, the present data seem to suggest that PCB 153 induces degradation of phosphorylated forms of Cx43 in WB-F344 cells. Its degradation probably proceeds via lysosomes; however, both ERK1/2–regulated pathways and the proteasome seem to contribute to regulation of Cx43.
internalization and degradation. PCB 153 seems also to interfere with restoration of gap junction plaques following inhibition of Cx43 trafficking to plasma membrane. Degradation of Cx43 might contribute to prolonged inhibitory effects of NDL-PCBs on GJIC in epithelial cells. Moreover, it may also interfere with Cx43-mediated regulation of genes linked to regulation of cell proliferation (Kardami et al., 2007). As a recent study has suggested that some of nodular hepatic lesions may originate from the liver epithelial cells following a chronic treatment with organochlorine contaminants (Hailey et al., 2005), the impact of NDL-PCBs on GJIC in this cell population deserves further attention. Nevertheless, as principal liver cells—hepatocytes—express predominantly other connexin species, Cx26 and Cx32, their deregulation via similar mechanisms would be also of interest. However, only little is currently known about the mechanisms of degradation regarding these connexin species. Cx26 is not phosphorylated due to its lack of cytoplasmic tail, which might have a short half-life similar to Cx43, suggesting that the mechanisms regulating its degradation might differ from Cx43 (Laird, 2005; Lampe and Lau, 2004). Cx32 has been shown to be highly related to liver cancer development as Cx32 knockout mice show high levels of both spontaneous and chemically induced liver tumors (Temme et al., 1997). However, like Cx26, also Cx32 could be degraded through this connexin species–specific mechanism (Laird, 2005). Therefore, possible similar effects of NDL-PCBs on other liver connexins remain unclear. Future studies should also aim to establish a link between the PCB-induced Cx43 degradation observed in vitro and its in vivo relevance.

FUNDING

ATHON (EU Framework Programme 6, Priority 5: “Food Quality and Safety”; EU Contract No. FOOD-CT-2005-022923); Czech Science Foundation (grant No. 524/06/0517); The institutional support was provided by the Academy of Sciences of the Czech Republic (Research Plans AV0Z50040507 and AV0Z50040702) and the Czech Ministry of Agriculture (MZE0002716201).

ACKNOWLEDGMENTS

The authors thank Josef Slavik for his expert assistance with densitometry.

REFERENCES


Robertson, L. W., and Hansen, L. (2001). Recent Advances in the Environmental Toxicology and Health Effects of PCBs. The University Press of Kentucky, Lexington, KY.


