

Inhibition of Gap Junctional Intercellular Communication by Perfluorinated Compounds in Rat Liver and Dolphin Kidney Epithelial Cell Lines *in Vitro* and Sprague-Dawley Rats *in Vivo*

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Gap junctional intercellular communication (GJIC) is the major pathway of intercellular signal transduction, and is thus important for normal cell growth and function. Recent studies have revealed a global distribution of some perfluorinated organic compounds, especially perfluorooctane sulfonic acid (PFOS) in the environment. Because other perfluoroalkanes had been shown to inhibit GJIC, the effects of PFOS and related sulfonated fluorochemicals on GJIC were studied using a rat liver epithelial cell line (WB-F344) and a dolphin kidney epithelial cell line (CDK). *In vivo* effects on GJIC were studied in Sprague-Dawley rats orally exposed to PFOS for 3 days or 3 weeks. Effects on GJIC were measured using the scrape loading dye technique. PFOS, perfluorooctane sulfonamide (PFOSA), and perfluorohexane sulfonic acid (PFHA) were found to inhibit GJIC in a dose-dependent fashion, and this inhibition occurred rapidly and was reversible. Perfluorobutane sulfonic acid (PFBS) showed no significant effects on GJIC within the concentration range tested. A structure activity relationship was established among all 4 tested compounds, indicating that the inhibitory effect was determined by the length of fluorinated tail and not by the nature of the functional group. The results of the studies of the 2 cell lines and the *in vivo* exposure were comparable, suggesting that the inhibitory effects of the selected perfluorinated compounds on GJIC were neither species- nor tissue-specific and can occur both *in vitro* and *in vivo*.

Key Words: GJIC; PFOS; perfluorinated chemicals; rodents; QSAR.

Perfluorinated fatty acids (PFFAs) and their sulfonic acid analogues characteristically have an aliphatic chain in which all the hydrogen atoms are replaced by fluorines. PFFAs are commonly used in industrial materials such as wetting agents, lubricants, corrosion-inhibitors, stain and moisture resistant treatments for leather, paper and clothing, as well as in foam fire extinguishers (Sohlenius *et al.*, 1994). Since PFFAs are chemically stabilized by the strong covalent bond between carbon and fluorine, they were historically considered to be

metabolically inert and nontoxic (Sargent and Seffl, 1970). However, recent accumulating evidence indicates that they are biologically active and can induce effects such as peroxisome proliferation, increased lipid metabolizing enzyme activities and induce xenobiotic metabolizing enzyme activities (Obourn *et al.*, 1997; Sohlenius *et al.*, 1994). Treatment with some PFFAs has also been associated with the induction of hepatic necrosis, hepatocellular carcinomas, Leydig cell adenomas, and pancreatic tumors (Obourn *et al.*, 1997).

Perfluorooctane sulfonic acid (PFOS) appears to be the ultimate degradation product of a number of perfluorinated compounds used in commercial applications (Giesy and Kannan, 2001). The concentrations of PFOS found in wildlife are greater than other perfluorinated compounds (Giesy and Kannan, 2001; Kannan *et al.*, 2001a,b). However, to date, most toxicological studies have been conducted using perfluorinated fatty acids, such as perfluorooctanoic acid (PFOA) and perfluorodecanoic acid (PFDA), rather than the more environmentally prevalent sulfonated compounds. Whether PFOS can cause similar effects as PFOA and other PFFAs is still under investigation, and its possible mechanism(s) of action remains to be elucidated.

Gap junctions are plaque-like features on the cell plasma membrane formed by connexin proteins (Yamasaki *et al.*, 1995). Each connexin protein is composed of 6 subunits, forming a pipeline-like structure with a center pore of about 17 Å in diameter (Yeager and Nicholson, 1996). When these protein complexes from adjacent cells join, they form a continuous channel structure, and allow electronic and metabolic signaling molecules to pass through the channel to synchronize tissue function, a process called gap junctional intercellular communication (GJIC; (Bruzzone *et al.*, 1996).

Of the various forms of intercellular connection, GJIC is the only one that allows direct exchange of chemicals from the interior of one cell to that of adjacent cells without passage through the extracellular space (Pitts and Finbow, 1986). The cytosolic molecules that can be exchanged through GJIC include ions, second messengers, and low molecular weight metabolites (Yamasaki, 1996). GJIC is considered to play an

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essential role in maintaining the homeostasis of tissues, therefore disruption of GJIC results in abnormal cell growth and function (Trosko *et al.*, 1998). Because tumor formation requires loss of homeostasis and abolition of contact inhibition, it has been hypothesized that the inhibition of GJIC is associated with tumor promotion (Trosko and Ruch, 1998). A recently developed quantitative structure activity relationship (QSAR) model has demonstrated that inhibition of GJIC is strongly linked to tumor development in rodents, uncontrolled cell proliferation and differentiation, embryonic lethality or teratogenesis (Ketcham and Klaunig, 1996). Chronic disruption of GJIC could also lead to neurological, cardiovascular, reproductive, and endocrinological dysfunction (Trosko *et al.*, 1998).

Previous studies have shown that PFFAs with carbon chain lengths of 7–10 can rapidly and reversibly inhibit GJIC in a dose-dependent fashion *in vitro* (Upham *et al.*, 1998). To compare the effects of the sulfonic acid class of PFFAs with various chain lengths on GJIC to those of other PFFAs, and to evaluate possible species and organ differences, the inhibition of GJIC was studied in the WB F-344 rat liver cell line and the CDK dolphin kidney cell line treated with PFOS and related perfluorinated compounds. The dolphin cell line was used here in an effort to develop a marine mammalian model for testing the effect of PFOS since relatively great concentrations of PFOS have been measured in marine mammal tissue samples, particularly liver samples (Kannan *et al.*, 2001b). In addition, an *in vivo* study with subchronic exposure of Sprague-Dawley rat to PFOS was conducted to determine whether effects on GJIC observed *in vitro* might be relevant *in vivo*.

MATERIALS AND METHODS

Chemicals. Perfluorooctane sulfonic acid (PFOS; 68% straight chain, 17% branched chain), perfluorohexane sulfonic acid (PFHS), and perfluorobutane sulfonic acid (PFBS) were obtained from 3M company (St. Paul, MN). PFOS (potassium salt) used in the *in vivo* experiments was purchased from Fluka Chemicals (Switzerland); chemical analysis revealed that it was essentially the same as the product obtained from 3M. Perfluorooctanoic sulfonamide (PFOSA) was purchased from Sigma (St. Louis, MO).

Cell culture. Rat liver epithelial cells (WB-F344) were obtained from J. W. Grisham and M. S. Tsao, University of North Carolina. This cell line has been well characterized for its expression of gap junction proteins (Ruch and Trosko, 2001) and oval cell characteristics (Tsao *et al.*, 1984). Carvan dolphin kidney (CDK) cell line was obtained from D. Busbee, Texas A & M University. The CDK line is an epithelial cell line isolated from a prematurely born female-bottle-nose dolphin (Carvan *et al.*, 1994). WB-F344 and CDK cells were cultured in 75 cm² flasks (Corning 430720) in a humidified incubator at 37°C, with a 5/95% CO₂/air atmosphere. WB-F344 cells were cultured in Dulbecco's Modified Eagle Medium (Formula 78-5470EF, Gibco, Rockville, MD), supplemented with 5% Fetal Bovine Serum (FBS; Gibco, Rockville, MD). CDK cells were cultured in Dulbecco's Modified Eagle Medium and Ham's F12 medium (Sigma, St. Louis, MO), supplemented with 10% FBS (Gibco, Rockville, MD), and other nutrients (Carvan *et al.*, 1994).

Animals and treatment. Sixty-day-old Sprague-Dawley rats (males 294 ± 4 g; females 209 ± 2 g) were obtained from Charles River Laboratories (Raleigh, NC), and housed at 20–24°C and humidity-controlled (40–60%)

facilities at the U.S. EPA Reproductive Toxicology Division. Rats were randomly assigned to either block 1 with 6 males and 6 females or block 2 with 4 males and 4 females. Block 1 was exposed to PFOS for 21 days, block 2 was exposed for 3 days. Within each block, half of the males and half of the females were randomly assigned to treatment or control groups. Rats received PFOS (5 mg/kg) or vehicle control (0.5% Tween-20) daily by gavage at a rate of 1 ml/kg body weight. Food and water were provided *ad libitum*.

GJIC *in vitro* assay. After reaching 90–100% confluence, cells were harvested with 1× trypsin-EDTA (Gibco, Rockville, MD) and the resulting cell suspension was diluted to approximately 1 × 10⁶ cells/ml for WB-F344 cells, and 1 × 10⁵ cells/ml for CDK cells. Two-ml aliquots of the diluted cell suspension were transferred to 35 mm diameter tissue culture plates, and cells were incubated for approximately 72 h until confluence was reached. Test compounds, dissolved in acetonitrile, were added to culture medium to assess effects on GJIC. Doses used and exposure durations are discussed in the results section for each experiment.

GJIC *in vitro* was measured using the scrape loading dye transfer technique (Weis *et al.*, 1998). Briefly, following the exposure to compounds of interest, the cells were washed 3 times with phosphate buffered saline (PBS). The fluorescent dye, lucifer yellow (Sigma, St. Louis, MO) dissolved in PBS (1 mg/ml) was applied to cover the cells. Three parallel scrapes were made in the cell monolayer using a surgical blade to allow passage of the membrane impermeable dye into ruptured cells. After a 3-min incubation, the cells were washed with PBS to remove excess dye and were fixed with 4% formalin. Dye migration was observed and photographed at 200X using a Nikon epifluorescence microscope illuminated with an Osram HBO 200W lamp and equipped with a COHU video camera. The program, Gel-Expert (Nucleotech, San Mateo, CA), was used to quantify GJIC by determining the intensity and distance of dye migration. The distance of dye migration perpendicular to the scrape (i.e., between adjacent cells linked only by gap junctions) represents the ability of cells to communicate via GJIC. Dye migration data are reported as a percentage of the corresponding mean control value. All treatments were tested in triplicate. NOEL and EC₅₀ values were determined by one-way ANOVA and linear regression analyses. Differences among compounds and between cell types were determined using two-way ANOVA, followed by Tukey's multiple range test.

GJIC *in vivo* assay. GJIC activity after *in vivo* exposure was measured using the incision loading/dye transfer technique (Krutovskikh *et al.*, 1991; Sai *et al.*, 2000). At the end of exposure period, rats were sacrificed by decapitation, the left lobe of the liver was excised immediately and rinsed with PBS. Lucifer Yellow (1 mg/ml in PBS) was applied onto the tissue surface. Four incisions (~1 cm long, 1 mm deep) were made on each of the tissue samples with a surgical blade. Additional dye solution was loaded into the incisions with a pipette tip, and the specimen was incubated for 5 min at room temperature. After incubation, the specimen was washed 3 times with PBS, and fixed in 10% buffered formalin overnight. Specimens were trimmed, mounted in tissue processing cassettes, and paraffin embedded. Sections, 5 μm, were prepared by cutting the paraffin block perpendicular to the incision lines on the liver specimen. Dye migration was quantitated using the same optical and data processing systems used for the *in vitro* assay. Three incisions were analyzed for each specimen; results were analyzed using nested ANOVA. Samples of each liver were also collected, and stored at –80°C for chemical analysis.

Chemical analysis. PFOS in the rat liver tissue samples was extracted and analyzed based on slight modifications of previously described methods (Hansen *et al.*, 2001). Extractions were carried out on homogenate volumes equivalent to 10–50 mg of the original liver tissue samples. Homogenates, prepared in nanopure water, were mixed with an equal volume of 0.5 M tetrabutylammonium (TBA) hydrogen sulfate, pH 10 and 0.25 M sodium carbonate buffer. After mixing, the sample was extracted twice with methyl-*tert*-butyl ether (MTBE). The MTBE was evaporated to dryness and the extract was resuspended in 1 ml methanol for transfer to injection vials. After transfer, methanol was removed by evaporation and the extract was resuspended in 200 μl of 50% methanol in 2 mM ammonium acetate. PFOS was analyzed using a Hewlett Packard 1100 HPLC system (Hewlett Packard, Palo Alto, CA)

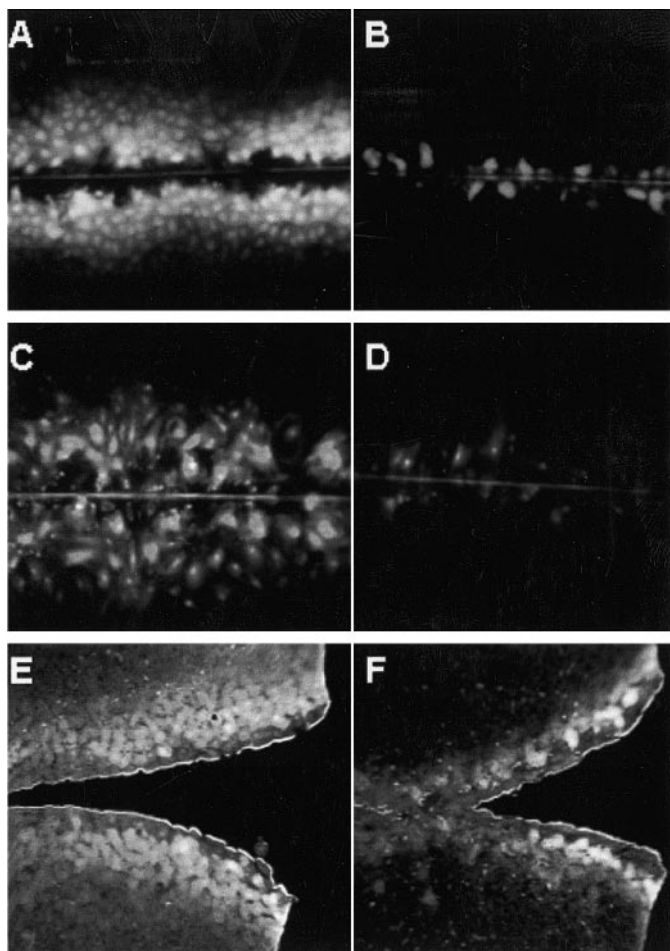


FIG. 1. GJIC inhibition in WB-F344 rat liver cells and CDK dolphin kidney cells, and Sprague-Dawley rat *in vivo*, photographed by fluorescence microscopy. Cells were exposed to either solvent control (DMSO) or 160 μM PFOS for 30 min prior to the measurement of GJIC. (A) WB cells treated with solvent (DMSO); (B) WB cells treated with 160 μM PFOS; (C) CDK cells treated with solvent (DMSO); (D) CDK cells treated with 160 μM PFOS; (E) Sprague-Dawley rat treated with vehicle control; (F) Sprague-Dawley rat treated with PFOS (5 mg/kg/day) for 21 days.

interfaced to a Micromass Platform II mass spectrometer (Micromass, Beverly, MD). Chromatography was conducted on a 150 \times 4 mm Betasil C₁₈ column (Keystone Scientific, Bellefonte, PA). Concentrations were calculated based on a standard curve generated with at least 5 PFOS concentrations that were run 3 times at the start, middle, and end of the analytical run. All calculations and curve fitting were performed with MassLynx software (Micromass, Beverly, MD).

RESULTS

Dose Response *in Vitro*

Cells treated with perfluorinated compounds at concentrations of 3.1, 6.25, 12.5, 50, 100, and 160 μM for 30 min showed a dose-dependent decrease in the migration of the membrane impermeable dye (Figs. 1A–D, Fig. 2). The distance from the front of the dye to the scrape line is directly propor-

tional to the degree of cell-cell communication (Weis *et al.*, 1998). The dye migrated the greatest distance in cells treated with solvent alone; exposure to increasing concentrations of PFOS reduced the dye migration distance. PFOS, PFOSA, and PFHS inhibited dye migration in a dose-dependent manner, while PFBS showed no significant effect on GJIC in the concentration range tested (Fig. 2, top). No indications of cytotoxicity were observed for any of the chemicals within the concentration ranges tested.

PFOS and PFOSA were equally potent at GJIC inhibition with a NOEL concentration of 6.25 μM equivalent to 3.1 $\mu\text{g}/\text{ml}$ medium (Table 1). EC₅₀ concentrations for PFOS and PFOSA were also similar with values of 30.0 and 36.6 μM respectively equivalent to 15.0 and 18.3 $\mu\text{g}/\text{ml}$ medium, respectively. In contrast the no observed effect level (NOEL) and EC₅₀ values for PFHS were 50 μM (20 $\mu\text{g}/\text{ml}$ medium) and 121.5 μM (48.6 $\mu\text{g}/\text{ml}$ medium), respectively. PFBS was essentially inactive in the assay with a NOEL equal to the highest concentration tested, 160 μM (48 $\mu\text{g}/\text{ml}$ medium). To determine whether differences existed in species sensitivity to in-

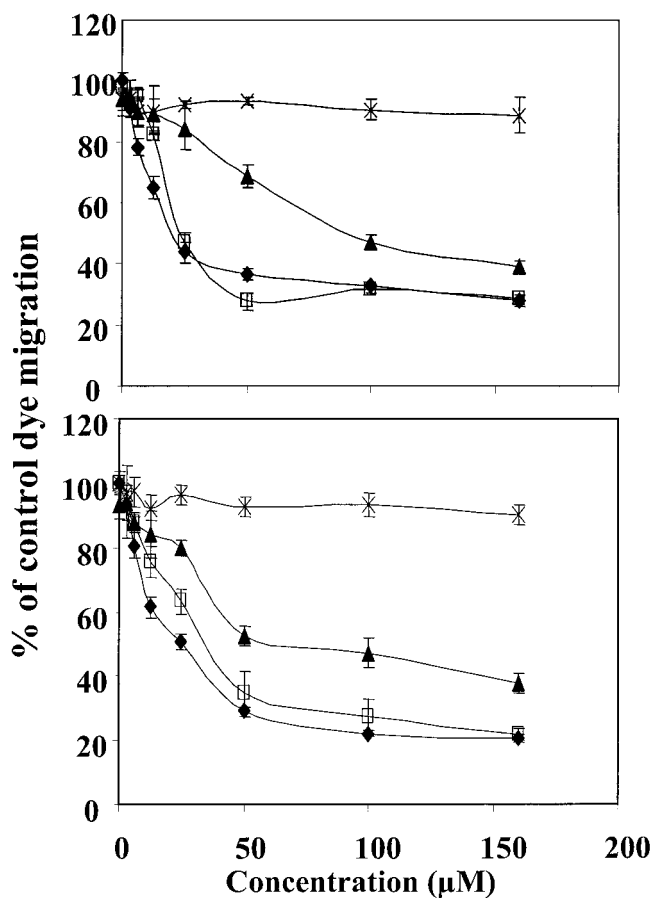


FIG. 2. Dose response effects of PFOS (diamonds), PFOSA (squares), PFHS (triangles), and PFBS (stars) on WB-F344 cells (top panel) and CDK cells (bottom panel) gap junctional intercellular communication (with exposure time of 30 min). Error bars indicate SEM.

TABLE 1
NOEL and EC₅₀ Values for *in Vitro* Dose-Response Experiment

Compound	NOEL	EC ₅₀
Rat WB-534 cells		
PFOS (C8)	6.25 μ M (3.1 μ g/ml)	29.96 μ M (14.98 μ g/ml)
PFOSA (C8)	6.25 μ M (3.1 μ g/ml)	36.60 μ M (18.3 μ g/ml)
PFHS (C6)	50 μ M (20 μ g/ml)	121.5 μ M (48.6 μ g/ml)
PFBS (C4)	>160 μ M (48 μ g/ml)	None
Dolphin kidney cells		
PFOS (C8)	6.25 μ M (3.1 μ g/ml)	25.51 μ M (12.8 μ g/ml)
PFOSA (C8)	6.25 μ M (3.1 μ g/ml)	35.84 μ M (17.92 μ g/ml)
PFHS (C6)	25 μ M (10 μ g/ml)	85.63 μ M (34.25 μ g/ml)
PFBS (C4)	>160 μ M (48 μ g/ml)	None

inhibition of GJIC, dose response curves were also determined for CDK dolphin kidney cells (Fig. 2, bottom). As with the rat cells, GJIC was inhibited in the CDK cells by exposure to perfluorinated chemicals (Figs. 1C–D). The NOEL and EC₅₀ for the CDK cells were essentially the same as for the rat cells with the exception of lesser NOEL and EC₅₀ values for PFHS, which had a NOEL of 25 μ M (10 μ g/ml medium) and an EC₅₀ of 85.6 μ M (34.3 μ g/ml medium; Table 1).

Time Course of Response

To determine the time course of GJIC inhibition rat WB-F344 cells were treated with 50 μ M PFOS or PFOSA for 2, 5, 10, 15, 20, or 30 min. GJIC inhibition was essentially complete after a 10 min exposure for both PFOS and PFOSA (Fig. 3, top). A 50% inhibition was observed after the WB cells were exposed to PFOS for only 2 min, and the maximum inhibition of 90% occurred within 10 to 30 min. These results were similar for cells treated with PFOSA. When the WB cells were exposed for 1 h or 24 h, no additional inhibition of GJIC was observed (results not shown).

To determine the time required for reversal of GJIC inhibition, WB cells were treated with 50 μ M PFOS for 30 min, then exposure was stopped by washing with PBS and replacement with fresh medium. GJIC was measured at 2, 5, 10, 15, 30, 60, and 90 min after the addition of fresh medium. The inhibition of GJIC by PFOS was rapidly reversible after removal of PFOS from the medium (Fig. 3, bottom). The time for cells to reach complete recovery was 60 to 90 min.

In Vivo Results

Exposure of rats to PFOS for 3 days did not alter the animals' body weight gain; in contrast, significant deficits were seen after 21 days of treatment (Table 2). Although low, measurable levels of PFOS were detected in control rat liver, after 3 days of treatment, about 125 μ g PFOS/g liver was detected; after 21 days of treatment an average of 725 μ g PFOS/g liver was measured (Table 3). The low concentrations of PFOS measured in control rats are presumably due to

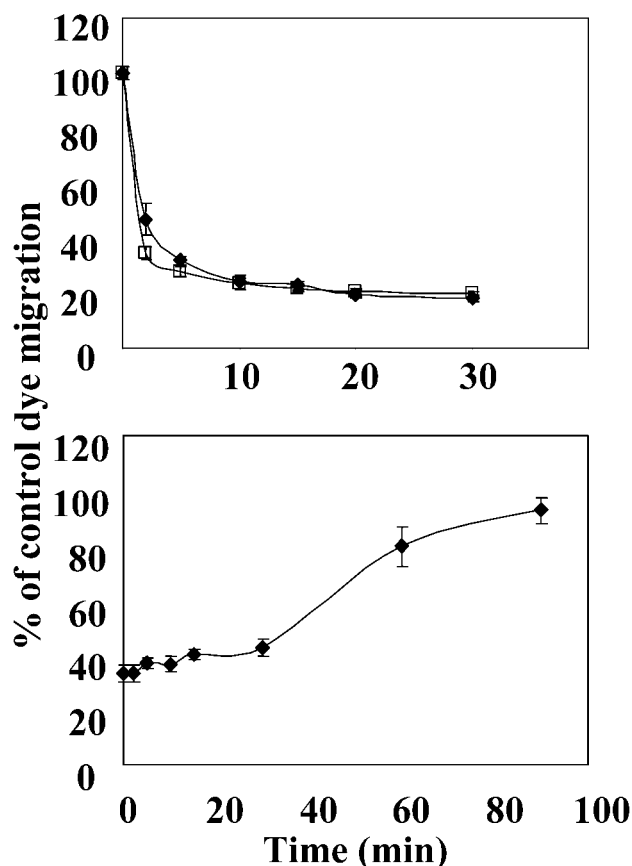


FIG. 3. Time course of the inhibitory effects (top panel) and recovery (bottom panel) from exposure to PFOS (diamonds) and/or PFOSA (squares) on GJIC in WB cells. Cells were exposed to PFOS or PFOSA at a concentration of 50 μ M. The inhibition of GJIC by PFOS was rapidly reversible after removal of PFOS from the medium (lower panel). See text for details of exposure and recovery experiments.

sources of contamination associated with the rats before purchase. GJIC was significantly reduced in liver tissue from PFOS-treated rats after 3 days of exposure (Figs. 1E–F, Fig. 4). The magnitude of inhibition was the same for the extended exposure up to 21 days. Since only a single dose concentration of PFOS was assessed it was not possible to develop a dose-response relationship for the *in vivo* exposure. No significant

TABLE 2
Effects of PFOS Treatment on Body Weight Gains in the Rat

	Male		Female	
	Control	PFOS	Control	PFOS
3-day treatment	12.5 \pm 3.5g	12.5 \pm 3.5g	12.5 \pm 3.5g	6.5 \pm 0.5g
21-day treatment	93.3 \pm 4.5g	70.0 \pm 6.1g*	65.0 \pm 9.3g	18.0 \pm 2.5g*

Note. Two-way ANOVA indicates an interaction between treatment durations, and a gender effect and interaction in the 21-day treatment group.

* $p < 0.05$ vs. controls.

TABLE 3

Analysis of PFOS Concentration in Liver Samples from Vehicle Control-Exposed Rats or Rats Exposed to 5 mg/kg/day PFOS *in Vivo*

Sample	Treatment	PFOS ($\mu\text{g/g}$)
A1	Control, 21 days, female	8.8
A2	Control, 21 days, female	5.9
A3	Control, 21 days, female	3.2
A4	Control, 21 days, male	2.2
A5	Control, 21 days, male	1.3
A6	Control, 21 days, male	1.3
A7	Control, 3 days, female	0.18
A8	Control, 3 days, female	0.35
A9	Control, 3 days, male	0.15
A10	Control, 3 days, male	0.36
C1	PFOS, 21 days, female	758
C2	PFOS, 21 days, female	582
C3	PFOS, 21 days, female	721
C4	PFOS, 21 days, male	720
C5	PFOS, 21 days, male	810
C6	PFOS, 21 days, male	763
C8	PFOS, 3 days, female	112
C9	PFOS, 3 days, female	116
C10	PFOS, 3 days, male	134
C11	PFOS, 3 days, male	142

Note. Concentration is expressed as $\mu\text{g/g}$ liver. See text for details of exposure and analytical procedures.

difference was detected between males and female rats in either the control or treatment groups.

DISCUSSION

These studies have demonstrated that perfluorinated sulfonic acid analogues of fatty acids inhibit GJIC at similar concentrations to perfluorinated carboxylic fatty acids. These findings are of significance given the recent discovery of PFOS in a variety of wildlife species. GJIC was chosen as the endpoint of interest because it has been linked to several other toxicological endpoints and is known to be affected by other classes of perfluorinated compounds at relatively small concentrations (Rosenkranz *et al.*, 2000; Upham *et al.*, 1998). Using this bioassay procedure also provides an opportunity to compare the relative biological potencies of several perfluorinated compounds recently detected in environmental samples. This provides a new opportunity to assess the relative potential risks of the different compounds and prioritization of future research efforts. However, these studies, based on *in vitro* cell culture exposures, cannot be used to directly assess possible risks to organisms *in vivo*. It is difficult to assess the biological and environmental significance of alterations in GJIC activity since few studies have related regulation of GJIC activity to endpoints other than tumor promotion.

The regulation of GJIC occurs at different levels of cellular control (Yamasaki *et al.*, 1995) including mutation of connexin

genes, reduced connexin gene expression, increased mRNA degradation, altered connexin protein translational control, posttranslational phosphorylation as well as binding of chemicals to the connexin proteins. The connexin molecules that constitute gap junctions are produced in the golgi apparatus. Therefore, the translocation of connexin from the golgi apparatus to cell membrane could also be a point of modulation of GJIC activity. Previous studies have shown that the peroxisome proliferator activated receptor (PPAR) mediates many of the effects of peroxisome proliferators, including perfluorinated compounds (Issemann and Green, 1990). Whether this receptor is potentially involved in this process is still under investigation; however, to date there is no direct evidence of a relationship between GJIC inhibition and PPAR.

While the mechanism of GJIC inhibition by perfluorinated compounds is not fully understood, the results of these experiments showed that exposure to these compounds resulted in a rapid inhibition and, after removal of chemical agents, rapid recovery of GJIC occurs within a period of minutes that is not sufficient for the expression of adverse effects at the transcriptional level to occur. Connexins are integral membrane proteins with 4 transmembrane domains. The C terminal of connexin has a protein kinase motif, which suggests possible regulation by phosphorylation mechanisms (Kimura *et al.*, 2000; Speisky *et al.*, 1995). However, previous results have indicated that no alteration in the phosphorylation of connexins is caused by PFFAs (Upham *et al.*, 1998) so that such a phosphorylation mechanism seems not to apply in this case (Hii *et al.*, 1995). Furthermore, induced changes in the phosphorylation of connexins do not always correlate with GJIC inhibition (Hossain *et al.*, 1999; Upham *et al.*, 1997). There are

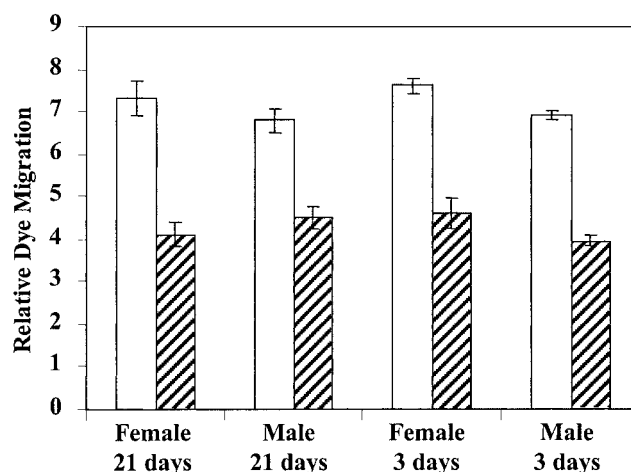


FIG. 4. Dye migration measured in Sprague-Dawley rats receiving oral gavage with vehicle control (open bars) or PFOS at 5 mg/kg (hatched bars) for 3 days or 21 days. A1–A3 female (21-day control), A4–A6 male (21-day control), A7–A8 female (3-day control), A9–A10 male (3-day control); C1–C3 female (21-day treatment), C4–C6 male (21-day treatment), C8–C9 female (3-day treatment), C10–C11 male (3-day treatment). Error bar indicates SE of 3 measurements in each rat.

also several other examples where various compounds known to inhibit GJIC do not alter the phosphorylation status of the connexins and their underlying mechanism of action remains unknown (Sai *et al.*, 2000; Suzuki *et al.*, 2000; Upham *et al.*, 2000).

The results from the current study support a structure-activity relationship for the inhibitory effects of perfluorinated sulfonic acids on GJIC. Previous studies have shown that PFFAs, such as PFOA and PFDA, can inhibit GJIC in a dose-dependent manner. The inhibitory potency of PFFAs depends on the length of the carbon chain, PFFAs with carbon chain lengths less than 5 or more than 16 did not inhibit GJIC (Upham *et al.*, 1998). In contrast, PFFAs with carbon chain lengths of 7, 8, 9, or 10 completely inhibit GJIC at concentrations of 50 μM (25 mg/l; Upham *et al.*, 1998). Our data are consistent with these previously published results. PFOS, which has an 8-carbon chain effectively inhibits GJIC, with an EC_{50} value of 36 μM (18 mg/l). PFOSA, the amide derivative of PFOS, inhibited GJIC with a similar potency to PFOS. However, it should be noted that the optimum chain length for the carboxyl fatty acids is 10 carbons while that for the sulfonic acids is 8 carbons. PFHS and PFBS, 6-carbon and 4-carbon chains respectively, but with the same functional group, do not inhibit GJIC. This indicates that the critical feature that determines GJIC inhibition for the PFFAs is the length of the carbon chain, not the nature of the functional group. This result suggests that the mode of action is based on a specific binding site for the ligands on the proteins of the gap junction since only ligands of a certain structure and size can elicit the observed effects.

It is significant that the structure-activity relationship for GJIC inhibition by endogenous fatty acids is different from that for the fluorinated analogues. As well as being less potent than the PFFAs, the optimum chain length for inhibition of GJIC by native fatty acids is 16–18 carbons compared to the 8–9 optimum carbon chain length for PFFAs (Boger *et al.*, 1998). In addition, the native fatty acids require a terminal carbonyl group capable of accepting a hydrogen bond so that the underivatized free fatty acids are essentially inactive (Boger *et al.*, 1998). This compares to the inhibition caused by the PFFAs, which is relatively insensitive to the nature of the functional group. Optimum activity of fatty acids also requires a delta-9 double bond and a hydrophobic methyl terminal group (Boger *et al.*, 1998). Together these observations indicate that the site of action of PFFA and native fatty acids for GJIC inhibition are different and that PFFAs are not simply acting as fatty acid analogues. A similar situation is observed with the binding of PFFAs to serum albumin where it appears PFFAs are bound to the protein at sites other than the fatty acid binding sites (Jones, unpublished results).

To date, most studies of GJIC inhibition have been conducted using the well-developed rat liver cell model. In this study dolphin kidney cells CDK were also used to test species specificity. Since inhibition of GJIC was observed in both cell lines the inhibitory effect of PFFAs on GJIC is neither species-

nor tissue-specific. However, since the dolphin cells were from a different species and a different organ than the rat cells it is not possible to make direct comparisons. Thus dolphin kidney cells CDK could be used as an effective model for effects of perfluorinated compounds on GJIC in marine mammal species.

To understand more completely the environmental relevance and effects of PFOS, it is necessary to evaluate the same endpoints using *in vivo* exposure systems. Since it is not possible to conduct *in vivo* exposure on bottlenose dolphin, we conducted PFOS exposure in Sprague-Dawley rats. Although effects on GJIC in the parenchymal tissue of liver are primarily expressed through gap junctions, which contain Cx32 and 26, most tumor promoting compounds (e.g., phthalate esters, 12-O-tetradecanoylphorbol-13-acetate, butylated hydroxytoluene, DDT, lindane, Aroclor 1254, clofibrate, trichloroethylene) that inhibit GJIC through Cx43 gap junctions are also known to inhibit GJIC in hepatocytes isolated from mouse and rat liver (Guan and Ruch, 1996; Jansen *et al.*, 1996; Klaunig *et al.*, 1988; Leibold *et al.*, 1994; Ruch *et al.*, 1987). Consistent with these *in vitro* studies, PFOS significantly inhibited GJIC in the livers of both treatment groups relative to the control, indicating that PFOS not only inhibits GJIC in Cx43 gap junctions but also in Cx32/26 gap junctions. This suggests the potential for PFOS to affect GJIC in multiple organisms. Oval cells, which have Cx43 gap junctions, are a major target for tumor promoting chemicals (Ruch and Trosko, 1999), so it is unfortunate that there is no *in vivo* technique to measure GJIC in these cells that exist as small populations in the periportal regions of the liver. However, it is not unreasonable to suspect that these cells would also be affected *in vivo* since PFOS inhibits GJIC in these cells under *in vitro* conditions. The *in vivo* results also suggest that PFOS is a robust inhibitor of GJIC. The final mean accumulated doses of PFOS in rat liver samples after 3-day or 21-day exposure were 125.6 $\mu\text{g/g}$ and 725.5 $\mu\text{g/g}$, respectively. However, no significant difference was observed in GJIC between short-term and long-term exposure. This could be explained by the fact that even after short-term exposure the accumulated dose was sufficient to cause maximum GJIC inhibition. Therefore extended exposure cannot cause further inhibition of GJIC. Even though the toxicokinetics of PFOS accumulation were expected to be different between male and female rats, the final dose of PFOS detected in samples of both male and female rat liver were similar. Furthermore, there was no significant difference in the measured inhibitory effects of PFOS on GJIC between male and female rats. This indicates that the effect of PFOS on cell-cell communication is not gender-related. Overall, these *in vivo* results suggest that PFOS poses a risk to the health of mammalian systems by interrupting GJIC, which is crucial in the maintenance of homeostasis within a tissue. Whether PFOS would pose a risk to human health cannot be determined, particularly, since many peroxisome proliferating compounds affect only rodents and not humans (Cattley *et al.*, 1998).

Together the results of this study demonstrate that, of the

compounds tested, PFOS is the most potent inhibitor of GJIC activity and that its potency is equivalent to PFDA, the most potent GJIC-inhibitor of the carboxylic-PFFAs. Inhibition of GJIC was observed both *in vitro* and *in vivo*, demonstrating the relevance of GJIC inhibition to organisms exposed *in vivo*.

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