been shown to support HIV-1 replication (*24*), are among the first virus targets in primates after oral exposure and play a key role in subsequent virus spread.

In sum, cell-free SIV is significantly more transmissible through the oral route as compared with the intrarectal route. The use of omeprazole, which results in the neutralization of gastric acid, was not required for infection. Because formal oral titrations were not conducted to establish the minimal infective dose in neonatal macaques (*10*), we do not know whether the oral exposure route is significantly more permissive of SIV entry in neonates as compared with adults. Our data, together with the case reports of HIV-1 seroconversion after oral-genital sex only (*5*), have implications for HIV-1 transmission. We conclude that oral exposure to infectious virus in the absence of mucosal lesions carries the risk of infection and AIDS. Thus, unprotected receptive oral intercourse should be added to the list of risk behaviors for HIV-1 transmission.

REFERENCES AND NOTES

1. J. A. Levy, *HIV and the Pathogenesis of AIDS* (American Society of Microbiology Press, Washington, DC, 1994).
2. T. W. Baba *et al.*, *J. Women's Health* **2**, 231 (1993).
3. D. Gibb and D. Wara, *AIDS* **8**, S275 (1994).
4. D. Williams-Hermann *et al.*, *International Conference on AIDS*, 6 to 11 June 1993 (Abstract Number PO-B05-1064).
5. A. R. Lifson *et al.*, *Am. J. Public Health* **80**, 1509 (1990); I. P. M. Keet *et al.*, *AIDS* **6**, 223 (1992); W. S. Rozenbaum *et al.*, *Lancet* **i**, 1395 (1988); A. B. Murray *et al.*, *Ibid.* **338**, 830 (1991); H. C. Lane, S. D. Holmberg, H. W. Jaffe, *Am. J. Public Health* **81**, 658 (1991).
6. M. D. Daniel *et al.*, *Science* **228**, 1201 (1985); J. Zhang *et al.*, *J. Infect Dis.* **158**, 1277 (1988).
7. G. Franchini and M. L. Bosch, *Ann. N.Y. Acad. Sci.* **554**, 81 (1989); V. M. Hirsch *et al.*, *Nature* **339**, 389 (1989); R. C. Desrosiers, *Annu. Rev. Immunol.* **8**, 557 (1990).
8. N. L. Letvin *et al.*, *Science* **230**, 71 (1985).
9. P. N. Fultz *et al.*, *J. Infect. Dis.* **154**, 896 (1986).
10. T. W. Baba *et al.*, *AIDS Res. Hum. Retroviruses* **10**, 351 (1994).
11. G. B. Baskin *et al.*, *J. Nat. Cancer Inst.* **77**, 127 (1986); G. B. Baskin *et al.*, *Vet. Pathol.* **25**, 456 (1988); A. M. Amedee *et al.*, *J. Virol.* **69**, 7982 (1995).
12. H. Larsson *et al.*, *Gastroenterology* **85**, 900 (1983); T. Lind *et al.*, *Gut* **24**, 270 (1983); D. McTavish, M. M. T. Buckley, R.C. Heel, *Drugs* **42**, 138 (1991).
13. To expose seven young adult macaques [*M. mulatta*; seronegative for SIV, simian retrovirus type D, and simian T lymphotropic virus type I; median age at inoculation 37 months (range 31 to 41 months)] orally to cell-free SIV, animals were allowed to drink water ad libitum, without food, during the night before exposure. Immediately before inoculation, macaques were sedated with ketamine and underwent a careful exam of the oral cavity, and the virus inoculum (diluted with medium consisting of RPMI 1640, 15% fetal bovine serum, penicillin-streptomycin, and l-glutamine to achieve a total volume of 3.0 ml) was dripped without trauma onto the back of the tongue.
14. J. L. Spouge, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7581 (1992).
15. C. D. Pauza, M. Malkovsky, M. S. Salvato, *AIDS Res. Hum. Retroviruses* **10**, S7 (1994); C. D. Pauza *et al.*, *J. Med. Primatol.* **22**, 154 (1993).
16. C. J. Miller *et al.*, *J. Med. Primatol.* **19**, 401 (1990); C. J. Miller *et al.*, *J. Virol.* **63**, 4277 (1989); C. J. Miller *et al.*, *ibid.* **68**, 6391 (1994); C. J. Miller, *Am. J. Reprod. Immunol.* **31**, 52 (1994); ———, *Curr. Top. Microbiol. Immunol.* **188**, 107 (1994); M. L. Marthas *et al.*, *J. Med. Primatol.* **21**, 99 (1992).
17. M. D. Conway *et al.*, *J. Med. Primatol.* **20**, 152 (1991).
18. W. Winkelstein Jr. *et al.*, *Am. J. Public Health* **77**, 685 (1987); *ibid.* **78**, 1472 (1988); L. A. Kingsley *et al.*, *Lancet* **i** (8529), 345 (1987); G. J. P. van Griensven *et al.*, *Am. J. Epidemiol.* **125**, 1048 (1987).
19. A. S. Berthier *et al.*, *Lancet* **ii**, 598 (1986); G. Friedland *et al.*, *AIDS* **4**, 639 (1990); M. F. Rogers *et al.*, *Pediatrics* **85**, 210 (1990).
20. D. D. Ho, T. Moudgil, M. Alam, *N. Engl. J. Med.* **321**, 1621 (1989).
21. D. J. Anderson *et al.*, *J. Am. Med. Assoc.* **267**, 2769 (1992); M. S. Borzy, R. S. Connell, A. A. Kiessling, *J. Acquired Immune Defic. Syndr.* **1**, 419 (1988); D. D. Ho *et al.*, *Science* **226**, 451 (1984); J. A. Levy, *J. Am. Med. Assoc.* **259**, 3037 (1988); B. J. Van Voorhis *et al.*, *Fertil. Steril.* **55**, 588 (1991); D. Zagury *et al.*, *Science* **226**, 449 (1984); K. A. Hamed *et al.*, *J. Infect. Dis.* **167**, 798 (1993); J. N. Krieger *et al.*, *ibid.* **163**, 386 (1991).
22. P. N. Fultz, *Lancet* **ii**, 1215 (1986); D. W. Archibald and G. A. Cole, *AIDS Res. Hum. Retroviruses* **6**, 1425 (1990); D. H. Coppenhaver *et al.*, *N. Engl. J. Med.* **330**, 1314 (1994); D. Malamud *et al.*, *AIDS Res. Hum. Retroviruses* **9**, 633 (1993); M. Pourtois *et al.*, *AIDS* **5**, 598 (1991); H. Su and R. J. Boackle, *Mol. Immunol.* **28**, 811 (1991); J. Amory *et al.*, *Clin. Res.* **40**, 51A (1992); E. J. Bergey *et al.*, *J. Acquired Immune Defic. Syndr.* **7**, 995 (1994); T. B. McNeely *et al.*, *J. Clin. Invest.* **96**, 456 (1995).
23. S. Glushakova, B. Baibakov, L. B. Margolis, J. Zimmerberg, *Nature Med.* **1**, 1320 (1995).
24. S. S. Frankel *et al.*, *Science* **272**, 115 (1996).
25. T. W. Baba *et al.*, *ibid.* **267**, 1820 (1995).
26. M. D. Lairmore *et al.*, *AIDS Res. Hum. Retroviruses* **9**, 565 (1993).
27. L. N. Martin *et al.*, *J. Infect. Dis.* **168**, 825 (1993); S. Ohkawa, L. Martin, M. Murphey-Corb, *FASEB J.* **4**, A2264 (1990).
28. L. N. Martin *et al.*, *Antimicrob. Agents Chemother.* **38**, 1277 (1994).
29. J. S. Allan *et al.*, *J. Virol.* **65**, 2816 (1991).
30. All animal experiments were approved by the Animal Care and Use Committees at the Tulane Regional Primate Research Center and at the Dana-Farber Cancer Institute, which take responsibility for humane care and use of laboratory animals. We are committed to comply with the Principles for Use of Animals, the *Guide for the Care and Use of Laboratory Animals*, the Provisions of the Animal Welfare Act, and other applicable laws and regulations. The center's statement of assurance is on file with the USPHS, Office for Protection from Research Risks. These facilities are accredited by the American Association for Accreditation of Laboratory Animal Care. Animals are anesthetized with ketamine before all procedures that require the removal of animals from their cages. No restraining devices are necessary during these procedures. When necessary, moribund animals are euthanized by iv inoculation of a lethal dose of sodium pentobarbital.

31. We thank L. Corey for sharing unpublished data; A. Rosowsky for reviewing the manuscript; C. Lanclos, E. deHaro, E. Pecunia, and S. Kraus for technical assistance; and L. Geronimo for preparing the manuscript. Supported in part by NIH grants U01 AI24345, RO1-AI32330, RO1-AI34266, and RO1-AI35533 to R.M.R.; RR-00164, providing base support for the Tulane Regional Primate Research Center; AI-15093 and AI-35550 to M.M.-C.; and AI-15119 to L.N.M.; and by the Center for AIDS Research Core Grant no. IP30 28691 awarded to the Dana-Farber Cancer Institute as support for the iInstitute's AIDS research efforts. T.W.B. is a Scholar of the Pediatric AIDS Foundation and A.M.T. is a Fellow of the Minnie Burke Foundation.

15 February 1996; accepted 13 May 1996

Synergistic Activation of Estrogen Receptor with Combinations of Environmental Chemicals

Steven F. Arnold, Diane M. Klotz, Bridgette M. Collins, Peter M. Vonier, Louis J. Guillette Jr., John A. McLachlan*

Certain chemicals in the environment are estrogenic. The low potencies of these compounds, when studied singly, suggest that they may have little effect on biological systems. The estrogenic potencies of combinations of such chemicals were screened in a simple yeast estrogen system (YES) containing human estrogen receptor (hER). Combinations of two weak environmental estrogens, such as dieldrin, endosulfan, or toxaphene, were 1000 times as potent in hER-mediated transactivation as any chemical alone. Hydroxylated polychlorinated biphenyls shown previously to synergistically alter sexual development in turtles also synergized in the YES. The synergistic interaction of chemical mixtures with the estrogen receptor may have profound environmental implications. These results may represent a previously uncharacterized level of regulation of estrogen-associated responses.

Reports of abnormal sexual development in reptiles (*1*, *2*) or birds (*3*) as well as feminized responses in male fish (*4–6*) have suggested an association with environmental chemicals functioning as estrogens. Similar hypotheses have been advanced in relation to an increased risk for breast cancer in women (*7*, *8*) and an observed decrease in human semen quality (*9*, *10*). A model for the developmental effects of estrogen is based, to a large extent, on studies with the synthetic estrogen diethylstilbestrol (DES) in animals and humans (*11–13*). Most estrogenic environmental compounds have potencies 1/50th to 1/10,000th those of DES or the natural estrogen 17β-estradiol; for example, the pesticides dieldrin, toxaphene, or endosulfan have an affinity for hER that is ~1/10,000th that of estradiol (*14*, *15*). The relatively low potencies of each of these compounds have suggested that these chemicals alone are unlikely to

Table 1. Concentrations of environmental chemicals required to achieve 50% β-Gal activity (EC_{50}) in YES. β-Galactosidase assays were done as described in Fig. 1. The EC_{50} values were generated by plotting the percent β-Gal activity versus the concentration of environmental chemical. The values for the combinations represent the total concentration of environmental chemicals as they were mixed at equal molar concentrations. The data shown are representative of at least three independent experiments. The EC_{50} values represent the concentration required to achieve half-maximal β-Gal activity for that chemical or combination of chemicals. The maximal β-Gal activity induced by the combined environmental chemicals appeared to be ~65% that of 17β-estradiol. ND, not determined.

Chemical	β-Gal EC_{50} (μM)
17β-Estradiol	0.0001
Endosulfan	>33
Dieldrin	>33
Toxaphene	>33
Chlordane	ND*
Endosulfan + dieldrin	0.092
Endosulfan + toxaphene	0.121
Endosulfan + chlordane	0.189
Dieldrin + toxaphene	0.210
Dieldrin + chlordane	0.286
Toxaphene + chlordane	0.306

*The EC_{50} for chlordane was not measured because it did not exhibit β-Gal activity at any concentration tested.

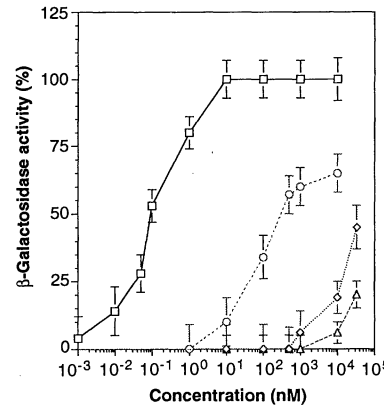
produce adverse health effects in humans (*16*, *17*). However, these compounds occur as mixtures in the environment, and their combined action has not been well studied.

To investigate the interaction of mixtures of chemicals with hER, we compared the transcriptional activation of hER in yeast in response to environmental chemicals alone and in combination (*18*). Dieldrin, endosulfan, or toxaphene alone weakly increased β-galactosidase (β-Gal) activity even at high concentrations (greater than 10 μM) (Fig. 1 and Table 1). However, a combination of any two of these chemicals produced a synergistic increase in β-Gal activity as compared with the individual compounds. For example, to increase β-Gal activity to a similar extent, the concentration of the mixture of endosulfan and dieldrin required was 1/160th to 1/1600th that of either chemical alone (that is, the mixture was 160 to 1600 times more potent than the individual chemicals). Chlordane, which had no measurable activity in the YES, also significantly enhanced the potency of the other environmental chemicals (Table 1).

Because other signaling pathways, such as those involving protein kinases activated by growth factors, play a role in estrogen action (*19*, *20*), we studied the binding of [^{3}H]17β-estradiol to hER with the environmental chemicals alone or in combination as a measure of the direct interaction of these compounds with hER (*21*). Consistent with the results in the YES, dieldrin, endosulfan, or toxaphene alone only weakly inhibited the binding of [^{3}H]17β-estradiol to hER (Fig. 2 and Table 2). To inhibit [^{3}H]17β-estradiol binding to the same extent, the concentration of the combined chemicals was at most 1/200th that required for either chemical alone (that is, the mixture was at least 200 times more potent than the individual chemicals). Chlordane, which did not inhibit binding of [^{3}H]17β-estradiol, also enhanced the competitive binding activity of the other environmental chemicals.

The synergistic action of combinations

Fig. 1. Estrogenic activity of environmental chemicals in YES. A single yeast colony was grown overnight at 30°C in synthetic media supplemented with Trp and Ura, then 50 μl was added to 950 μl of fresh media with vehicle, 17β-estradiol (□), endosulfan (◇), dieldrin (△), or endosulfan and dieldrin (○) and grown overnight at 30°C. Equivalent molar concentrations of endosulfan and dieldrin were used for the combinations (for example, 5 nM of endosulfan and dieldrin for a total of 10 nM). After treatment, the yeast cells were collected by centrifugation, the A_{600} measured, and the activity of β-Gal determined as described (*18*). The data are representative of at least three separate experiments. The increase in β-Gal activity is greater at the lower concentrations of mixtures of environmental chemicals.



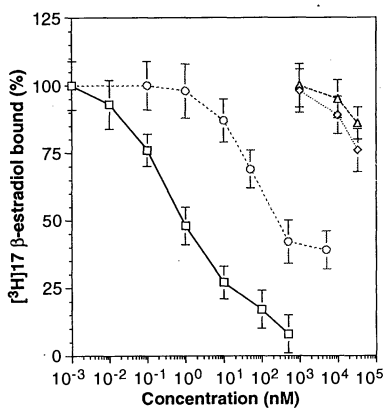


Fig. 2. Inhibition of [^{3}H]17β-estradiol binding to hER by environmental chemicals. Recombinant hER at a concentration of ~0.4 nM was dissolved in the binding buffer [10 mM tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 10% glycerol, γ-globulin (10 mg/ml), 0.5 mM PMSF, and 0.2 mM leupeptin] for 1 hour at 25°C with 2.5 nM[^{3}H]17β-estradiol in the absence or presence of the indicated concentrations of radioinert environmental chemicals or 17β-estradiol. Equivalent molar concentrations of endosulfan and dieldrin were used for the combination. Free [^{3}H]17β-estradiol was removed by incubation with Chardex for 10 min at 4°C and centrifugation for 3 min at 15,000*g*. The data shown are representative of at least three independent experiments. The decrease in [^{3}H]17β-estradiol binding is greater at the lower concentrations of mixtures of environmental chemicals. Symbols as in Fig. 1.

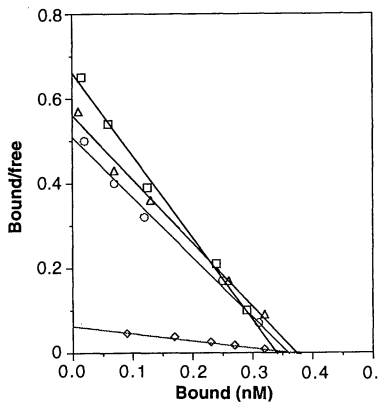


Fig. 3. A Scatchard analysis of hER binding to [^{3}H]17β-estradiol in the absence or presence of environmental chemicals. Recombinant hER was dissolved in binding buffer for 1 hour at 25°C (see Fig. 1) with 0.5, 1, 2.5, 5 or 10 nM [^{3}H]17β-estradiol and a 300-fold molar excess of unlabeled 17 β-estradiol (□), 100 nM endosulfan (○), 100 nM dieldrin (△), or 100 nM endosulfan and 100 nM dieldrin (◇). The data were analyzed by the method of Scatchard (*31*) and are representative of at least three independent experiments.

S. F. Arnold, Department of Environmental Health Sciences, Tulane University of Public Health and Tropical Medicine, 1501 Canal Street, New Orleans, LA 70112, USA and Tulane-Xavier Center for Bioenvironmental Research, 1430 Tulane Avenue, New Orleans, LA 70112, USA.
D. M. Klotz, B. M. Collins, P. M. Vonier, Program in Molecular and Cellular Biology, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112 USA and Tulane-Xavier Center for Bioenvironmental Research, 1430 Tulane Avenue, New Orleans, LA 70112, USA.
L. J. Guillette Jr., Department of Zoology, University of Florida, 223 Bartam Hall, Gainesville, FL 32611, USA and Tulane-Xavier Center for Bioenvironmental Research, 1430 Tulane Avenue, New Orleans, LA 70112, USA.
J. A. McLachlan, Department of Environmental Health Sciences, Tulane University of Public Health and Tropical Medicine, 1501 Canal Street, New Orleans, LA 70112, USA; Department of Pharmacology, Tulane University Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA; and Tulane-Xavier Center for Bioenvironmental Research, 1430 Tulane Avenue, New Orleans, LA 70112, USA.

*To whom correspondence should be addressed at Tulane-Xavier Center for Bioenvironmental Research, 1430 Tulane Avenue, New Orleans, LA 70112, USA. E-mail: jmclach@mailhost.tcs.tulane.edu

of environmental chemicals suggested the possibility of additional binding sites on hER (*22–24*). Linear slopes observed in Scatchard analyses of binding experiments with combined environmental chemicals demonstrated competitive inhibition (Fig. 3), suggesting that the chemicals interact in combination with hER at the hormone binding site (*25*).

Hydroxlyated polychlorinated biphenyls (PCBs) activated estrogen-dependent reporter activity in the YES and in Ishikawa cells, an endometrial cancer cell line transiently transfected with hER, and an estrogen-sensitive luciferase reporter (Table 3). A mixture of the two PCBs synergistically activated reporter gene activity in the YES and in Ishikawa cells. In vitro binding experiments confirmed that PCBs interacted with the hER and when mixed displayed a synergistic reduction in [^{3}H]17β-estradiol binding to hER. Compound F demonstrated a median inhibitory concentration (IC_{50}) of 55 nM, whereas compound G had an IC_{50} of 120 nM; both compounds added together resulted in an IC_{50} of 5 nM.

Table 2. Concentrations of environmental chemicals required to achieve 50% displacement or inhibition (IC_{50}) of hER binding of [^{3}H]17β-estradiol. Competitive binding assays were done as described (Fig. 2). The IC_{50} values were calculated by plotting the percent [^{3}H]17β-estradiol remaining versus the concentration of environmental chemical as shown in Fig. 2. The values for the combinations represent the total concentration of environmental chemicals as they were mixed at equal molar concentrations. The data shown are representative of at least three independent experiments. The standard error was less than 7.5% for the competition binding assays.

Chemical	hER binding IC_{50} (μM)
Endosulfan	>50
Dieldrin	>50
Toxaphene	>50
Chlordane	ND*
17β-Estradiol	0.001
Endosulfan + dieldrin	0.324
Endosulfan + toxaphene	0.339
Endosulfan + chlordane	0.363
Dieldrin + toxaphene	0.498
Dieldrin + chlordane	0.514
Toxaphene + chlordane	0.533

*The IC_{50} value was not determined because chlordane did not appear to demonstrate competitive binding activity at any concentration tested. It has been reported that the IC_{50} values for endosulfane and toxaphene are 631 and 470 μM, respectively (*15*).

Table 3. Concentrations of environmental chemicals required to achieve 50% β-Gal activity in YES (*18*) or luciferase activity in Ishikawa cells (*32*). The PCB compounds included F (2′,4′,6′-trichloro-4-biphenylol) and G (2′,3′,4′,5′-tetrachloro-4-biphenylol). The difference in the EC_{50} values between the β-Gal and luciferase assays is probably due in part to a higher concentration of hER in Ishikawa cells compared with the YES. The EC_{50} values were generated by plotting the percent β-Gal or luciferase activity versus the concentration of environmental chemical. The values for the combinations represent the total concentration of environmental chemicals as they were mixed at equal molar concentrations. The data shown are representative of at least two independent experiments. The standard error was less than 7.5% for the assays.

Chemical	β-Gal EC_{50} (μM)	Luciferase EC_{50} (μM)
Estradiol	0.0001	0.0002
F	0.0070	0.0120
G	0.0180	0.0300
F + G	0.0015	0.0025

Our results provide a molecular explanation for studies in vivo that described synergistic effects of environmental estrogens. For example, the addition of weakly estrogenic PCB congeners to developing turtle eggs resulted in sex reversal of male-determined eggs; addition of two PCBs at subthreshold concentrations were effective (*26*). Likewise, dieldrin, endosulfan, or toxaphene, although only marginally effective in stimulating proliferation of human breast cancer cells in culture, exhibited greater than additive effects when administered together (*15*). Apparently synergistic estrogenic effects of environmental chemicals were observed in cultures of fish hepatocytes (*27*). Our data and the above reports suggest that the estrogenic potency of some environmental chemicals, when tested singly, may be underestimated.

In addition to their environmental implications, these findings may also have general biological significance, because ovarian and phytoestrogens act synergistically in the YES (*28*). Transcriptional activation of hER through interaction with two chemicals is consistent with the observation that the active site of a cytochrome P_{450} has the potential to recognize two substrates (*29*). The possibility for synergistic action of apparently inactive chemicals functioning as hormones may represent a previously uncharacterized level of receptor-mediated gene regulation. The interaction of multiple chemicals with the estrogen receptor suggests a complex interplay between environmental signals and biological systems.

REFERENCES AND NOTES

1. L. J. Guillette Jr. *et al.*, *Environ. Health Perspect.* **102**, 680 (1994).
2. L. J. Guillette Jr., T. S. Gross, D. Gross, A. A. Rooney, H. F. Percival, *ibid.* **103** (suppl. 4), 31 (1995).
3. D. M. Fry, *ibid.* **103** (Suppl. 7), 165 (1995).
4. R. White, S. Jobling, S. A. Hoare, J. P. Sumpter, M. G. Parker, *Endocrinology* **135**, 175 (1994).
5. S. Jobling and J. P. Sumpter, *Aquat. Toxicol.* **27**, 361 (1993).
6. S. Jobling, T. Reynolds, R. White, M. G. Parker, J. P. Sumpter, *Environ. Health Perspect.* **103**, 582 (1995).
7. F. J. Falck, A. J. Ricci, M. S. Wolff, J. Godbold, P. Deckers, *Archives Environ. Health* **47**, 143 (1992).
8. M. S. Wolff, P. G. Toniolo, E. W. Lee, M. Rivera, N. Dubin, *J. Natl. Cancer Inst.* **85**, 648 (1993).
9. E. Carlsen, A. Giwercman, N. Keiding, N. E. Skakkebaek, *Br. Med. J.* **304**, 609 (1992).
10. J. Auger, F. Czyglik, J. M. Kunstmann, P. Jouannet, *N. Engl. J. Med.* **332**, 281 (1995).
11. R. R. Newbold, B. C. Bullock, J. A. McLachlan, *Cancer Res.* **50**, 7677 (1991).
12. J. A. McLachlan, R. R. Newbold, C. T. Teng, K. S. Korach, in *Chemically-Induced Alterations in Sexual and Functional Development: The Wildlife/Human Connection*, T. Colborn and C. Clement, Eds. (Princeton Scientific, Princeton, 1992), vol. 21, pp. 107–112.
13. H. Bern, *ibid.*, pp. 9–15.
14. J. A. McLachlan, *Environ. Health Perspect.* **101**, 386 (1993).
15. A. M. Soto, K. L. Chung, C. Sonnenschein, *ibid.* **102**, 380 (1994).
16. S. H. Safe, *Hum. Ecol. Risk Assessment* **1**, 17 (1995).
17. R. J. Sherins, *N. Engl. J. Med.* **332**, 327 (1995).
18. YES was developed by transforming yeast strain BJ2407 (*Mata/Matα ura3-52, trp1, leu2, srb1-1122, prc1-407, pep4-3*) with an expression plasmid containing the complementary DNA of hER and a reporter plasmid containing two estrogen response elements linked to the *lacZ* gene. A single yeast colony was grown overnight at 30°C in synthetic media supplemented with Trp, Ura, then 50 μl was added to 950 μl of fresh media with vehicle, 17β-estradiol, endosulfan, dieldrin, or endosulfan and dieldrin and grown overnight at 30°C. The environmental chemicals and estradiol were dissolved in dimethyl sulfoxide (DMSO), and the concentration of DMSO in the media did not exceed 1%. The solubility limit for the environmental chemicals was between 33 and 50 μM, and therefore concentrations greater than 33 μM were not used. After treatment, the yeast cells were collected by centrifugation and permeabilized by the addition of 700 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 35 mM β-mercaptoethanol), 4 μl of 0.1% SDS, 6 μl of $CHCl_3$ and mixed for 25 s. The reactions were then placed at 30°C with the addition of 160 μl of *o*-nitrophenyl β-D-galactopyranoside (4 mg/ml in Z-buffer). The reactions were terminated by the addition of 400 μl of 1 M $NaCO_3$, and the absorbance at 420 nm (A_{420}) was measured. β-Galactosidase activity was determined by the formula [A_{420}/(A_{600} of 1/10 dilution of cells × volume of culture × length of incubation)] × 1000 and expressed as a percentage of the maximal response to estradiol (S. F. Arnold, M. K. Robinson, A. C. Notides, L. J. Guillette Jr., J. A. McLachlan, *Environ. Health Perspect.* **104**, 544 (1996).
19. S. F. Arnold, J. D. Oburn, H. Jaffe, A. C. Notides, *J. Steroid Biochem. Mol. Biol.* **55**, 163 (1995).
20. D. M. Ignar-Trowbridge *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4658 (1992).
21. We produced recombinant hER in Sf9 insect cells by using the baculovirus expression system and prepared the hER as ammonium sulfate precipitates. The hER produced in Sf9 cells has approximately the same affinity for estradiol as hER produced in mammalian and yeast cells (*30*). Recombinant hER at a concentration of ~0.4 nM was dissolved in the binding buffer [10 mM tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 1 mM $NaVO_4$, 10% glycerol, γ-globulin (10 mg/ml), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mM leupeptin for 1 hour at 25°C with 2.5 nM [^{3}H]17β-estradiol in the absence or presence of increasing concentrations of radioinert environmental chemicals or 17β-estradiol. [^{3}H]17β-estradiol, endosulfan, and dieldrin were dissolved in DMSO at 10 mM and added to the reaction so that the concentration of DMSO did not exceed 2.5%. Equivalent molar concentrations of endosulfan and dieldrin were used for the combination. Endosulfan or dieldrin were not tested at concentrations greater than 33 μM. Nonspecific binding of [^{3}H]17β-estradiol was

assessed by addition of 300-fold molar excess of radioinert 17β-estradiol. Free [^{3}H]17β-estradiol was removed by incubation with Chardex (contains 5% activated charcoal and 0.5% dextran dissolved in phosphate-buffered saline) for 10 min at 4°C and centrifugation for 3 min at 15,000*g*. The bound [^{3}H]17β-estradiol was measured by scintillation counting. The data shown are representative of at least three independent experiments (D. M. Klotz, J. A. McLachlan, S. F. Arnold, in preparation).
22. T. S. Ruh and L. J. Baudendistel, *Endocrinology* **100**, 420 (1977).
23. A. Fargin *et al.*, *Chem. Biol. Interactions* **66**, 101 (1988).
24. P. M. Martin, Y. Berthois, E. V. Jensen, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2533 (1988).
25. V. Kumar, S. Green, A. Staub, P. Chambon, *EMBO J.* **5**, 2231 (1986).
26. J. M. Bergeron, D. Crews, J. A. McLachlan, *Environ. Health Perspect.* **102**, 780 (1994).
27. J. P. Sumpter and S. Jobling, *ibid.* **103**, 173 (1995).
28. S. F. Arnold, B. M. Collins, W. A. Toscano, J. A. McLachlan, in preparation.
29. M. Shou *et al.*, *Biochemistry* **33**, 6450 (1994).
30. S. F. Arnold *et al.*, unpublished data.
31. G. Scatchard, *Ann. NY Acad. Sci.* **51**, 660 (1949).
32. Ishikawa cells grown in a 35-mm plate were transfected with 2 μg of pERE-luc (containing two copies of the vitellogenin estrogen response element linked to the luciferase gene), 1 μg of a control β-Gal plasmid, and 20 ng of hER in a mammalian expression vector with 10 μl of lipofectamine (GIBCO BRL) for 5 hours. The cells were treated with vehicle, estradiol, or 2′,4′,6′-trichloro-4-biphenylol or 2′,3′,4′,5′-tetrachloro-4-biphenylol (or both) in Dulbecco's modified Eagle's medium with 5% charcoal-stripped fetal bovine serum for 18 hours. All chemicals were dissolved in DMSO and added to the media so that the concentration of DMSO did not exceed 1%. The cells were collected by incubation with lysis buffer (Analytical Luminescence, Ann Arbor, MI) for 15 min at 25°C, the protein concentration determined with the Bio-Rad protein assay reagent, and the β-Gal activity measured as described (*18*). The amount of extract used in the luciferase assay was normalized for protein and β-Gal activity. The luciferase assay was performed in the Monolight 2010 as recommended by the manufacturer (Analytical Luminescence).
33. We thank Tulane University, the W. Alton Jones Foundation, and the Louisiana Breast Cancer Foundation for support; W. A. Toscano, N. Masahiko, and J. R. Ostberg for critical reading of the manuscript; and M. K. Robinson for help with the YES and S. Safe for providing the PCBs.

22 February 1996; accepted 8 May 1996

Regulation of the Inositol 1,4,5-Trisphosphate Receptor by Tyrosine Phosphorylation

Thottala Jayaraman, Karol Ondriaš, Elena Ondriašová, Andrew R. Marks*

Tyrosine kinases indirectly raise intracellular calcium concentration ($[Ca^{2+}]_i$) by activating phospholipases that generate inositol 1,4,5-trisphosphate (IP_3). IP_3 activates the IP_3 receptor (IP_3R), an intracellular calcium release channel on the endoplasmic reticulum. T cell receptor stimulation triggered a physical association between the nonreceptor protein tyrosine kinase Fyn and the IP_3R, which induced tyrosine phosphorylation of the IP_3R. Fyn activated an IP_3-gated calcium channel in vitro, and tyrosine phosphorylation of the IP_3R during T cell activation was reduced in thymocytes from *fyn*$^{-/-}$ mice. Thus, activation of the IP_3R by tyrosine phosphorylation may play a role in regulating $[Ca^{2+}]_i$.

The IP_3R forms the IP_3-gated Ca^{2+} release channel on the endoplasmic reticulum in many cell types, including neurons (*1*) and T cells (*2*). The human type 1 IP_3R (IP_3R1) is a 308-kD polypeptide that contains two potential tyrosine phosphorylation sites, at residues 482 (Glu-Asp-Leu-Val-Tyr) and 2617 (Asp-Ser-Thr-Glu-Tyr) (*2*). Amino acid 482 is adjacent to the IP_3-binding site (*3*), and amino acid 2617 is near the COOH-terminus (*4*).

The IP_3R is autophosphorylated on serine and is phosphorylated by protein kinases A, C, and G and by Ca^{2+}-calmodulin–dependent kinase II (CaMkII) in vitro (*5*). Members of the Src family of nonreceptor protein tyrosine kinases function in signal transduction in the brain (*6*) as well as in T cells (*7*, *8*). To determine whether tyrosine phosphorylation of the IP_3R occurred, we immunoprecipitated the Ca^{2+} release channel with an IP_3R1-specific antibody (anti-IP_3R1) from both brain and T lymphocytes and subjected it to kinase assays with two nonreceptor protein tyrosine kinases, Src and Fyn. Both kinases induced tyrosine phosphorylation of the brain IP_3R (Fig. 1A). Similar results were obtained with canine, rabbit, and murine brain microsomes (*9*) and with purified IP_3R (Fig. 1A), which suggests that the kinase acts on the channel directly, not through an intermediary molecule.

Fyn and Src induced tyrosine phosphorylation of the IP_3R from unstimulated Jurkat cells (Fig. 1B) in vitro. Tyrosine phosphorylation of the IP_3R from unactivated Jurkat cells by Fyn was greater than that of the IP_3R from cells activated by incubation with monoclonal antibody to CD3 (CD3 mAb) (Fig. 1B). This result indicated that Fyn induces tyrosine phosphorylation of the IP_3R during T cell receptor (TCR) stimulation, and therefore that exogenous Fyn does not induce further tyrosine phosphorylation of the IP_3R in vitro.

To determine whether tyrosine phosphorylation of the IP_3R occurred in vivo, we prepared anti-phosphotyrosine immunoblots of IP_3R immunoprecipitated from unstimulated and from TCR-stimulated Jurkat cells. IP_3R was detected in both activated and unactivated cells, but tyrosine-phosphorylated IP_3R was detected only in TCR-stimulated cells (Fig. 1C). This finding indicated that tyrosine phosphorylation of the IP_3R occurs in intact cells.

During T cell activation, Fyn physically associates with the TCR (*10*) and the IP_3R cocaps with the TCR (*11*). To determine whether Fyn physically associated with the IP_3R during T cell activation, we used anti-IP_3R1 or Fyn mAb to immuno-

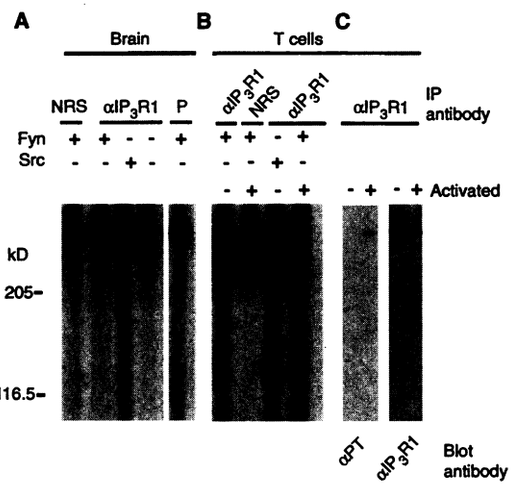


Fig. 1. Tyrosine phosphorylation of the ~300-kD IP_3R in brain and T lymphocytes (Jurkat) by Src and Fyn. IP_3R was immunoprecipitated from canine brain microsomes (**A**) and from lysates of unactivated Jurkat cells or cells activated with CD3 mAb (**B**). Immunoprecipitated proteins or purified IP_3R (P) were used in kinase assays with or without exogenous Fyn or Src (*21*). Preimmune serum (NRS) or affinity-purified anti-IP_3R1 (αIP_3R1) were used for immunoprecipitations (*21*). (**C**) Immunoprecipitated IP_3R from activated or unactivated Jurkat cells was immunoblotted with anti-phosphotyrosine (αPT) (4G10) and then, after stripping, with anti-IP_3R1 as described (*2*).

Laboratory for Molecular Cardiology, Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, USA.

*To whom correspondence should be addressed.